DRUG DEVELOPMENT – A CASE STUDY BASED INSIGHT INTO MODERN STRATEGIES

Edited by **Chris Rundfeldt**

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Preface

The term "drug development" depicts the process leading from a selected chemical structure as drug development candidate to the application for marketing authorization. During the development process three different aspects need to be elaborated: the chemical and pharmaceutical quality, the safety and toxicity profile, and the efficacy in patients. The majority of work conducted during the formal development process aims at generating the data required to submit a marketing application dossier, which will be reviewed by local authorities around the world for approval. The most important authorities are the Food and Drug Administration (FDA) in the US, the European Medicines Agency (EMA) and the Ministry of Health, Labor and Welfare (MHLW) in Japan. These agencies represent the major markets for pharmaceuticals.

Data generated during a drug development process should be suitable to be included into a dossier for marketing application in each of these countries and also in other regions. This, however, has not always been possible. In the 1980s, what is today the European Union began harmonizing regulatory requirements. In 1989, Europe, Japan, and the United States began creating plans for harmonization; and only in April 1990 a structure was established which aimed at generating standards which are acceptable in all respective regions. This institution was called "International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use" (ICH). It is composed from the three regulatory agencies representing US, EU and Japan, but it also included participants from the pharmaceutical industry of these regions. The purpose of ICH is to reduce or obviate the need to duplicate the testing carried out during the research and development of new medicines by recommending ways to achieve greater harmonization in the interpretation and application of technical guidelines and requirements for product registration.

Since its establishment, the ICH has generated several guidelines and standards strictly regulating the requirements for successful drug development. Respective guidelines are often very detailed and describe the required data set and often even the way how such data are to be interpreted. At the same time, the ICH aims at being up to data to represent the best standard of current research. This requires constant update of guidelines and in part generation of novel regulations. Numerous guidelines have been generated and revised as needed in all three sections of drug development, i.e. the pharmaceutical section describing quality aspects of the drug substance and the

drug product (guidelines Q1-Q11), safety aspects (guidelines S1-S9) and efficacy aspects (E1-E16). Multidisciplinary guidelines are M1-M8. All guidelines are accessible online.

The backbone of drug development is described in the guideline ICH M3 where preclinical safety studies are described which are needed at the different stages of clinical development. The guideline M4 describes the required format of a dossier for marketing application, the so called technical document where all data generated during a development process need to be compiled and evaluated by experts.

As can be seen from the description above, the formal drug development process is a highly regulated procedure and this strict regulation is required to ensure safety of all medicinal products. From a scientific point of view, the most interesting step within the drug development process is the selection of the right development candidate. The available book focusses on this bridging step, where basic research, chemical optimization, and scientific genius cumulate in the selection of a single molecule or at most 2-3 different molecules to be put through the formal process of drug development. This early drug development stage is most critical for the later success. At this stage, future efficacy in patients is predicted by well selected disease models and early risk mitigation procedures are required to reduce the development failure rate. At this stage creativity is not limited by regulatory agencies and the target audience for each data set generated is the management of a company or a group of investors, which need to be convinced that the respective molecule is worth investing several hundred million dollar, since this amount of money is often needed to conduct all the studies and generate all the data required to submit a full marketing authorization dossier. Indeed, drug development is very expensive.

A given development candidate will most likely only enter the development pipeline of a pharmaceutical company, if it can be shown that the development risks are below average and that the revenues which can be generated from future marketing of the drug are capable of paying back the investment. The requirement that a drug candidate must ultimately generate income makes a development difficult for diseases which affect only few people or for diseases which affect mainly poor people which cannot afford expensive medicines. However, if the costs of a specific development project can be reduced compared to the standard, the cost-revenue balance can be accomplished even if the expected sales are not sufficient for a standard development program.

Regulatory agencies in the major markets have already introduced measures to encourage drug development for rare diseases. Companies developing drugs for such rare diseases can get market exclusivity for a defined period, the clinical development program to show efficacy may be reduced and development support is also offered. But no such tools are currently available to encourage development for diseases which mainly affect poor people such as the so called neglected diseases or tuberculosis. Measures to reduce the costs to enable drug development for these diseases are for example to re-use available medicinal products. Indeed, as laid out by Dr. Nguyen et

al. in this book, established antibiotics may be useful for the treatment of tuberculosis, if the mechanisms of resistance are attacked by well selected combination treatment. A different method to counterbalance a limited revenue potential is to reduce the development risks. Adequate studies showing that a given compound has a high chance to be active in a patient population, has a pharmacokinetic property which is in line with a once or twice daily treatment regime, and has a lower than average potential for toxicity, can attract decision makers to invest in the project.

In this book, this early drug development stage is described giving interesting examples in a number of disease areas.

1st section: Cancer treatment

Despite extensive research conducted in the past, the fight against cancer is an ongoing battle with significant, but still only small advances in chemotherapy. In five different chapters, five different novel potential targets for cancer treatment are presented.

An enzyme critically involved in modulation of intracellular signaling by methylation of DNA is presented as potential target, directly affecting cell growth and differentiation (Jakyung Yoo and José L. Medina-Franco).

Several cancer cell types utilize sex hormones as growth stimulus and reducing this stimulation retards or blocks the growth of prostate and breast cancer. But these cells often escape the initial pharmacological intervention. Rahul Aggarwal and Charles J. Ryan describe novel strategies to overcome the hormone resistance.

A different strategy is described by Karolina Gluza and Paweł Kafarski. A solid cancer is increasing its malignancy if it is capable to grow invasive. To penetrate cell barriers, the intercellular matrix needs to be dissolved requiring proteinases which are released by invasive cancer cells. The inhibition of these enzymes can reduce invasiveness. An irreversible (mechanism based) inhibition is considered to be most effective.

Vanina A. Medina et al. describe findings linking the histamine receptor to cancer treatment, which seems to be quite unexpected, but the data are intriguing.

Histone deacetylase (HDAC) inhibitors are an emerging class of therapeutic agents that induce tumor cell cytostasis, differentiation, and apoptosis in various hematologic and solid malignancies. But currently available chemical scaffolds did not reach the stage of formal drug development. Ethirajulu Kantharaj and Ramesh Jayaraman describe the pharmacokinetics and metabolic properties to show which scaffold could be best suited for further structure optimization.

2nd section: Anti-infectives

While antibacterial treatments did revolutionize the medical treatment of bacterial infections in the early years of rational drug development with the development of the sulfonamides in the years between 1910 and 1940, followed by penicillin in 1942, we

are now focusing the problem, that many bacteria are escaping the currently available antibiotics and becoming resistant, even to multiple antibiotics. To combat such multi-resistant bacteria, novel strategies are required.

Mario Zucca et al. describe novel antibacterial peptides which could help overcome the growing resistance. But peptides are difficult to develop since they are sensitive to metabolic degradation and may also have problems to penetrate tissues. These aspects are discussed.

Elizabeth Hong-Geller and Nan Li describe microRNA based treatment strategies for infective diseases, utilizing current knowledge of microRNA as cellular signaling tool.

Treatment of tuberculosis is the topic of the next 3 chapters. Mycobacterium tuberculosis is a bacterium which is difficult to treat. Due to its extremely dense cell membrane consisting of multiple layers, and it's very slow life cycle, a treatment requires dosing for many months, which is not only a compliance problem, but can be a commercial problem for the affected person, which often is found among the poor people. Multi drug resistant forms are spreading, requiring the combination of two or even three different drugs. To improve treatment without having to identify novel antibacterial drugs, Kerstin A. Wolff et al. evaluate currently available antibiotics for the treatment of tuberculosis by addressing strategies to combat resistance of mycobacteria. Virgilio Bocanegra-García et al. review potential targets of mycobacteria and evaluate the current development pipeline, and Auradee Punkvang et al. give insight in the technology of chemical structure optimization based on computer aided molecular design, focused on an emerging target of mycobacteria, the enzyme enoyl acyl carrier protein reductase.

3rd section: Novel targets and technologies leading to improved treatment options

Novel technologies may be useful for diverse diseases. RNA interference is such a novel technology, however several hurdles have to be overcome before successful treatments can emerge, including stability of RNA molecules and targeting of the right cells. Tamara Martinez et al. have compiled and excellent overview of this technology.

If the physiology of a novel receptor or a receptor sub-family is explored, the utility of such a receptor as target for different diseases can be deducted. Examples are presented by Liang Xin et al., reviewing the P2X receptor family, and by Sue Sien Ong et al., reviewing the pregnane X receptor.

The elucidation of physiology of specific functions is the basis of the contribution from Takekazu Kubo et al. They describe the interplay between the central nervous system and the immune system, leading to a novel target idea for the treatment of neuroinflammatory diseases such as multiple sclerosis or traumatic brain injury.

A different strategy for drug development was selected by Rui Ting Liu et al., focusing on a natural product extracted from traditional herbal medicine. The flavonoide

Liquiritigenin, which is having estrogen-like function, is shown to have potent its activity in a mouse model of Alzheimer's disease.

The evaluation of the activity of natural products is also the basis of the next chapter. Chagas disease is a tropical disease which has not attracted active drug development in the recent years. Alane Beatriz Vermelho et al. compare the activity of currently available medicines with the effects of novel lead structures, in part derived from natural products.

The interaction of pharmacological agents with a biotope which is often not considered to be of importance, the gut microbiota, is discussed by Joan Vermeiren et al. The composition of the gut bacteria community can have influence on stability, pharmacokinetics, and metabolism of xenobiotics. A better knowledge of this possible interaction can improve drug development. This is explored using inflammatory bowel disease as case study.

The following two chapters explore novel sources for pharmacologically active compounds. While Luiz H. Rosa et al. focuss on endophytic fungi of tropical forests as a unique source for new chemical entities with potential for the treatment of diseases such as leishmaniasis, María Estrella Legaz et al. describe the utilization of the enzymatic properties of lichen to produce a compound class called depsidones. Preclinical data indicated that such compounds may have antiviral, antibacterial and other effects.

A different field of drug development is touched by the next two chapters, the pharmaceutical development of adequate dosage forms. Intranasal and transdermal drug administration is used to bypass the gastrointestinal tract and the metabolic capability of the liver. Successful application of these dosing routes requires that the administration results in adequate drug levels at the target organ without risk for the patient. A potential risk for intranasal application is bacterial contamination and hence infection from the nasal applicator. While preservatives can reduce the risk of bacterial growth in the container, such agents can induce irritation on the nasal mucosa and should be avoided. Degenhard Marx and Matthias Birkhoff describe novel technologies which can avoid the use of preservatives in multidose intranasal containers. Transdermal application is often limited by the skin representing a diffusion barrier. A technology to reduce the barrier function by using microneedles prior to application of the transdermal patch is described by Stan L. Banks et al.

4th section: Drug development strategies

The final book section focusses on issues related to drug development in general. Drug development is very expensive and only if the potential revenues which can be generated by a drug candidate can payback the required investment, a development project can be started. Rare diseases or diseases predominantly affecting poor people are often not sufficiently covered by novel drug developments. Kristina M. Lybecker

describes different incentivation models developed to improve the access of poor people to modern drugs, with special focus on patent rights.

The first step in clinical drug development is the conduct of studies in healthy volunteers. Such clinical studies aim at exploring the safety profile and the pharmacokinetics of a new drug candidate. However no hints on efficacy can be obtained in these studies. Giovanni Gori et al. describe modern tools to expand the options of phase I studies in healthy volunteers. Using biomarkers, modern imaging technology, and specific challenge protocols, hints for efficacy can be obtained and the safety evaluation can be improved.

The final chapter of this section focuses on drug development for children. In former times, drugs were developed in adults and the use of pharmaceutical agents in children was based in most cases on empiric information obtained from the use in children. Regulatory agencies have realized that this procedure can be very dangerous since the juvenile organism differs considerably from the adult organism. In 2007 the pediatric regulations were introduced in the EU requiring now stringent criteria on quality, safety, and efficacy of medicinal products to be used in the pediatric population. Adriana Ceci et al. evaluate how the drug development environment has changed after the introduction of these regulations.

Conclusion

This book represents a case study based overview on many different aspects of drug development, ranging from target identification and characterization to chemical optimization for efficacy and safety, as well as bioproduction of natural products utilizing for example lichen. In the last section special aspects of the formal drug development process are discussed. Since drug development is a highly complex multidisciplinary process, case studies are an excellent tool to obtain insight in this field. While each chapter gives specific insight and may be read as independent source of information, the whole book represents a unique collection of different facets giving insight in the complexity of drug development.

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Part 1

Novel Approaches to Cancer Treatment

1

Discovery and Optimization of Inhibitors of DNA Methyltransferase as Novel Drugs for Cancer Therapy

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1. Introduction

The genome contains genetic and epigenetic information. While the genome provides the blueprint for the manufacture of all the proteins required to create a living thing, the epigenetic information provides instruction on how, where, and when the genetic information should be used (Robertson, 2001). The major form of epigenetic information in mammalian cells is DNA methylation that is the covalent addition of a methyl group to the 5-position of cytosine, mostly within the CpG dinucleotide (Robertson, 2001). DNA methylation is involved in the control of gene expression, regulation of parental imprinting and stabilization of X chromosome inactivation as well as maintenance of the genome integrity. It is also implicated in the development of the immune system, cellular reprogramming and brain function and behaviour (Jurkowska et al., 2011). DNA methylation is mediated by a family of DNA methyltransferase enzymes (DNMTs). In mammals, three DNMTs have been identified so far in the human genome, including the two *de novo* methyltransferases (DNMT3A and DNMT3B) and the maintenance methyltransferase (DNMT1), which is generally the most abundant and active of the three (Goll and Bestor, 2005; Robertson, 2001; Yokochi and Robertson, 2002). DNMT3L is a related protein that has high sequence similarity with DNMT3A, but it lacks any catalytic activity owing to the absence of conserved catalytic residues. However, DNMT3L is required for the catalytic activity of DNMT3A and 3B (Cheng and Blumenthal, 2008). The protein DNMT2 can also be found in mammalian cells. Despite the fact that the structure of DNMT2 is very similar to other DNMTs, its role is comparably less understood (Schaefer and Lyko, 2010). It has been reported that DNMT2 does not methylate DNA but instead methylates aspartic acid transfer RNA ($tRNA^{Asp}$) (Goll et al., 2006). Recent evidence suggests that DNMT2 activity is not limited to $tRNA^{Asp}$ and that DNMT2 represents a noncanonical enzyme of the DNMT family (Schaefer and Lyko, 2010).

DNMT1 is responsible for duplicating patterns of DNA methylation during replication and is essential for mammalian development and cancer cell growth (Chen et al., 2007). These enzymes are key regulators of gene transcription, and their roles in carcinogenesis have been the subject of considerable interest over the last decade (Jones and Baylin, 2007; Robertson, 2001). Therefore, specific inhibition of DNA methylation is an attractive and novel approach for cancer therapy (Kelly et al., 2010; Lyko and Brown, 2005; Portela and

Esteller, 2010; Robertson, 2001). It is worth noting that DNA methylation inhibitors have also emerged as a promising strategy for the treatment of immunodeficiency and brain disorders (Miller et al., 2010; Zawia et al., 2009).

The structure of mammalian DNMTs can be divided into two major parts, a large N-terminal regulatory domain of variable size, which has regulatory functions, and a C-terminal catalytic domain which is conserved in eukaryotic and prokaryotic carbon-5 DNMTs. The N-terminal domain guides the nuclear localization of the enzymes and mediates their interactions with other proteins, DNA, and chromatine. The smaller C-terminal domain harbors the active center of the enzyme and contains ten amino acids motifs diagnostic for all carbon-5 DNMTs (Jurkowska et al., 2011). Motifs I-III form the cofactor binding pocket, motif IV has the catalytic cysteine, motifs VI, VIII, and X compose the substrate binding site, and motifs V and VII form the target recognition domain (Sippl and Jung, 2009). Human DNMT1 has 1616 amino acids for which limited three-dimensional structural information is available. For example, just recently a crystal structure of human DNMT1 bound to duplex DNA containing unmethylated cytosine-guanine (CG) sites was published (Song et al., 2011). Further details of the structure of DNMTs and other available crystal structures of DNMTs are extensively reviewed elsewhere (Cheng and Blumenthal, 2008; Lan et al., 2010; Sippl and Jung, 2009).

The proposed mechanism of DNA cytosine-C5 methylation is summarized in Fig. 1 (Schermelleh et al., 2005; Sippl and Jung, 2009; Vilkaitis et al., 2001). DNMT forms a complex with DNA, and the cytosine which will be methylated flips out from the DNA. The thiol of the catalytic cysteine from motif IV acts as a nucleophile that attacks the 6-position of the target cytosine to generate a covalent intermediate. The 5-position of the cytosine is activated and conducts a nucleophilic attack on the cofactor S-adenosyl-L-methionine (AdoMet) to form the 5-methyl covalent adduct and *S*-adenosyl-L-homocysteine (AdoHcy). The attack on the 6-position is assisted by a transient protonation of the cytosine ring at the endocyclic nitrogen atom N3, which is stabilized by a glutamate residue from motif VI. The same residue also contacts the exocyclic N4 amino group and stabilizes the flipped base. The carbanion may also be stabilized by resonance, where an arginine residue from motif VIII may participate in the stabilization of the cytosine base. The covalent complex between the methylated base and the DNA is resolved by deprotonation at the 5-position to generate the methylated cytosine and the free enzyme.

DNA methylation inhibitors have been well characterized and tested in clinical trials (Issa and Kantarjian, 2009). To date, only 5-azacytidine and 5-aza-2′-deoxycytidine (Fig. 2) have been developed clinically. These two drugs are nucleoside analogues, which, after incorporation into DNA, cause covalent trapping and subsequent depletion of DNA methyltransferases (Schermelleh et al., 2005; Stresemann and Lyko, 2008). Aza nucleosides are approved by the Food and Drug Administration of the United States for the treatment of myelodysplastic syndrome, where they demonstrate significant, although usually transient improvement in patient survival and are currently being tested in many solid cancers (Issa et al., 2005; Schrump et al., 2006). Despite the clinical successes achieved with DNA methylation inhibitors, there is still need for improvement since aza nucleosides have relatively low specificity and are characterized by substantial cellular and clinical toxicity (Stresemann and Lyko, 2008). Their exact mechanism of antitumor action – demethylation of aberrantly silenced growth regulatory genes, induction of DNA damage, or other mechanism also remains unclear (Fandy et al., 2009; Issa, 2005; Palii et al., 2008).

Consequently, there is clear need to identify novel and more specific DNMT inhibitors that do not function via incorporation into DNA.

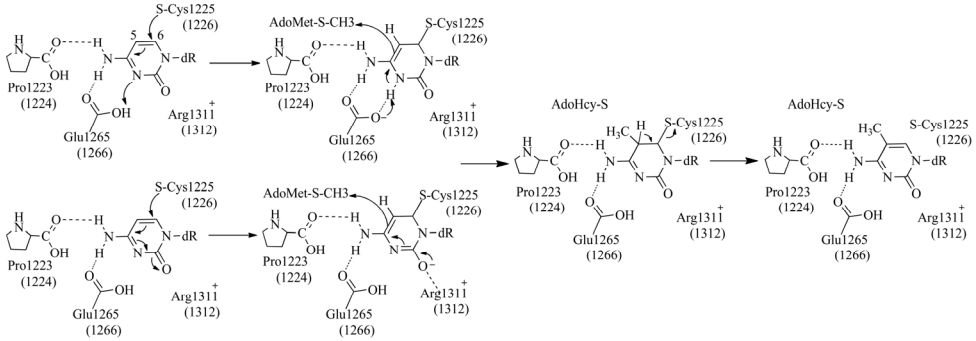

Fig. 1. Mechanism of DNA cytosine-C5 methylation. Amino acid residue numbers are based on the homology model. Equivalent residue numbers in parentheses correspond to the crystal structure.

There is now an increasing number of substances that are reported to inhibit DNMTs (Lyko and Brown, 2005). Selected DNMT inhibitors and other candidate demethylating agents are depicted in Fig. 2. Some of these compounds are approved drugs for other indications; i.e., the antihypertensive drug hydralazine (Segura-Pacheco et al., 2003), the local anaesthetic procaine (Villar-Garea et al., 2003), and the antiarrhythmic drug procainamide (Lee et al., 2005a). Others like the L-tryptophan derivative RG108, NSC 14778 (Fig. 2) have been identified by docking-based virtual screening (Kuck et al., 2010a; Siedlecki et al., 2006). Several natural products have been implicated in DNA methylation inhibition. Selected examples are the main polyphenol compound from green tea, (-)-epigallocathechin-3-gallate (EGCG) (Fang et al., 2003; Lee et al., 2005b), other tea polyphenols such as catechin and epicatechin, and the bioflavonoids quercetin, fisetin, and myricetin. Curcumin, the major component of the Indian curry spice turmeric, has been reported to inhibit the DNA cytosine C5 methyltransferase M.SssI, an analogue of DNMT1 (Liu et al., 2009). However, more recent studies showed that curcumin did not cause DNA demethylation in three arbitrarily chosen human cancer cell lines (Medina-Franco et al., 2011). Mahanine, a plant-derived carbazole alkaloid, and a fluorescent carbazole analogue, has been reported to induce the Ras-association domain family 1 (RASSF1) gene in human prostate cancer cells, presumably by inhibiting DNMT activity (Jagadeesh et al., 2007; Sheikh et al., 2010). Nanaomycin A, a quinone antibiotic isolated from a culture of *Streptomyces*, has been described as the first non *S*-adenosyl-L-homocysteine (AdoHcy/SAH) analogue acting as a DNMT3B-selective inhibitor that induces genomic demethylation. Nanaomycin A treatment reduced the global methylation levels in three cell lines and reactivated transcription of the RASSF1A tumor suppressor gene (Kuck et al., 2010b). These and several other natural products as putative demethylating agents are extensively reviewed elsewhere (Gilbert and Liu, 2010; Hauser and Jung, 2008; Li and Tollefsbol, 2010; Medina-Franco and Caulfield, 2011). While the substantial number of recent reports may suggest that many natural products inhibit DNA methylation, it should be noted that only a few reports provide compelling evidence for DNMT inhibition in biochemical and in cellular assays. As such, it

remains possible that many of these compounds have an indirect and fortuitous effect on DNA methylation, but do not show a pharmacologically relevant activity that can be developed further for therapeutic purposes (Medina-Franco et al., 2011).

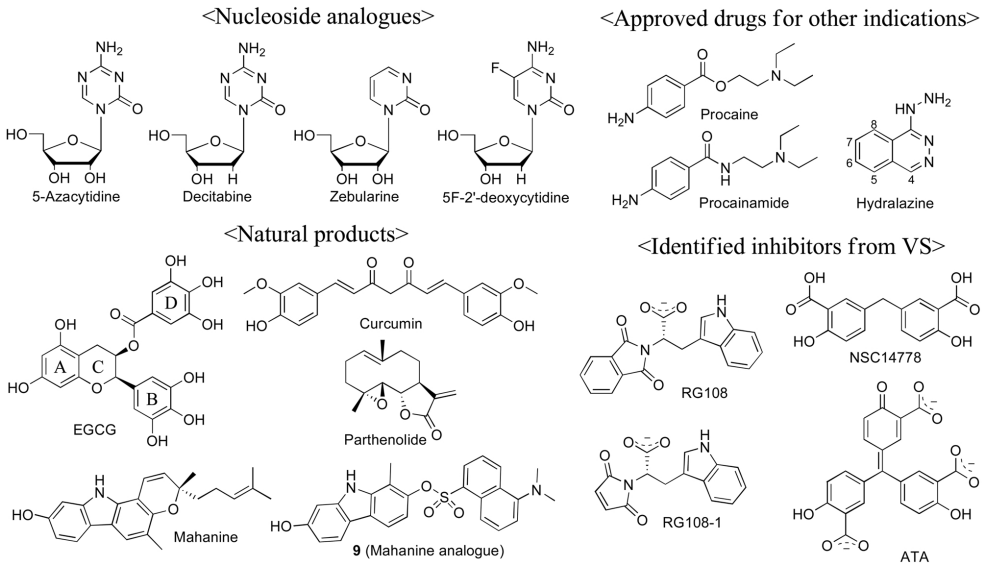

Fig. 2. Chemical structures of selected DNA methyltransferase inhibitors and other compounds with putative demethylating activity.

Until now most compounds associated with DNA methylation inhibition have been identified fortuitously. Remarkable exceptions are RG108 and 5,5′-methylenedisalicylic acid (NSC 14778) that were identified by computational screening followed by experimental evaluation (Kuck et al., 2010a; Siedlecki et al., 2006). In order to accelerate the discovery and optimization of new DNMT inhibitors, rational approaches are increasingly being used. To this end, *in silico* studies have significantly helped to understand the structure and function of DNMTs and the mechanism of DNMT inhibition (Medina-Franco and Caulfield, 2011). This chapter focuses on the different strategies ongoing in our and other research groups for the discovery and optimization of inhibitors of DNMTs with particular emphasis on *in silico* screening (section two) and *in silico* design (section three).

2. *In silico* screening of compound collections to identify novel inhibitors

In silico screening, also called in the literature, computational or virtual screening, consists of the computational evaluation of databases aiming to select a small number of reliable and experimentally testable candidate compounds that have a high probability of being active (Muegge, 2008; Shoichet, 2004). *In silico* screening is one of the most common rational approaches to guide the identification of new hits from large compound libraries. Hit identification using this approach requires several interactive steps that include (1) the compound collection, (2) the computational methods used for screening, and (3) the analysis of the output (López-Vallejo et al., 2011).

2.1 Screening databases

A number of compound databases from different sources can be used in *in silico* screening. These libraries may contain existing or hypothetical; i.e., virtual compounds. Libraries of existing compounds may be proprietary; e.g., in-house libraries, commercial, or public. The sources of screening libraries, with emphasis on libraries in the public domain, have been reviewed (Bender, 2010; Scior et al., 2007). Currently, the ZINC database is one of the most used libraries (Irwin and Shoichet, 2005). The type of screening library utilized should be closely associated with the objective of the particular screening campaign (Shelat and Guy, 2007). Chemically diverse libraries are particular attractive for identifying novel scaffolds for new or relatively unexplored targets such as DNMTs. If the goal is lead optimization, e.g., optimize the activity of known DNMT inhibitors (Fig. 2), focused libraries or collections with high inter-molecular similarity (highly dense libraries) are an attractive source.

2.1.1 Natural product databases

The presence of DNMT inhibitors in dietary products and commonly used herbal remedies (Gilbert and Liu, 2010; Hauser and Jung, 2008; Li and Tollefsbol, 2010) demonstrates the feasibility of identifying additional inhibitors of natural origin. Natural products have unique characteristics attractive for drug discovery. For example, the chemical structures of natural products are, in general, different from the chemical structures of synthetic compounds occupying different areas of chemical space (Ganesan, 2008; Medina-Franco et al., 2008; Singh et al., 2009b). In addition, natural products may be drug candidates themselves or may be the starting point for an optimization program (Ganesan, 2008; Hauser and Jung, 2008). Indeed several natural products are bioavailable, and the rationale of these observations has been recently provided (Ganesan, 2008). Fig. 3 shows a visual representation of the chemical space of natural products, drugs, and DNMT inhibitors. To compare the chemical space, a subset of 1,000 compounds was randomly selected from each database. The visual representation was obtained with principal component analysis (PCA) of the similarity matrix of the databases computed using Molecular ACCess System (MACCS) keys (166 bits) and the Tanimoto coefficient (Maggiora and Shanmugasundaram, 2011). The first three principal components are displayed in Fig. 3 and account for 79% of the variance. This figure clearly shows that most of the DNMT inhibitors, e.g., nucleoside analogues, RG108, RG108-1, procaine, procainamide, SG1027, and hydralazine, share the same chemical space of drugs. This observation is expected from inhibitors such as procaine, procainamide, and hydralazine. In contrast, NSC14778 and DNMT inhibitors from natural origin, EGCG and curcumin, are in a less-dense populated area of the chemical space of drugs. These compounds are characterized by containing one or more hydroxyl groups. Fig. 3b shows that most of the compounds in the natural product database also occupy this second region. Before conducting virtual and experimental screening, it is feasible to filter out natural products with potential toxicity issues using drug- or lead-like filters (Charifson and Walters, 2002).

2.1.2 Combinatorial libraries

Combinatorial libraries, either existing or virtual, are important sources of compound collections that can be used for *in silico* screening (López-Vallejo et al., 2011). Advances in synthetic approaches can generate *libraries from libraries*, *target-oriented* libraries, and *diversity-oriented* libraries which explore the chemical space in different ways (López-Vallejo

et al., 2011) and can be used in lead optimization or hit-identification, depending on the goals of the screening campaign.

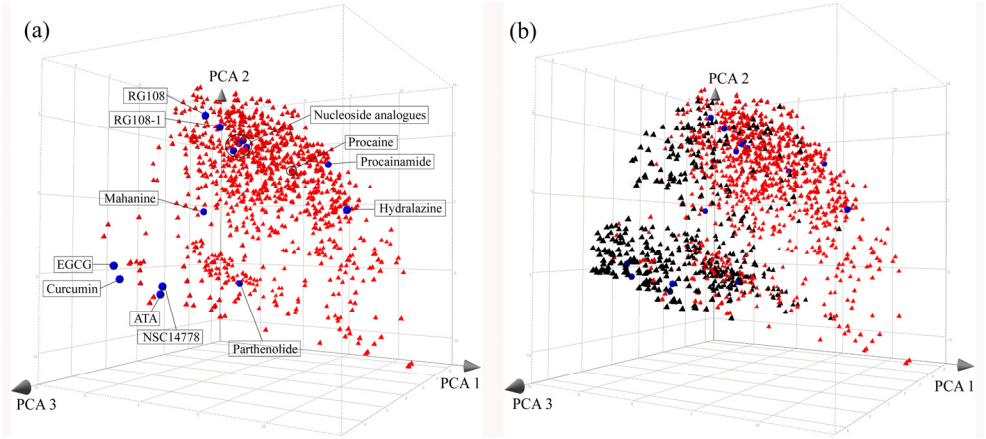

Fig. 3. Comparison of 486 natural products (black triangles), 1,000 drugs (red triangles), and 14 DNMT1 inhibitors (blue spheres). Depiction of a visual representation of the chemical space obtained by PCA of the similarity matrix computed using MACCS keys and Tanimoto similarity. The first three PCs account for 79% of the variance. (a) Comparison of drugs and DNMT1 inhibitors. (b) Comparison of drugs, natural products, and DNMT inhibitors.

2.2 Development and validation of computational approaches

In silico screening can be divided into two general strategies: ligand-based and structure-based (Medina-Franco et al., 2006; Ooms, 2000). Ligand-based approaches use the structural information and biological activity data from a set of known active compounds to select promising candidates for experimental screening. When the three-dimensional structure of the target is known, structure-based methods can be used. Three-dimensional structure information of the target is usually obtained from X-ray crystallography or nuclear magnetic resonance. In the absence of three-dimensional structural information of the receptor, homology models have been successfully used (Grant, 2009; Villoutreix et al., 2009). Perhaps the most common structure-based approach is molecular docking. Docking aims to find the best position and orientation of a molecule within a binding site and gives a score for each docked pose (Hernández-Campos et al., 2010; Kitchen et al., 2004; Villoutreix et al., 2009). Ligand- and structure-based methods can be combined if information for both the experimentally active compounds and the three-dimensional structure of the target are available (Sperandio et al., 2008). The selection of a particular method is generally based on the goal of the project, the information available for the system, and the computational resources available. For structure-based and ligand-based methods, it is highly advisable to validate the virtual screening protocol prior to the selection of compounds for experimental testing. However, the experimental results of the tested candidates will provide full validation of the virtual screening approach.

2.2.1 Structure-based screening

Structure-based screening for novel DNMT inhibitors performed so far has been conducted with homology models of the catalytic domain (Kuck et al., 2010a; Siedlecki et al., 2006). The

construction of useful homology models has been facilitated by the extensive conservation of the catalytic domain of DNMTs (Kumar et al., 1994). Crystal structures of other methyltransferases such as bacterial DNA cytosine C5 methyltransferase from *Haemophilus hemolyticus* (M.HhaI), bacterial cytosine C5 methyltransferase M.HaeIII, and the human DNMT2 (Siedlecki et al., 2003; Yoo and Medina-Franco, 2011) have been used as templates (Medina-Franco and Caulfield, 2011).

We have recently developed two homology models of the catalytic domain of DNMT1. In one model (Yoo and Medina-Franco, 2011), the crystal structures of the DNMTs M.HhaI, M.HaeIII, and DNMT2 were used as templates. The first structure is a ternary complex of M.HhaI, *S*-adenosyl methionine (AdoMet), and DNA containing flipped 4′-thio-2′-deoxcytidine with partial methylation at C5. The crystal structure of M.HaeIII is bound covalently to DNA. In this complex, the substrate cytosine is extruded from the DNA and it is inserted into the active site. The structure of human DNMT2 complexed with *S*-adenosyl-L-homocysteine (AdoHcy/SAH) has high similarities to methyltransferases of both prokaryotes and eukaryotes. A second homology model was developed using only the structure of M.HhaI as template. Both models contain DNA and the conserved residues which are involved in the catalytic mechanism. The target cytosine which is flipped out of the embedded DNA is inserted into the active site. The catalytic loop containing the catalytic cysteine is located above the cytosine as an active site "lid". The target cytosine lies between the nucleophile cysteine residue (Cys1225) and the sulfur atom of AdoHcy. The distance of cytosine C6 to the sulfur atom of Cys1225 is 3.3 Å. The cytosine C5 atom is 3.0 Å away from the sulfur atom of AdoHcy. In the reactive state of Cys1225, the distance between $O^{\varepsilon 1}$ of Glu1265 and N3 of cytidine is 2.8 Å, where the N3 atom is proposed to be protonated making a hydrogen bond with the acidic side chain of Glu1265. In addition, the N3 protonated form of cytosine can make hydrogen bonds with Arg1311 and Pro1223. These key interactions in the catalytic site are commonly observed in both homology models. More specifically, in the first homology model, the α-phosphate backbone and 3′-OH of the sugar moiety of deoxycytidine make a hydrogen bond network with Arg1311, Arg1461, Ser1229, Gly1230, and Gln1396; in the second model, the interactions are observed with the following residues: Gln1226, Ser1229, Gly1230, Arg1268, and Arg1310.

Fig. 4 shows a superimposition of the first homology model of the catalytic site of hDNMT1 (Yoo and Medina-Franco, 2011) with the recently published crystal structure of unmethylated human DNMT1 (Song et al., 2011). The catalytic cores of their methyltransferase domains have similar features, but unmethylated DNA in the crystallographic structure is positioned further away from the active site. In the crystal structure of the human DNMT complex, the key amino acid residues Glu1265 and Arg1311 are positioned in very similar place. In contrast, the catalytic loop adopts a different conformation with respect to the homology model. The catalytic loop has an open conformation, and the catalytic cysteine is far from the binding site, e.g., the distance of superimposed cytosine C6 to the sulfur atom of Cys1225 is 9.5 Å. Taken together, the structural characterization of the catalytic site supports that our homology model is in full agreement with the proposed catalytic mechanism of DNA methylation.

In silico screening has been successfully used to identify novel small molecule inhibitors of DNMT1. In one study, 1990 compounds in the Diversity Set available from the National Cancer Institute were the starting point of a screening using docking with a validated homology model of human DNMT1. Compounds with undesirable size, hydrophobicity, and uncommon atom types were filtered out. Two of the top scoring compounds were

tested experimentally showing activity both *in vitro* and *in vivo*, probably by binding into the DNMT1 catalytic pocket (Siedlecki et al., 2006). In that work, RG108 (Fig. 2) showed an IC_{50} = 0.60 μM with M.SssI (Siedlecki et al., 2006). Additional characterization showed that this L-tryptophan derivative did not cause covalent enzyme trapping and that the carboxylate group plays an essential role in the binding with the enzyme since the analogue without this moiety is inactive (Brueckner et al., 2005).

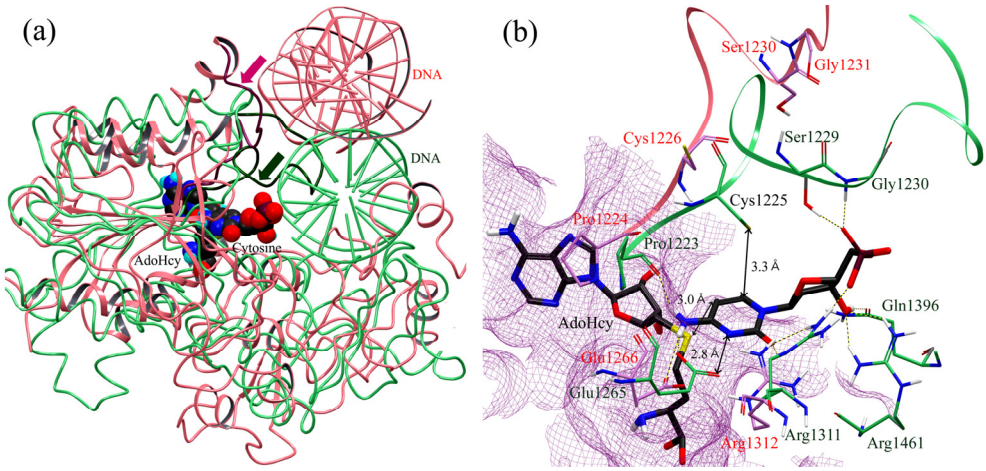

Fig. 4. (a) Superposition of the homology model (green) of the catalytic domain of human DNMT1 with the crystallographic structure (pink) of the unmethylated human DNMT1. The catalytic loops are marked with arrows. AdoHcy and the flipped cytosine in the homology model are shown in space-filling view. (b) Binding model of deoxycytidine (black) with key amino acid residues of homology model (carbon atoms in green) and crystal structure (carbon atoms in pink). Hydrogen bonding interactions are represented by dotted lines.

In a follow-up study, our group screened a larger set of the National Cancer Institute database containing more than 260,000 compounds (Kuck et al., 2010a). In order to focus the screening on compounds that could be promising for further development, we selected a subset of approximately 65,000 lead-like molecules (Charifson and Walters, 2002). The lead-like set was further filtered using a high-throughput *in silico* screening. As part of the screening, three docking programs were used. Favorable docking scores from all three docking approaches were combined to create a total of 24 consensus compounds. Of the 24 molecules that were identified, thirteen were obtained for experimental testing. Seven out of the thirteen consensus hits had detectable DNMT1 inhibitory activity in biochemical assays. Further experimental characterization of active compounds showed that six out of the seven inhibitors appeared selective for DNMT1. The methylenedisalicylic acid derivative, NSC 14778 (Fig. 2), showed an IC_{50} = 92 μM with DNMT1 and an IC_{50} = 17 μM with DNMT3B. The observed potency was comparably low for most test compounds, which was partially attributed to the high amount of protein used in the biochemical assay. In fact, it is well-known that DNMTs are weak catalysts and are difficult to assay (Hemeon et al., (2011 - ASAP)). Despite the low potency, the *in silico* screening was successful in that it identified diverse scaffolds that were not previously reported for DNMT inhibitors. The new scaffolds represent excellent candidates for optimizing their inhibitory activity and selectivity.

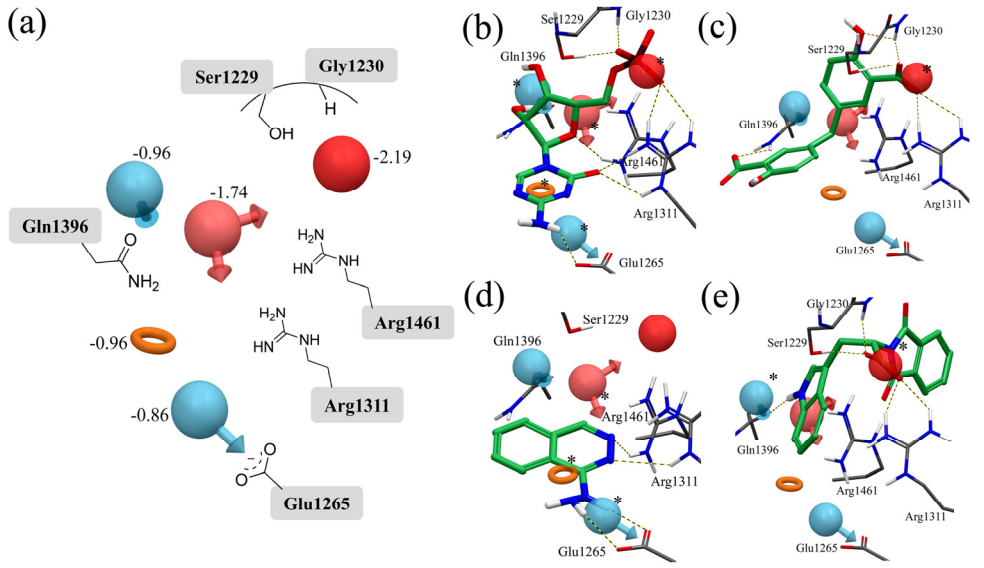

Fig. 5. (a) Structure-based pharmacophore model proposed for human DNMT1 inhibitors. *Red sphere*: negative ionizable, *pink sphere*: hydrogen bond acceptor, *blue sphere*: hydrogen bond donors, and *orange ring*: aromatic ring. Selected amino acid residues in the catalytic site of homology model are schematically depicted for reference. Comparison between the binding mode and pharmacophore hypothesis for representative DNMT inhibitors, (b) 5-azacytidine, (c) NSC14778, (d) hydralazine, and (e) RG108.

Recently, our group developed a structure-based pharmacophore hypothesis for inhibitors of DNMT1 (Yoo and Medina-Franco, 2011). Using the energy optimized hypothesis, 'e-pharmacophore' method (Salam et al., 2009) the pharmacophore model was developed based on the scores and predicted binding modes of 14 known DNMT1 inhibitors docked with a homology model of DNMT1. Fig. 5a shows the pharmacophore model for the 14 DNMT1 inhibitors. The model contains five features which represent the most important interactions of the inhibitors with the catalytic domain. The energetic value assigned to each pharmacophoric feature is displayed in the figure. Nearby amino acids are schematically depicted for reference. The best-scoring feature is a negative charge which is close to the side chains of Ser1229, Gly1230, and Arg1311. The second most favorable feature is an acceptor site that is in close proximity with the side chains of Arg1311 and Arg1461. The third ranked features are an aromatic ring that stabilizes the binding conformation of ligands between AdoHcy and Cys1225, and a donor site that is close to the side chain of Gln1396. The fifth-ranked feature is a donor site that is nearby the side chain of Glu1265 which is a residue implicated in the methylation mechanism. Fig. 5b shows the alignment of representative DNMT inhibitors to the pharmacophore hypothesis. It remains to evaluate the performance of the pharmacophore model in prospective *in silico* screening.

2.2.2 Ligand-based screening

Ligand-based screening can be performed as an alternative approach when the relevant crystal structures are not available on the molecular target. Ligand-based approaches

include similarity searching, substructure, clustering, quantitative structure-activity relationships (QSAR), pharmacophore-, or three-dimensional shape matching techniques (Villoutreix et al., 2007). Several ligand-based methods, including similarity searching and QSAR, can roughly be divided into two- or three-dimensional approaches. Ligand-based virtual screening may be applied even if a single known-ligand has been identified through similarity-based screening. Interestingly, although many more successful structure-based than ligand-based virtual screening applications are reported to date, recent reviews indicate that the potency of hits identified by ligand-based approaches is on average considerably higher than for structure-based methods (Ripphausen et al., 2011; Ripphausen et al., 2010).

If multiple active compounds are known, it is possible to apply QSAR using two- or three-dimensional information of the ligands. One of the main goals of QSAR is to derive statistical models that can be used to predict the activity of molecules not previously tested in the biological assay. Despite the fact that QSAR is a valuable tool, there are potential pitfalls to develop predictive QSAR models (Scior et al., 2009). A major pitfall can occur when the compounds were assayed using different experimental conditions. Other major pitfall is due to the presence of "activity cliffs," i.e., compounds with very high structural similarity but very different biological activity (Maggiora, 2006). Activity cliffs give rise to QSAR with poor predictive ability (Guha and Van Drie, 2008).

3. *In silico* design and optimization of established inhibitors

Concerns about severe toxicity of nucleoside analogues have strongly encouraged not only identifying novel DNMT inhibitors but also developing further established non-nucleoside inhibitors. To this end, medicinal chemistry approaches, either alone or in combination with *in silico* strategies, are being pursued.

3.1 Optimization of RG108, procaine, and mahanine

As mentioned above, procaine, a local anesthetic drug, and procainamide, a drug for the treatment of cardiac arrhythmias, have been reported as inhibitors of DNA methylation (Fig. 2). In a recent report, constrained analogues of procaine were synthesized and tested for their inhibition against DNMT1 (Castellano et al., 2008). Procaine as a lead structure was modified to partially reduce the high flexibility which can have a detrimental effect for drug-likeness. The most potent inhibitor in an *in vitro* methylation assay also showed demethylation activity in HL60 human myeloid leukemia cells, and it was suggested as a lead compound for further studies (Castellano et al., 2008).

In a separate work, a series of maleimide derivatives of RG108 were reported (Suzuki et al., 2010). In that work, design, chemical synthesis, inhibitory activity assays, and automated docking methods were used. The most active compound of the series was RG108-1 (Fig. 2). A binding model of RG108-1 with the crystal structure of bacterial M.HhaI suggested that this compound could be a covalent blocker of the catalytic cysteine. A more recent molecular modelling study using a model of human DNMT1 (Yoo and Medina-Franco, 2011) supported this hypothesis. Interestingly, in the model obtained with human DNMT, the maleimide moiety of RG108-1 interacts with Arg1311, Arg1461, and lies next to Cys1225, where the conjugate addition of the thiol group of the catalytic cysteine to the maleimide can occur. In addition, the carboxylate anion of RG108-1 overlaps with that of RG108 and

has the same interaction with Arg1311, Ser1229, and Gly1230 (Yoo and Medina-Franco, 2011).

The natural product mahanine (Fig. 2) has the ability to restore RASSF1A expression, and it is a potent inhibitor of androgen dependent (LNCaP) and androgen independent (PC-3) human prostate cancer cell proliferation (Jagadeesh et al., 2007). The antiproliferative activity of mahanine is associated with inhibition of the DNMT activity. Recently, fluorescent carbazole analogues of mahanine were designed and synthesized to find a novel and more potent small molecule with a mechanistic profile similar to that of the parent compound. Compound '9' in Fig. 2 inhibited human prostate cancer cell proliferation at 1.5 μM and also showed DNMT inhibition activity without the cytotoxic effects seen with mahanine treatment. Inhibition of DNMT was proposed as the event leading to the restoration of RASSF1A expression (Sheikh et al., 2010).

3.2 Structure-based optimization of hydralazine

Hydralazine, a potent arterial vasodilator, has been used for the management of hypertensive disorders and heart failure. Using a drug repurposing strategy (Duenas-Gonzalez et al., 2008), clinical trials have demonstrated the antitumor effect of the combination of hydralazine with valporic acid (a histone deacetylase inhibitor). Hydralazine and procainamide were first reported to have DNA methylation inhibition effect in 1988. Despite the fact that numerous studies were conducted with hydralazine, its molecular mechanism has remained unknown. In order to help understand the activity of hydralazine at the molecular level, we developed a binding mode of this compound with a validated homology model of the catalytic domain of DNMT1 using docking and molecular dynamics (Singh et al., 2009a; Yoo and Medina-Franco, 2011).

In molecular modeling studies, hydralazine showed similar interactions within the binding pocket as nucleoside analogues including a complex network of hydrogen bonds with arginine and glutamic acid residues that play a major role in the mechanism of DNA methylation (Yoo and Medina-Franco, 2011). Fig. 6 shows the comparison of the binding modes of hydralazine with 5-azacytidine. The amino group of hydralazine matched well with the amino group of 5-azacytidine, and it is capable of making hydrogen bonds with Glu1265 and Pro1223. The nitrogen of the phthalazine ring overlapped with the carbonyl oxygen of 5-azacytidine and formed hydrogen bonds with Arg1311 and Arg1461. We also identified that the small structure of hydralazine could not occupy the site of the sugar ring and phosphate backbone of nucleoside analogues. This result also suggests that hydralazine can be substituted at the C4 position to yield analogues with enhanced affinity with the enzyme. In contrast, there is a small empty pocket nearby the carbocyclic aromatic ring of hydralazine (C5-C8) that can be occupied by a substituent (Yoo and Medina-Franco, 2011).

The molecule shown in Fig. 6 was designed based on the structure and binding mode of hydralazine. Molecular modeling indicates that the addition of a phenyl group in the C4 position of hydralazine improves the calculated binding affinity with DNMT1. Moreover, adding polar substituents at various positions of the phenyl group can provide additional favorable interactions with the catalytic site. Also based on our molecular modeling analysis, the binding affinity is expected to increase by the addition of polar groups to the carbocyclic aromatic ring of phthalazine. It is *expected* that new compounds with increased calculated affinity with the enzyme will show increased potency in the DNMT1 enzyme inhibition assays.

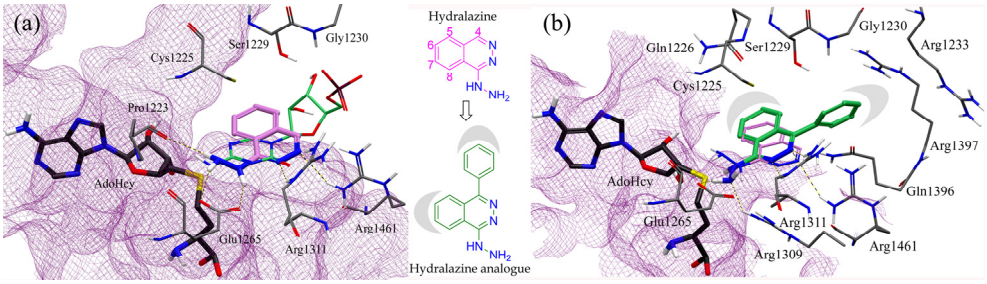

Fig. 6. Design of analogues of hydralazine. (a) comparison of the binding modes of hydralazine (carbon atoms in pink) with 5-azacytidine (carbon atoms in green) (b) structure-guided design of a representative hydralazine analogue (carbon atoms in green).

3.3 Design of focused combinatorial libraries

Computer-assisted combinatorial library design is a powerful tool frequently used in the discovery and optimization of new lead compounds. Molecular diversity has played a critical role in designing combinatorial libraries for screening (Tommasi and Cornella, 2006; Zheng and Johnson, 2008). However, the core chemical scaffolds of some currently used diverse libraries might be inadequate to provide drug-like compounds for new targets. Library design based on bioisosteric replacement or scaffold hopping methods can be used as an alternative to diversity oriented synthesis. Bioisostere searching involves swapping functional groups of a molecule with other functional groups that have similar biological properties. Scaffold hopping is an approach to discover structurally novel compounds starting from known active compounds by modifying the central core structure of the molecule (Brown and Jacoby, 2006). Scaffold hopping is an important drug design strategy to develop novel molecules with potent activity, altered physicochemical attributes, and Absorption, Distribution, Metabolism, Excretion and Toxicity –ADMET- properties. An example of application is phosphodiesterase 5 inhibitors for the treatment of erectile dysfunction. Sildenafil and vardenafil represent a case of heteroaromatic core scaffolds hopping with a small change in the scaffold (Jordan and Roughley, 2009). In contrast, tadalafil has a very different core scaffold, but it has the same biological activity. Computational design of focused libraries or compounds designed using any other strategy has to be in agreement with the experimental synthetic feasibility of the compounds proposed. Ideally, synthetic routes should follow short and easy steps.

Fig. 7 shows additional hydralazine analogues that have been proposed based on the knowledge gained in our previous molecular modeling studies of DNMT inhibitors. Starting from the 1-hydrazinyl-4-phenylphthalazine, polar groups are introduced into the carbocyclic aromatic ring of phthalazine. Based on molecular modeling analysis, substitution at ortho-, meta-, and para- position of the phenyl group with e.g., carboxyl, cyanide, and acetyl, showed a significant improvement in the calculated binding of the new compounds with DNMT1. A carboxyl group introduced into the ortho position plays a key role to make hydrogen bonds with Ser1229 or Gly1230. In contrast, substitution at C8 position of the phthalazine did not fit well into the catalytic site because of the narrow pocket size. Addition of polar groups to other positions slightly increases the predicted

binding affinity with the enzyme. Further chemical modifications to the structures of the lead DNMT inhibitors will be suggested toward the improvement of the *in vitro* and *in vivo* demethylating activity.

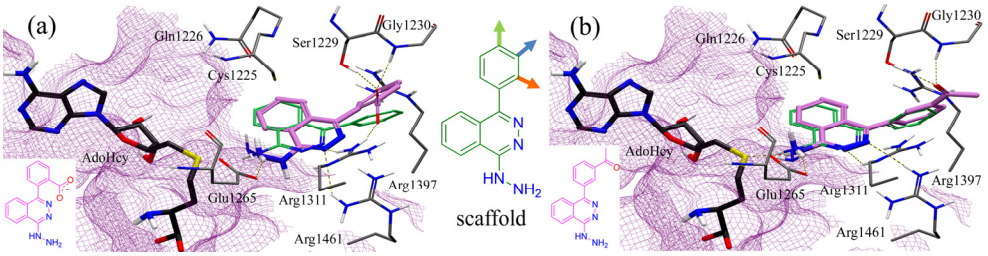

Fig. 7. Binding mode of hydralazine analogues (carbon atoms in pink) designed by scaffold hopping. The carbon atoms of new core scaffold are in green. Analogues with (a) ortho-carboxylate substitution and (b) meta-acetyl substitution on the phenyl moiety.

3.4 Characterization of structure-activity relationships

Currently, DNMT inhibitors have been screened in different assays using different conditions, and QSAR studies may not be reliable. However, once quality activity data has been gathered for several compounds assayed under comparable experimental conditions, it is feasible to conduct structure-activity relationships (SAR) of the compounds tested. When there is a significant amount of data, for example, activity data for more than 100 or 200 compounds, systematic analysis of the SAR can be performed via chemoinformatic approaches using the concept of "activity landscape modelling" (Wawer et al., 2010). The goal of activity landscape modeling of molecular data sets is to help rationalize the underlying SAR identifying key compounds and structural features for further exploration. The concept of activity landscape is strongly associated with the basic relationships between molecular structure and biological activity. While predictive SAR methods, such as pharmacophore modelling and traditional QSAR, focus on specific molecular descriptors or arrangements of substructures or functional groups associated with activity, descriptive activity landscape models rely on the "similarity property principle," i.e., similar structures should have similar biological properties (Bender and Glen, 2004; Maggiora and Shanmugasundaram, 2011) and employ whole-molecular similarity measures (Wawer et al., 2010). Systematic approaches to model activity landscapes and to detect "activity cliffs" using multiple representations are published elsewhere (Medina-Franco et al., 2009; Pérez-Villanueva et al., 2010; Pérez-Villanueva et al., 2011).

4. Conclusion

DNA methyltransferases are promising epigenetic targets for the treatment of cancer and other diseases. Clinical data demonstrates the potential of DNMT inhibitors for the therapeutic treatment of cancer. This is evidenced by the two DNMT inhibitors approved by the Food and Drug Administration of the United States for the treatment of patients with high-risk myelodysplastic syndrome. However, current approved drugs are nucleoside analogues that are not specific and still present issues such as cellular and clinical toxicity. A

wide range of computational approaches are being used to assist in the discovery and development of novel DNMT inhibitors. Molecular docking, pharmacophore modelling and molecular dynamics have been used to better understand the mechanism of action of established DNMT inhibitors; *in silico* screening of large compound libraries followed by experimental testing has been successful in identifying non-nucleoside inhibitors with novel chemical scaffolds; structure-based design is being used to guide the optimization of inhibitors such as hydralazine. Homology models of the catalytic domain of DNMT1 has played an important role to conduct the computational approaches that rely on the three dimensional structure of the target. It is expected that the recently published crystal structure of human DNMT1 bound to duplex DNA containing unmethylated CG sites will be the starting point of future structure-based studies with inhibitors of DNA methylation. It is also anticipated that the synergistic combination of computational approaches with combinatorial chemistry, and the systematic *in silico* and experimental screening of natural products will boost the discovery and optimization of inhibitors of DNMT for cancer therapy.

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Discussions with Dr. Fabian López-Vallejo and other members of the group are highly appreciated. Authors are also very grateful to Karen Gottwald for proofreading the chapter. This work was supported by the State of Florida, Executive Office of the Governor's Office of Tourism, Trade, and Economic Development. J.L.M-F. also thanks the Menopause & Women's Health Research Center for funding.

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2

Development of Novel Secondary Hormonal Therapies for Castrate-Resistant Prostate Cancer

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1. Introduction

Androgen deprivation therapy (ADT), initially via surgical orchiectomy and more contemporarily with medical castration through the use of luteinizing hormone-releasing hormone (LHRH) agonists, has been the mainstay of treatment for advanced prostate cancer for more than 60 years (Huggins and Hodges, 1941). Though initially effective in decreasing serum PSA, lessening pain from bone metastases, and delaying clinical progression, almost all men develop disease progression despite ADT within 2-3 years. Initially, this disease state was considered hormone-refractory and androgen-independent. However, more recent research has led to the understanding that many prostate cancers continue to depend on androgen receptor (AR) signalling in this state of low but still detectable circulating androgens. Thus, a more appropriate term for this disease state is castrate resistant prostate cancer (CRPC). In this chapter we will discuss the biology behind continued AR signalling in CRPC, traditional non-selective secondary hormonal therapies, and the development of novel secondary hormonal agents which selectively and potently target the AR axis in CRPC.

2. Androgen Receptor signalling in Castrate Resistant Prostate Cancer

In many cases, CRPC retains the ability to activate the AR to stimulate prostate cancer growth and progression, despite low circulating levels of testosterone induced by medical or surgical castration (i.e. less than 50 ng/dL). Various research efforts have sought to understand the mechanism through which this occurs, both as a means of understanding tumor biology and as a means of developing new targeted therapies exploiting the AR axis in CRPC. Signalling can be conceptually divided into efforts to understand ligand production and AR modification.

2.1 Ligand production in Castrate Resistant Prostate Cancer

2.1.1 Endocrine ligands

Current ADT strategies using LHRH agonists suppress gonadal androgen production, resulting in a decrease in circulating serum testosterone to castrate levels (less than 50 ng/dL). Despite gonadal androgen suppression, low levels of circulating androgens persist

in CRPC, often via production of adrenal androgens, such as dihydroepiandrostenedione (DHEA), DHEA-sulfate (DHEA-S), and androstenedione, which are then converted to testosterone in peripheral tissues. Figure 1 below displays the steroid biosynthetic pathway and several secondary hormonal agents which block various steps of steroidogenesis, to be discussed in the following sections.

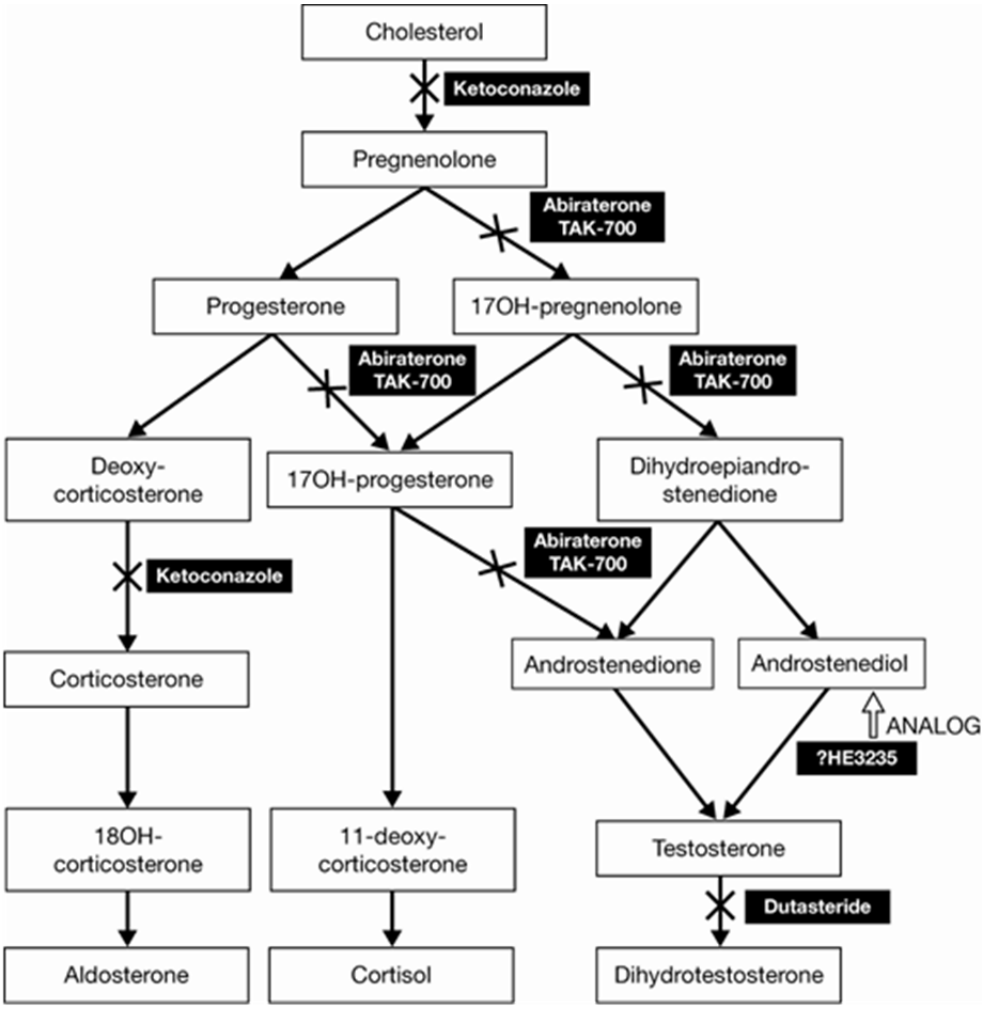

Fig. 1. Steroid Biosynthetic Pathway. Adapted from Aggarwal R and Ryan C, 2011.

Due to the peripheral conversion of adrenal steroids, low levels of circulating testosterone persists, and may account for levels up to 10% of that of pre-castrate levels (Puche, C et al. 2002). Low levels of circulating testosterone, along with circulating adrenal androgens, are hypothesized to subsequently stimulate CRPC progression through activation of the AR.

2.1.2 Intracrine ligands

More recently, research has shown that CRPC tissue has the ability to convert adrenal steroids to androgens, thereby creating an intracrine signalling system capable of converting steroid precursors to testosterone and dihydrotestosterone (DHT) which leads to continued stimulation of the AR and prostate cancer progression. The evidence for this comes from various lines of research. Direct measurements of intra-prostatic androgens including DHT demonstrates that levels of androgens in CRPC tissue is not significantly different compared with normal prostate tissue, despite significantly lower levels of circulating testosterone in the castrate men (Nishiyama T, et al. 2004). This finding implies that CRPC tissue acquires the ability to produce testosterone and DHT in an intracrine fashion, a finding which has been supported by further studies showing up-regulation of many of the steroid enzymes involved in androgen synthesis (see figure 1).

For example, Stanbrough et al. analysed oligonucleotide microarrays from 33 CRPC bone metastasis samples and compared their gene expression with samples from 22 hormone-sensitive primary cancers. The CRPC bone metastases demonstrated up-regulated expression of several enzymes involved in the steroid synthetic pathway: 17-beta hydroxysteroid dehydrogenase which converts androstenedione to testosterone; 3-beta hydroxysteroid dehydrogenase, which converts DHEA to androstenedione, and increased ratio of 5-alpha reductase isoform 1 to 2, which converts testosterone to DHT (Stanbrough M, et al 2006).

In a follow up study, Montgomery et al. evaluated androgen levels and transcripts encoding steroidogenic enzymes in benign prostate tissue, untreated primary prostate cancer, metastases from patients with castration-resistant prostate cancer, and xenografts derived from castration-resistant metastases. In this study, castrate-resistant tissues displayed increased expression of several key enzymes involved in androgen synthesis, including: CYP17A1 (C17,20 lyase), a key enzyme which converts progesterone and pregnenolone to 17-hydroxyprogesterone and 17-OH pregnenolone, as well as subsequent conversion of these steroids to androstenedione and DHEA respectively; 3-beta hydroxysteroid dehydrogenase as in the prior study. Furthermore, metastatic prostate cancers from CRPC patient samples express transcripts encoding androgen-synthesizing enzymes and maintain intratumoral androgens at concentrations capable of activating AR target genes and maintaining tumor cell survival in a xenograft model (Montgomery R et al, 2008). Finally, in an innovative study by Locke et al., it was demonstrated that tumor explants isolated from CRPC progression are capable of de novo conversion of [14C] acetic acid to dihydrotestosterone and that uptake of [3H] progesterone allows detection of the production of six other steroids upstream of dihydrotestosterone.

This cumulative body of evidence suggests that CRPCs are capable of adapting to lower circulating levels of androgens induced by castration, in which steroid enzymes involved in the synthetic pathway are upregulated, and thereby maintain high levels of intra-tumor androgens capable of stimulating the AR and driving prostate cancer progression. Understanding this mechanism of castration resistance has led to the development of targeted secondary hormonal therapies which specifically inhibit key enzymes of the androgen synthetic pathway, as will be discussed in the later section.

2.2 Androgen Receptor modification in Castrate Resistant Prostate Cancer

In addition to modification in the enzymes involved in steroid hormone production, the AR itself undergoes adaptation in the castrate state, and is implicated in disease progression to

CRPC. Mechanisms by which the AR adapts to the castrate state have been extensively studied in the past several decades, and include: (1) AR amplification and overexpression (2) heightened AR sensitivity to ligand activation through increased AR stabilization, enhanced nuclear localization, and overexpression of nuclear co-activators (3) increased AR promiscuity through various point mutations (4) ligand-independent activation of the AR through various signal transduction pathways, (5) AR splice variants with constitutive activity. In the following sections we will examine some of the evidence behind these modifications to the AR.

2.2.1 Androgen Receptor gene amplification and overexpression

In the late 1990s, research was starting to show that AR activation continued to play an important role in prostate cancer progression despite low circulating testosterone levels, and a potential mechanism through which this might occur was AR gene amplification and overexpression. In a study by Koivisto, et al, AR gene amplification was analyzed in 54 patient tumor samples at the time of recurrence after prior therapy, as well as in 26 cases, paired primary tumor samples prior to any therapy. In this study, 28% of the recurrent therapy-resistant tumors, versus none of the primary tumor samples, displayed AR gene amplification. Furthermore, through genomic analysis, the AR gene was wild type in all but one of the 15 AR gene amplified tumor samples. Interestingly, this study went on to show a clinicopathologic correlation between AR gene amplification and prior responsiveness to ADT, as well as improved subsequent prognosis (Koivisto P et al. 1997). In a follow up study by Linja et al., in which real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to analyze AR expression levels in eight benign prostate hyperplasias, 33 untreated and 13 castrate-resistant locally recurrent carcinomas, as well as 10 prostate cancer xenografts. All castrate-resistant tumors showed on average, 6-fold higher expression than androgen-dependent tumors or benign prostate hyperplasias ($P < 0.001$). Four of 13 (31%) castrate-resistant tumors contained AR gene amplification detected by fluorescence in situ hybridization. Finally, and equally as important, two of the ten prostate cancer xenograft models displayed AR overexpression, thus providing a key model for testing future drugs targeting the AR in the AR-amplified disease state (Linja MJ, et al. 2001).

Early studies such as these provided compelling evidence that AR gene amplification and thus overexpression may represent an important mechanism by which prostate cancers overcome low circulating androgen levels. Given this, a logical therapeutic strategy is the development of potent AR antagonists which would have activity in this AR-amplified disease state, and indeed, there are several novel potent, AR antagonists which are in clinical phase of drug development (see section below).

2.2.2 Androgen Receptor stabilization and heightened activity

In addition to numerical increase in the number of ARs per cancer cell, increased stabilization and nuclear localization of the AR may also factor into the mechanism of prostate cancer progression in the castrate resistant disease state. In a prior study by Gregory et al, recurrent prostate cancer cell lines had an AR degradation half-life that was 2-4 times longer than that of androgen-sensitive cancer cell lines. Furthermore, IHC staining showing that AR localization was entirely nuclear in the recurrent cancer cell lines; while localizing to both the cytoplasm and nucleus in the androgen sensitive cell lines (Gregory

CW, et al. 2001). This data suggests that AR activation and subsequent AR-mediated gene expression may in part be stimulated in CRPCs by mechanisms to prevent AR degradation and enhance localization to the nucleus. The mechanism of AR stabilization in CRPCs may in part related to increased cyclin-dependent kinase 1, which has been shown to phosphorylate and stabilize the AR and is also upregulated in castrate-resistant cell lines in prior pre-clinical study (Chen S, et al. 2006).

2.2.3 Androgen Receptor point mutations

Estimating the true frequency of acquired point mutations with functional significance in advanced prostate cancer has been difficult, due to various factors including patient selection, tumor heterogeneity, tissue source (prostate gland v metastases), method of tissue preservation, and molecular methods. They appear to be fairly uncommon in early prostate cancer and more prevalent in advanced prostate cancer. In a correlative analysis of bone marrow samples from patients with CRPC being treated with first generation anti-androgen withdrawal (CALGB study 9663), 10% of the patient samples had an AR point mutation, which was found within the hormone binding domain involved with transcription factor binding (Taplin M, et al. 2003). From a functional standpoint, it appears that certain AR point mutations lead to a more promiscuous AR, capable of being activated by a wider range of ligands. In a prior study of a mutant AR transfected into various cell lines, the adrenal androgen DHEA was capable of inducing greater AR-mediated transcriptional activity in the mutant AR cell line compared with wild type AR (Tan J, et al. 1997).

In this way, the increase in AR promiscuity may complement the changes in ligand production as outlined in the previous section, in which point mutations in the AR confer a greater ability for the AR to be activated by alternative ligands in the presence of low circulating testosterone levels, including the adrenal androgens such as DHEA. Mutations in the AR may also lead to partial agonistic activity of the first generation anti-androgens, such as flutamide, nilutamide, and bicalutamide, as will be discussed in the following section.

2.2.4 Ligand-independent activation of the Androgen Receptor

There is a wide-ranging body of evidence which suggests that for a subset of prostate cancers, ligand-independent activation of the AR, via activation from other signal transduction pathways, can independently activate the AR and lead to disease progression in the absence of hormone binding to the AR. Though not the focus of the current book chapter, the various signaling pathways that have been implicated in such a manner include the insulin-like growth factor pathway, epidermal growth factor receptor, and keratinocyte growth factor pathways, among others (Feldman B & Feldman D, 2001).

2.2.5 Androgen Receptor splice variants

Over the past several years a growing body of research implicates the generation of AR splice variants as a potential mechanism of driving disease progression to CRPC. Such AR splice variants have “hidden exons” within introns that are not normally transcribed in the wild type AR. The alternate splicing that incorporates such hidden exons into the variant mRNA transcripts creates pre-mature stop codons prior to the translation of the C-terminal ligand binding domain. Thus, variant AR proteins are created which lack the traditional ligand binding domain (see figure 2 below). In a seminal paper by Hu et al. prostate cancer tissue from primary hormone-sensitive and metastatic CRPC cancer tissue was analyzed by

in silico DNA sequencing for the presence of AR splice variants. In total, 7 variant AR transcripts were discovered, AR-V1 through AR-V7. The two most abundantly expressed were AR-V1 and AR-V7. On average, there was 20-fold higher expression of these two variant transcripts in CRPC as opposed to hormone-sensitive prostate cancer. Functionally, AR-V7 was found to localize to the nucleus of prostate cancer cell line under androgen depleted conditions, and most importantly, was constitutively active in driving the expression of androgen-responsive genes (Hu, R, et al. 2009).

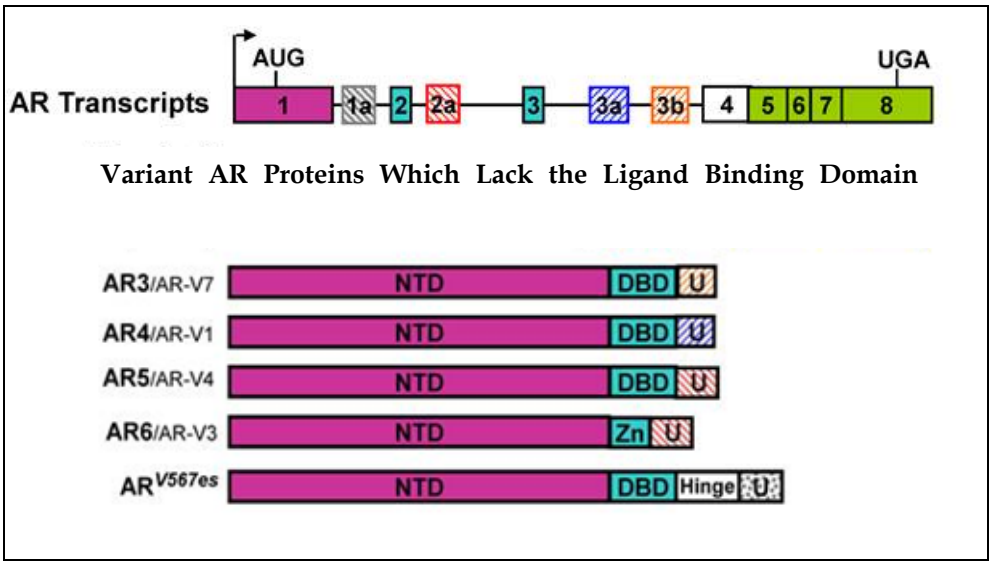

Fig. 2. Androgen Receptor Transcript and Splice Variants. NTD = N terminal domain. DBD = DNA binding domain. The hatched areas represent "hidden" exons spliced into the DNA biding domain exons (2 and 3), thus creating variant AR transcripts. The hidden exons of the variant AR transcripts encode stop codons, leading to premature termination and exclusion of the C-terminal ligand binding domain (exons 5-8 in green). Figure adapted from Guo, Z & Qiu, Y, 2011.

The exciting discovery of AR splice variants represents another potential mechanism by which cancer cells modify AR processing to adapt to a low circulating testosterone environment, creating AR splice variants which are not dependent on hormone binding to drive gene expression and cancer cell division and metastasis. Targeting the variant AR proteins, perhaps at the more ubiquitous N-terminal domain, represents a potential therapeutic approach to overcome this mechanism of resistance.

3. Traditional secondary hormonal therapies for Castrate Resistant Prostate Cancer

Traditional hormonal manipulations can be of some benefit to patients with CRPC; however significant responses are not seen in the majority of patients, and responses tend to be short-lived. Furthermore, the response duration and magnitude of benefit tend to diminish with

each successive hormonal manipulation. Chemotherapy has traditionally been the mainstay of treatment for CRPC patients who have failed secondary hormonal therapy; however the median increase in overall survival with first line docetaxel chemotherapy is a modest 3 months, and fewer than 20% of patients with CRPC live beyond 3 years (Tannock, et al. 2004; Petyrlak DP, et al. 2004). Clearly, novel therapies are needed which applied together or in succession can lead to meaningful improvement in the quality and quantity of time for patients with CRPC. In the following sections we will first discuss the traditional secondary hormonal agents which have been used to treat CRPC. We will then continue onwards with a discussion of the novel secondary hormonal therapies currently in clinical development, which more selectively and potently inhibit either steroid ligand production or AR activation.

3.1 First generation antiandrogens

First generation antiandrogens, which competitively inhibit the binding of androgens to the ligand binding doman of the AR, remain in widespread use in the treatment of prostate cancer of various disease stages. The addition of a first generation antiandrogen (i.e. flutamide, nilutamide, or bicalutamide) to medical castration (combined androgen blockade) demonstrates only modest benefits in the hormone-sensitive disease population, with a small absolute survival benefit of less than 5% in most studies and meta-analyses. Similarly, the addition of an anti-androgen after ADT fails has demonstrated only modest benefit in prior clinical studies. In a prior clinical trial of flutamide 250 mg orally three times daily versus prednisone 5 mg orally four times per day, the median time to symptomatic progression on flutamide was only 2.3 months (as compared to 3.4 months with prednisone), and the proportion of patients with a greater than 50% decline in PSA or greater was 23% in the flutamide group vs. 21% in the prednisone group (Fossa SD, et al. 2001).

Similar rates of biochemical response were noted in a trial of 232 men who received either flutamide 375 mg/day or bicalutamide 80 mg/day after disease progression on combined androgen blockade. The percentage of men with a greater than 50% decline in PSA was 35.8%; the response duration was a little over 6 months (Suzuki H, et al. 2008). In another small trial of 31 men with CRPC treated with high dose bicalutamide 150 mg/day, only 22.5% of men had a PSA decline of > 50% for more than 2 months, almost all in men without prior treatment with flutamide (Joyce R, et al. 1998).

The modest efficacy and limited duration of response of first generation anti-androgens may in part be due to the fact that these molecules can act as partial agonists of the AR, especially AR which develop point mutations as a mechanism of resistance to these anti-androgens. Clinically, this partial agonist effect is observed with the phenomenon of anti-androgen withdrawal, a therapeutic maneuver in which the anti-androgen is discontinued in a patient who is progressing despite combined androgen blockade. In a prior study of anti-androgen withdrawal, 11% of patients demonstrated a decline of 50% or more in serum PSA after anti-androgen withdrawal (Small E, et al. 2004). Presumably, in these small subsets of patients who respond to antiandrogen withdrawal, the AR may have developed mutations which confer the ability to be activated by the antiandrogen.

Novel second generation antiandrogens which lack any agonist activity against the AR and demonstrate markedly more potent AR inhibition, including MDV-3100, will be discussed in the upcoming section.

3.2 Estrogens

Estrogens have long known to have been active in the treatment of prostate cancer; however the exact mechanism of actions remains uncertain. Putative mechanisms include inhibition of LH hormone release from the pituitary gland, inhibition of adrenal androgen production, and a direct cytotoxic effect on prostate cancer cells (Robertson CN, et al. 1996). In a prior phase randomized phase II trial comparing the estrogenic herbal compound PC-SPES with diethylstilbestrol, a greater than 50% decline in baseline PSA was noted in 40% and 24% of patients respectively; median time to progression was 5.5 vs. 2.9 months respectively (Oh W, et al. 2004).

There is clearly a modest degree of activity of estrogenic compounds in the treatment of CRPC; however current use of these agents (i.e. diethylstilbestrol, Premarin, etc.) is limited by the small but not insignificant risk of venous thromboembolic events and possibly increased risk of myocardial infarction and stroke; these particular co-morbidities are especially concerning in a disease population of elderly men. Concomitant prophylactic anticoagulation is recommended when using these agents.

3.3 Ketoconazole

Ketoconazole is a broad, non-specific inhibitor of multiple cytochrome p450 enzymes involved in androgen biosynthesis, including the conversion of cholesterol to pregnenolone, 11-beta hydroxylation, and 17-alpha hydroxylase/C17, 20 lyase (CYP17) activity. In a previously referred to randomized phase II study of 260 men with CRPC, with progressive disease despite combined androgen blockade, randomized to treatment with antiandrogen withdrawal alone or in combination with ketoconazole, 27% of patients assigned to the ketoconazole arm had a 50% or greater decline in serum PSA level, and 20% of patients had an objective response (Small EJ, et al. 2004). Interestingly, at the time of disease progression on ketoconazole, levels of adrenal androgens including DHEA, DHEA-S, and androstenedione had all increased compared to month 1 levels, which suggest that ketoconazole resistance may in part reflect inadequate androgen production suppression. This mechanism of resistance has implications for the development of novel androgen synthetic enzyme inhibitors such as abiraterone acetate. In an intriguing analysis of adrenal androgen hormone levels from the study by Small et al., patients who had higher baseline levels of androstenedione had a higher likelihood of response to treatment with ketoconazole (Ryan CJ, et al. 2007). This suggests that baseline adrenal androgen levels may be used as predictive biomarker for the use of adrenal androgen blockade as a therapeutic maneuver for CRPC; however this hypothesis requires prospective validation in larger studies.

Ketoconazole is a relatively non-specific inhibitor of multiple enzymes involved in the steroid synthetic pathway, and as such, as blocks normal corticosteroid production and causes iatrogenic adrenal insufficiency. Accordingly, side effects of this medication include lethargy, rash, nausea, and liver toxicity. Supplementation with physiologic replacement doses of hydrocortisone (i.e. 20 mg in the morning, 10 mg in the evening) is required while patients are taking ketoconazole. Furthermore, given the relatively non-specific CYPP450 inhibition, ketoconazole interacts with a wide number of other medications. Its oral absorption and bioavailability can be variable, depending on the acidity of the stomach and fed/fasting state and use of acid suppressing medications.

Despite these potential side effects and drug interactions, ketoconazole represents a viable and widely used secondary hormonal agent for CRPC, especially in the patient population

with asymptomatic or minimally symptomatic bone-only metastatic or rising PSA-only disease.

4. Novel secondary hormonal therapies for Castrate Resistant Prostate Cancer

Insights into the mechanisms of continued AR signaling in CRPC, as discussed above, including (1) adrenal and intra-tumoral androgen ligand production, and (2) modifications of the AR, including gene amplification, over-expression, point mutations, ligand-independent activation, and splice variants, have led to the development of novel secondary hormonal therapies for CRPC. These new therapies are more selective and potent than their traditional counterparts. In the following subsections we will discuss the clinical development of several of the new hormonal therapies for CRPC.

4.1 Selective inhibition of CYP17

As displayed in figure 1, CYP17 (17-alpha hydroxylase/C17, 20 lyase) catalyzes two key steps of androgen synthesis within the steroid biosynthetic pathway: the 17-hydroxylation of progesterone and pregnenolone and subsequent conversion to DHEA and androstenedione respectively. Inhibition of this enzyme would divert cholesterol derivatives away from androgen production, and towards mineralocorticoid production (corticosterone and aldosterone). As outlined above, intra-tumoral upregulation of CYP17 has been previously implicated in the progression to CRPC. Logically then, selective inhibition of CYP17 represents an attractive strategy for inhibiting adrenal and intra-tumor androgen production in CRPCs and thereby slowing disease progression.

4.1.1 Abiraterone acetate

Abiraterone acetate is the prodrug of abiraterone, a potent, highly selective, irreversible inhibitor of CYP17. In pre-clinical in vivo study using WHT mice, this compound was able to markedly decrease the level of serum testosterone to less than 0.1 nanomolar concentration, despite 3-4 fold increase in serum LH concentration (Barrie SE, et al. 1994). In the first phase 1 study of abiraterone, O'Donnell et al. studied various dosing schedules ranging from 10 to 500 mg x 1 dose in castrate resistant men. At a dose level of 500 mg, there was suppression of serum testosterone to less than lower limit of detection (< 0.14 nmol/L) with parallel reduction androstenedione levels, supporting its mechanism of action of CYP17 inhibition. The duration of testosterone suppression after a single dose was variable, but generally ranged from days 2-5 post-dose (O'Donnell A, et al. 2004). In a follow up phase I trial by Attard and colleagues, 21 patients with CRPC and progression through multiple prior traditional secondary hormonal therapies were treated with abiraterone with doses ranging from 250 mg to 2000 mg/day. Pharmacodynamic effects on serum hormone levels showed a plateau at a dose of 1000 mg/day, which was the dose level of an expanded cohort of 9 patients and the subsequent recommended phase II/III dose. There were no treatment-related grade 3 or 4 adverse events from this trial. As expected, increases in levels of ACTH, corticosterone, and 11-deoxycorticosterone were observed, and there were adverse events related to subsequent mineralocorticoid excess, namely hypokalemia and hypertension. This was effectively managed with the use of eplerenone, a mineralocorticoid antagonist. The median baseline serum testosterone level was 7 ng/mL in this study; at all

dose levels serum testosterone was decreased to < 1 ng/mL within 8 days of treatment initiation.

In a separate phase I dose escalation study of abiraterone acetate in 33 men, including 19 with prior ketoconazole treatment, daily dosing from 250 mg to 1000 mg was well tolerated with no dose-limiting toxicities (DLTs) (Ryan CJ, et al. 2010). Hypertension and hyperkalemia, signs of mineralocorticoid excess, as might be expected by the mechanism of action, were the most common serious toxicities (grade 3 or higher 12% and 9% respectively), which responded to medical management including low dose corticosteroids or mineralocorticoid receptor antagonists such as eplerenone. Spironolactone was avoided given its potential androgenic properties. Overall, 55% of patients in this study had a confirmed 50% or greater decline in serum PSA level at 12 weeks. In the subset of 19 patients with prior ketoconazole exposure, 46% had a greater than or equal to 50% decline in serum PSA at 12 weeks. Importantly, this data suggests that CRPCs which are resistant to ketoconazole may still be sensitive to the effects of abiraterone, which is a more potent and selective inhibitor of androgen synthesis compared to ketoconazole. In contrast to prior studies of ketoconazole in CRPC, in which adrenal androgens levels rose at the time of disease progression, serum hormone levels including testosterone and DHEA-S did not rise at the time of disease progression on abiraterone. This data suggests the mechanism of resistance to abiraterone may be unrelated to a rise in androgen levels. The phase II portion of this study included added prednisone 5 mg orally twice daily, and excluded patients with prior chemotherapy or ketoconazole (Ryan CJ, et al. 2009). Preliminary results indicated a 50% or greater decrease in PSA in 88% of patients; median time to PSA progression was 337 days.

Subsequent various phase II studies have evaluated abiraterone as monotherapy and combined with low dose prednisone in men with CRPC and prior docetaxel chemotherapy. In a two stage phase II trial by Reid and colleagues of 47 men with CRPC and previous treatment with docetaxel, treated with abiraterone 1000 mg/day monotherapy, 51% of patients demonstrated a 50% or greater decline in serum PSA level. Furthermore, the median time to PSA progression was 169 days; the objective response rate was 28% among men with measurable disease at baseline. 8 patients had prior ketoconazole treatment; all but one had prior treatment with a first generation antiandrogen. Adverse events were as expected due to secondary mineralocorticoid excess, including 55% with hypokalemia, 17% with hypertension, and 15% with fluid retention. In a phase II trial of abiraterone 1000 mg/day + prednisone 5 mg twice daily in 58 men with CRPC and prior docetaxel treatment, a confirmed ≥ 50% decline in PSA was observed in 36% of patients, including 27% of patients with prior ketoconazole treatment (Danila DC, et al. 2010). The median time to PSA progression was 169 days. The addition of prednisone decrease the incidence of clinical mineralocorticoid excess, and no patients required treatment with eplerenone while on study.

Results of the follow up confirmatory randomized phase III trial of abiraterone in the post-docetaxel CRPC population were recently reported (de Bono JS, et al. 2011). In this trial, 1195 patients with CRPC and prior docetaxel were randomized in a 2:1 fashion to receive either the combination of abiraterone 1000 mg/day + prednisone 5 mg twice daily versus placebo + prednisone 5 mg twice daily. After a median follow up of 12.8 months, overall survival was longer in the abiraterone group vs. the placebo group (median overall survival of 14.8 vs. 10.9 months; HR = 0.65, $p < 0.0001$). The data was unblinded at the time of interim analysis, as the results exceeded the pre-planned stopping rule for efficacy. All secondary

endpoints, including progression-free survival, objective response rate, and PSA response rate favored the abiraterone treatment arm. Hypokalemia was noted in 17% of abiraterone group patients, and 10% of patients experienced hypertension of any grade severity. As a result of the overall survival benefit demonstrated in this phase III trial, abiraterone acetate was granted FDA approval on April 28th, 2011 for use in men with metastatic CRPC who had received prior chemotherapy containing docetaxel. An ongoing phase III trial of prednisone with or without abiraterone in men with metastatic CRPC without prior chemotherapy has finished accrual; study results are expected within the next year (NCT00887198).

The drug development of abiraterone acetate has unfolded rapidly over the past decade, based on a strong scientific rationale, pre-clinical and early clinical phase data indicating potent blockade of CYP17, a rational phase II/III dose selection, and the selection of clinically relevant endpoints for confirmatory phase III trials. Development of this drug remains ongoing, and many questions remain to be answered, including: (1) mechanisms of abiraterone resistance (2) optimal sequencing in the therapy of men with CRPC (e.g. before or after docetaxel?) (3) potential combination with other secondary hormonal agents (4) activity in patients with prior ketoconazole (patients treated with ketoconazole were excluded from the above mentioned phase III trials) (5) population pharmacokinetic analysis, and (6) development of predictive biomarkers that might allow for individualized patient selection for those most likely to benefit from abiraterone. This last issue is likely to become increasingly more relevant in an era of rising medical costs and the choice of multiple new agents for the treatment of CRPC. Preliminary data suggests that patients with higher levels of baseline adrenal androgen levels are more likely to respond to abiraterone, similar to the results obtained with prior studies of ketoconazole (Logothetis CJ, et al. 2008).

4.1.2 TAK-700 and TOK-001

Orteronel (TAK-700) is a selective CYP17 inhibitor which has reached clinical development in CRPC. Preliminary phase 1 data of 26 men with CRPC treated with dose levels ranging from 100 through 600 mg twice daily as well as 400 mg twice daily + low dose prednisone were recently presented (Dreicer R, et al. 2010). No dose limiting toxicities were seen. Fatigue was the most common adverse event, seen in 62% of patients, including 3 patients with grade 3 fatigue at the 600 mg twice daily dose. Other common adverse events included nausea, vomiting, anorexia, and constipation. Doses at or above 300 mg twice daily produced a 50% or greater decline in PSA in 70% of patients, of whom 29% had an impressive > 90% decline in serum PSA. Phase 3 trials of orteronel in men with metastatic CRPC pre and post docetaxel are ongoing (NCT01193244 and NCT01193257 respectively).

TOK-100, in a pre-clinical model, selectively inhibits CYP17 enzymatic activity and down regulates AR expression. In the LAPC4 xenograft model, TOK-100 combined with castration inhibited tumor growth and down-regulated AR expression, in contrast to treatment with castration or bicalutamide alone, in which AR expression was up-regulated (Vasaitis T, et al. 2008). Phase I/II trials of TOK-001 are underway in CRPC. The potential for down regulation of AR expression in addition to CYP17 inhibition may lead to more potent suppression of AR-mediated disease progression in CRPC, a hypothesis that warrants testing in current and future clinical trials of this compound.

4.2 Selective and potent inhibition of the Androgen Receptor

AR gene amplification and over-expression appears to be a relatively common phenomenon as tumors adapt to a low circulating testosterone environment, and may lead to progression to CRPC. First generation AR antagonists such as flutamide or bicalutamide inhibit ligand binding to the AR and thereby decrease nuclear localization and activation of AR-mediated gene expression. However, in the AR-amplified state, the first generation antiandrogens may not block the AR in a potent enough manner to block ligand-mediated AR activation. Furthermore, acquired point mutations in the AR may cause first generation antiandrogens to exhibit partial agonistic activity towards the AR, as supported by the clinical phenomenon of response to antiandrogen withdrawal. Pre-clinically, first generation antiandrogens exhibit partial agonist activity towards the AR in prostate cancer cell lines engineered to expression higher amounts of AR. More potent AR antagonists, which are capable of inhibiting the AR even in cells with AR overexpression, and do not possess any agonistic activity towards the AR, would be highly desirable as a hormonal therapy in CRPC, a potentially AR-amplified disease state.

4.2.1 MDV3100

MDV3100 was developed pre-clinically in an iterative screening process of various compounds that retain AR antagonistic activity in an AR-overexpressed cell line. MDV3100 binds to AR with 5-8 fold greater affinity compared to the first generation antiandrogen bicalutamide (Tran C, et al. 2009), impairs AR nuclear translocation, and inhibits AR binding to DNA, and blocks binding of AR to co-activators to a greater degree than bicalutamide. In tumor xenograft models known to overexpress AR, treatment with MDV3100 led to substantial tumor shrinkage.

MDV3100 was studied in a phase I/II clinical trial of 140 men with CRPC, including 45% of patients with prior ketoconazole and 54% with prior chemotherapy, in doses ranging from 30 mg to 600 mg/day. The maximum tolerated dose for ≥ 28 days was 240 mg/day. At doses of 360 mg/day and higher, 13% of patients discontinued treatment due to an adverse event, including three patients with seizures and one patient with a myocardial infarction. In contrast, at doses of 240 mg/day or lower, 1% of patients (1 out of 87 patients) discontinued treatment due to an adverse event. The most common grade 3 or higher dose-limiting toxicity was fatigue, which generally resolved with dose reduction. Anti-tumor activity was noted at all dose levels. In total, 56% of patients showed a 50% or greater reduction in serum PSA level; 22% of patients had an objective radiographic response among those with measurable disease at baseline, and conversion from unfavorable to favorable circulating tumor cell (CTC) count in 49% of patients. Similar PSA response rates were seen in patients with and without prior chemotherapy, though among patients with prior ketoconazole exposure, there was a lower percentage of patients with a 50% or greater decline in serum PSA (37% vs. 71% for those with and without prior ketoconazole respectively). The median time to radiographic progression was 47 weeks. Based on the encouraging results of this phase I/II clinical trial, ongoing phase III trials of MDV3100 vs. placebo, at a dose of 160 mg/day, are ongoing in patients with metastatic CRPC with and without prior docetaxel treatment (NCT00974311 and NCT01212991 respectively).

4.2.2 Other Androgen Receptor antagonists

Several other second generation, highly potent, pure AR antagonists have reached clinical development in CRPC. BMS-641988 is a highly potent AR inhibitor was designed based on

AR crystal structure. In pre-clinical study, this AR antagonist showed a > 1 log increase in potency of AR inhibition compared with bicalutamide, both in regards to AR binding and inhibition of AR-mediated gene expression (Attar RM, et al. 2009). Furthermore, in a human xenograft model, BMS-6419888 displayed greater growth inhibition compared with bicalutamide. Based on the encouraging pre-clinical data, this compound was subsequently tested in a phase I dose escalation study (Rathkopf D, et al. 2010). In this trial, doses of BMS-6419888 were escalated from 5 mg to 150 mg. In total, 61 men were treated. One patient experienced an epileptic seizure at a dose of 60 mg twice daily. Antitumor activity was limited to one partial response, and partial agonism was seen as evidenced by a decrease in serum PSA upon drug withdrawal. Based the limited anti-tumor activity despite achieving target therapeutic levels, as well as the epileptic seizure, the study was closed prematurely and further clinical development of this compound discontinued.

ARN-509 is a potent AR antagonist in the early phases of clinical development. It inhibits AR nuclear translocation and DNA binding, thereby modulating expression of genes which drive prostate cancer growth. It is currently being tested in a phase I/II clinical trial of men with metastatic CRPC with up to two prior chemotherapy regimens (NCT01171898). The primary endpoint is maximum tolerated dose; secondary endpoints include change in PSA, number of new bone lesions, and objective response by RECIST criteria. Enrollment began in July of 2010 and results are expected in 2012. Likely due to the several seizure events during prior clinical trials of MDV3100 and BMS-6419888, patients with a history of seizures or potentially lower seizure threshold are excluded from this phase I/II trial of ARN-509.

5. Future directions

The clinical activity of the novel secondary hormonal therapies which attack the AR axis continues to lend credence to the now widely held hypothesis that continued activation of the AR plays an important role in the progression of disease to CRPC and ultimately to prostate cancer death. Much progress has been made over the past several decades in the drug development of secondary hormonal therapies for CRPC. However, there are many questions that remain yet to be answered, including: (1) optimal timing and sequence of hormonal therapies in relation to chemotherapy and each other (2) relative risks and benefits of combination versus sequential hormonal monotherapy (3) mechanisms of resistance (4) patterns of disease progression on these novel therapies (5) potential predictive biomarkers to help individualize patient therapy, including the molecular characterization of circulating tumor cells (6) pharmacokinetic studies across various study populations and ethnicities (7) pharmacogenomics analysis of potential associations between germ line mutations and response (8) long term safety data, and (9) optimal phase II/III clinical trial endpoints to assess efficacy of these agents, including the potential use of surrogate markers such as change in number of circulating tumor cells.

Furthermore, there are new treatment strategies which target the AR axis that are in the infancy of drug discovery and development. Among them is EPI-001, a compound which inhibits transactivation of the N-terminal domain of the AR, without interacting with the AR ligand-binding doman, and thus may serve as a potential inhibitor of the AR splice variants that are hypothesized to play a role in the resistance to androgen ablation therapy (Andersen et al, 2010). Additionally, inhibitors of heat shock proteins, which act to stabilize the AR among other proteins, are also in clinical development.

6. Conclusions

AR activation continues to play a role in the progression of CRPC, despite low circulating serum testosterone levels in this disease state. This is accomplished through endocrine ligand production via adrenal androgen synthesis, intracrine ligand formation via up-regulation of the enzymes involved in androgen synthesis, including CYP17, AR overexpression and point mutations which confer receptor promiscuity and promote agonistic activity of traditional antiandrogen therapy, ligand-independent AR activation, and generation of constitutively active AR splice variants, among others. Pre-clinical drug discovery and development targeting specific steps in these mechanisms has led to the clinical development of numerous secondary hormonal agents which specifically and potently target the AR axis. Ongoing research is directed at optimizing and personalizing the use of the current novel secondary hormonal therapies as well as developing new therapeutic strategies to overcome treatment resistance in CRPC.

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3

Inhibitors of Proteinases as Potential Anti-Cancer Agents

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1. Introduction

Cancer is a collection of over 100 devastating diseases that share a number of characteristics, a primary hallmark of which is out-of-control growth. However, in reality there are significant differences among these diseases, a fact that underlies the difficulties in the past few decades in their chemotherapeutic intervention. It is becoming evident that there are multiple routes to development of cancer, in part because so many distinct metabolic and biochemical steps can be altered to give rise to uncontrolled cell growth.

There is a positive correlation between the aggressiveness of a tumor and the secretion of various proteinases. Using bioinformatic analysis approximately 600 proteinases have been determined in human and mouse genomes (2-4% of the genome), many of which are orthologous (Puente et al., 2003). Only some of them are involved in tumor progression and growth, both at the primary and metastatic sites. As tumor progresses towards increased malignancy, it passes through several important stages that require the action of proteinases. First, the induction of angiogenesis requires degradation of the vascular basement membrane and the release of matrix-bound proangiogenic growth factors. Second, invasion of cancer cells into the surrounding tissue involves the dissolution of cell-cell junctions, degradation of the epithelial basement membrane and remodeling of extracellular matrix to allow cancer cells to be released from the primary tumor mass. Third, at least two key steps in metastasis require proteolysis: intravasation of cancer cells into the blood or lymphatic circulation at the primary site and then extravasation at the secondary site, where proteinases can play a part in promoting the colonization and growth of cancer. Proteinases may co-operatively mediate these steps with individual ones having distinct roles. Therefore, inhibition of their activity might be one of the means to combat the development of cancer. Despite of the described facts, recent findings have revealed that the functions of proteinases in tumors are significantly more complex and varied. For example, they are now seen as extremely important signaling molecules that are involved in numerous vital processes. Proteinase signaling pathways are strictly regulated, and the deregulation of their activities can lead to various pathologies, including cancer. Thus, construction of the inhibitor, which should have an impact on tumor progression and metastasis, cannot be done without placing certain proteinase in the proper metabolic context. Inhibitor therapy design is further complicated because different types of cancers utilize diverse proteinases at varying stages of cancer development.

Through the evolution, proteinases have adapted to the wide range of conditions found in human organism (variations in pH, reductive environment and so on) and they use several catalytic mechanisms for substrate hydrolysis. Basing on the chemical mechanism of their action human proteinases may be classified as: cysteine, serine, threonine, aspartic acid and metallo proteinases. In most cases specific inhibitors for each class of these enzymes are being designed.
A number of reviews on various aspects of the use of proteinase inhibitors as a mean to combat cancer have been published recently (Castro-Guillen et el., 2010; Lee et al., 2004; Magdolen et al., 2002; Pandey et al., 2007; Puxbaum & Mach, 2009; Turk, 2006). Therefore, in this review the current trends in designing of such inhibitors will be presented. Special emphasis will be put on rational design using the techniques which are based either on the knowledge of detailed mechanism of enzymatic catalysis or on three-dimensional structure of active sites of chosen enzymes. Indeed, several small-molecule drugs targeting proteinases obtained in that manner are already on the market and many more are in development.

2. Cysteine proteinases

Despite mounting evidence in the last 30 years showing that expression, localization and activation of lysosomal cysteine proteinases are aberrant in tumor cells, when compared to normal cells, this class of proteases has received little attention. Studies on increased expression, elevated activity and mislocalization of certain enzymes have indicated that members of the cysteine proteinases have been implicated in cancer progression. In mammalian cells, cysteine proteinases are localized mainly in the cytoplasm (calpain and caspase families) and lysosomal compartments (cathepsin and legumain families). Cathepsins are the most directly involved in tumor progression. There are 11 human cathepsins: B, C, F, H, K, L, O, S, W, V and X. These enzymes alongside with aspartic proteinases - cathepsins D and E are mainly involved in intracellular proteolysis within lysosomes. Their increased expression correlates with more aggressive tumors and poorer prognoses for patients (Berdowska, 2004). Cathepsins B and L expression is increased in many human cancers and these enzymes have been investigated most intensively (Bell-McGuinn, et al., 2007; Koblinski et al, 2000). In addition, the predominant expression of cathepsin K in osteoclasts has rendered this enzyme as a major target for the development of novel drugs against bone tumors (Lindeman et al., 2004).
The common belief is that cathepsin-mediated degradation of he extracellular matrix is primarily extracellular at the invasive front of tumor cells. This proteolytic process is associated both with early tumor development, affecting tumor cell proliferation and angiogenesis, and with dissemination of malignant cells from primary tumors (Turk et al 2004). Therefore inhibitors of cathepsins are most intensively studied.
Recent evidence reveals that tumor-promoting proteinases act as part of an extensive multidirectional network of proteolytic interactions. These networks involve various constituents of the tumor microenvironment, with cathepsin B being one of the best examples. An aspartic enzyme - cathepsin D converts pro-cathepsin B into cathepsin B. Cathepsin B can be also activated by a series of other proteinases with cathepsins C and G, urokinase-type plasminogen activator and tissue-type plasminogen activator being the most active ones. Finally, cathepsin B may undergo auto-activation under certain conditions. Activated cathepsin B cleaves a wide variety of targets depending on its subcellular

localization in the tumor microenvironment. Some of its best-known substrates are proteins of extracellular matrix, as well as several important proteinases and their inhibitors (Skrzydlewska et al., 2005; Mason & Joyce, 2011). This complicated pattern of activity emphasizes the central role of cathepsin B in tumor progression simultaneously showing that design of its inhibitors as anticancer agents is a difficult task.

2.1 Cystatins

Cystatins are a superfamily of endogenous inhibitors of proteinases of papain family. So far, 25 representatives of these proteins have been determined. Their main function is to ensure protection of cells and tissue against the proteolytic activity of lysosomal peptidases that are released during normal cell death, or intentionally by proliferating cancer cells or by invading organisms, such as parasites. They exhibit low specificity towards their target proteases, meaning that one cystatin can inhibit several cathepsins. This is because they have apparently similar three-dimensional structure. In some types of cancers, the changes in cysteine cathepsin epression or activity have diagnostic or prognostic value with imbalance between cathepsins and cystatins being associated with tumor phenotype. Since the latter ones are able to inhibit cathepsins tumor-associated activity many studies have indicated their potential use in therapeutic approaches (Keppler, 2006; Kopitz et al, 2005; Palermo & Joyce, 2007). Indeed, one of these inhibitors, cystatin C (mostly the one isolated from egg white) has been used in preclinical research studies for more than 20 years, however, it has been introduced into clinical practice quite scarcely. Despite some isolated promising results (Saleh at al., 2006) this approach is also highly criticized (Keppler, 2006; Mussap & Plebani, 2004) with the greatest problems being high cost of the inhibitor (140 $ USA per milligram), its low bioavailability and short circulation time, and general skepticism amongst clinicians.

Despite the fact that cystatins of different families posses different biochemical properties their inhibitory properties are rather common. They are tight and reversible inhibitors of cathepsins and interact with the active sites of these proteinases via their inhibitory reactive site, made up of the juxtaposition of three regions of the molecule, which form a wedge-shaped edge that is highly complementary to the active site of papain family of proteinases (Fig. 1).

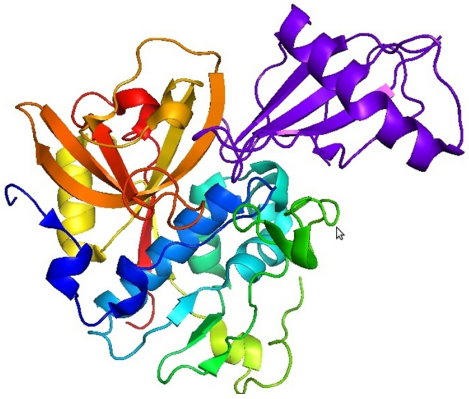

Fig. 1. Stefin A (violet) complexed with cathepsinB.

Thus, mimicking the segment of cystatin interacting with the cathepsin active site (Fig. 1) seems to be the method of choice. This approach is well represented by highly active inhibitor (N-1845, Fig. 2) of cathepsin B (K_i value of 0.088nM) containing an azaglycine residue in place of evolutionary conserved glycine residue in the N terminal part of cystatin (Wieczerzak et al., 2002). Further modification of this molecule, enforced by the use of molecular dynamic and NMR, afforded next potent and selective inhibitor of cathepsin B (K_i of 0.48 nM, Fig. 2) (Wieczerzak et al.; 2007).

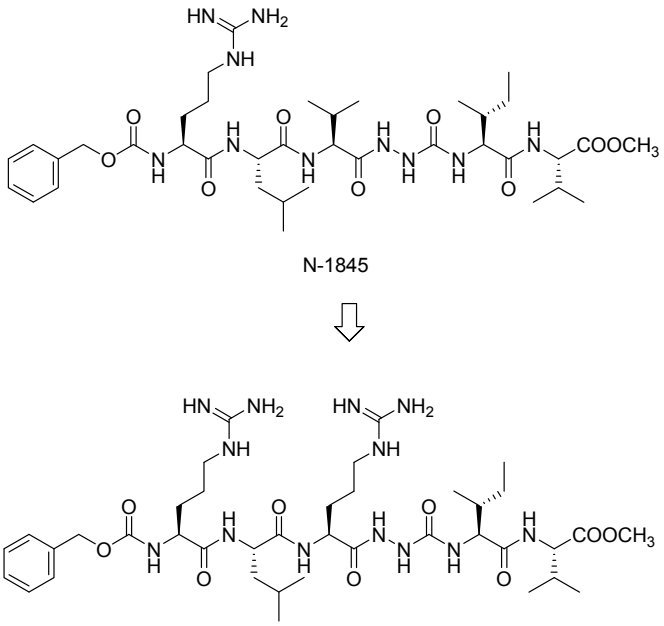

Fig. 2. Two potent azapeptide inhibitors of cathepsin B.

2.2 Inhibitors from natural sources

General strategy employed for discovery of a new drug relays on random screening of libraries of newly available compounds and selection of these, which exhibit desired activity at micromolar range. The leads are then being modified in order to obtain significantly more potent and selective inhibitors, which might be further introduced as drugs. Nature is strongly exploited as a source of lead substances. Isolated in 1978 from *Aspergillus japonicus*, non-specific, irreversible inhibitor of cysteine proteinases, E-64 can serve as a good example (Hanada et al, 1978). The epoxysucciante fragment of this molecule reacts with active-site cysteine and binds covalently to the enzyme. By using this inhibitor as a frame and applying X-ray crystal structures of cathepsins B and L, specific inhibitors of these enzymes were designed (Fig. 3), prepared and shown to have promising anticancer activity in animal studies (Katunuma, 2011).

Traditionally, secondary metabolites from streptomyces show a wide range of diversity with respect to their biological activity and chemical nature. Therefore it is not surprising that their secondary metabolites appear to be interesting lead compounds. A mixture of two

peptide metabolites from *Streptomyces* NCIM 2081 (Fig. 4) exhibited potent inhibitory action against papain and significantly inhibited tumor cell migration at subcytotoxic concentrations, indicating its remarkable potential to be developed as antimetastatic drug (Singh et al, 2010).

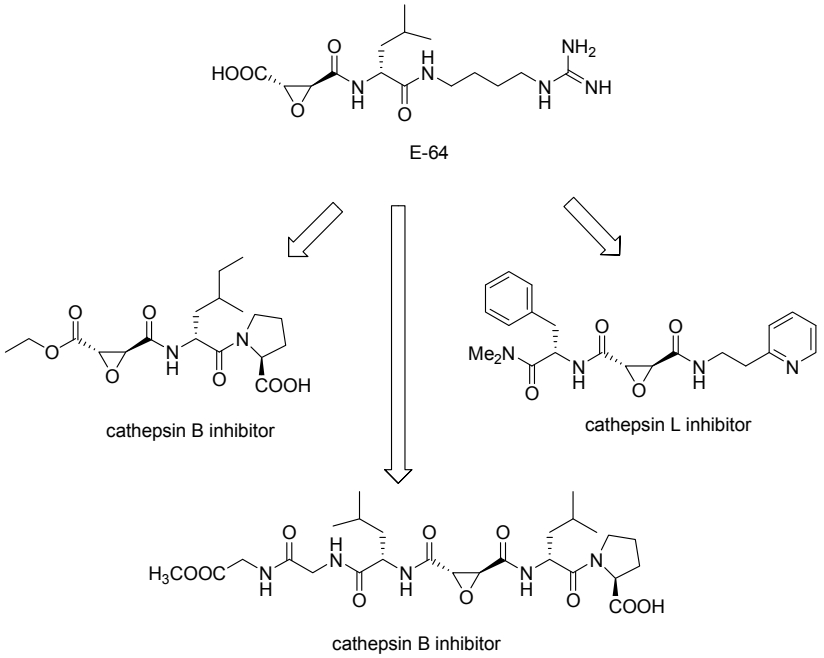

Fig. 3. Specific inhibitors of cathepsins B and L built up on the frame of E-64.

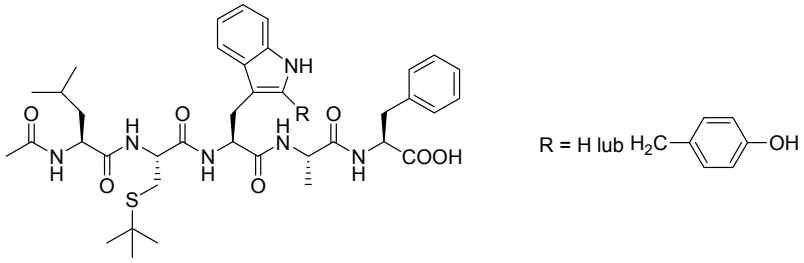

Fig. 4. Anticancer peptides produced by *Streptomyces* NCIM 2081.

2.3 Irreversible inhibitors

The majority of synthetic cysteine proteinase inhibitors contain a peptide segment for recognition by the chosen enzyme and an electrophilic functionality that is able to react with the thiolate moiety of active site cysteine. In most cases this results in covalent modification of the enzyme and irreversible inhibition. A wide variety of such reactive groups have been employed, including: azomethyl- or halomethyl ketone, acyloxymethyl ketone,

acylhydroxamate, vinyl sulfone and chloromethyl sufoxide functions. It is also worth to mention that epoxysuccinates, described earlier, also fall within this class of inhibitors. Representative examples of structurally variable inhibitors are shown in Figure 5.

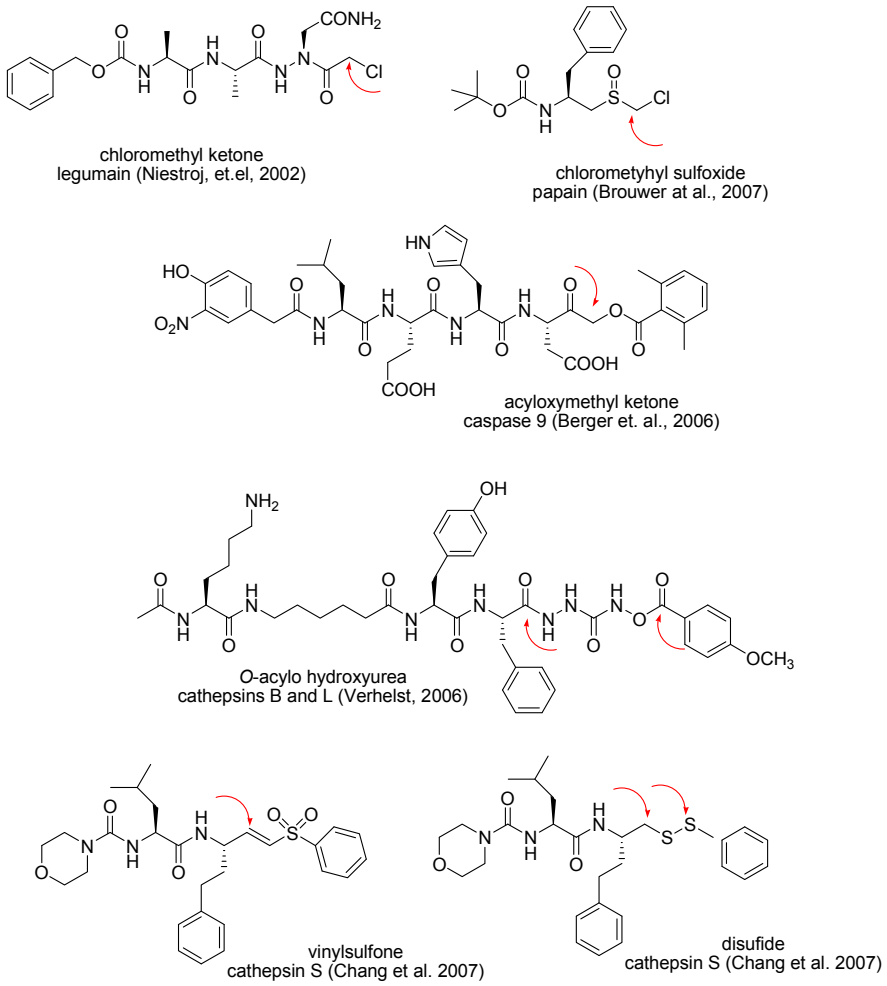

Fig. 5. Representative examples of irreversible inhibitors of cysteine proteinases. The curved arrows indicate possible sites of nucleophilic attack by the active site –SH of active site cysteine.

The reactivity of the electrophilic group greatly determines the selectivity and reaction rate of the formation of the covalent enzyme-inhibitor complex. With this respect halomethylketones are known to react not only with cysteine but also with serine proteinases, thus being non-selective. Although irreversible inhibitors possess high potency and selectivity, they are not considered to be viable drug candidates for treating diseases like cancer, osteoporosis or arthritis. This is because such inhibitors often react over time

with other cysteine proteinases, thus causing toxic side effects or generating immunogenic adducts (Joyce et al., 2004).

Rational design of the peptidyl or peptidomimetic part of inhibitor requires X-ray determination of either cysteine proteinase alone or complexed with already known inhibitors. This provides the detailed insight into the active site and binding pockets of certain enzyme and makes the design process viable. The knowledge of the architecture of the active site of cathepsin B and molecular docking studies were used to design the mechanism-based inhibitor of this enzyme with dual action (Lim et el., 2004)). First, active site Cys-29 is acylated by the inhibitor, which is followed by transfer of acetyloxy moiety of the inhibitor catalyzed by His-199. Thus, two vital active site amino acids are blocked irreversibly (Fig. 6).

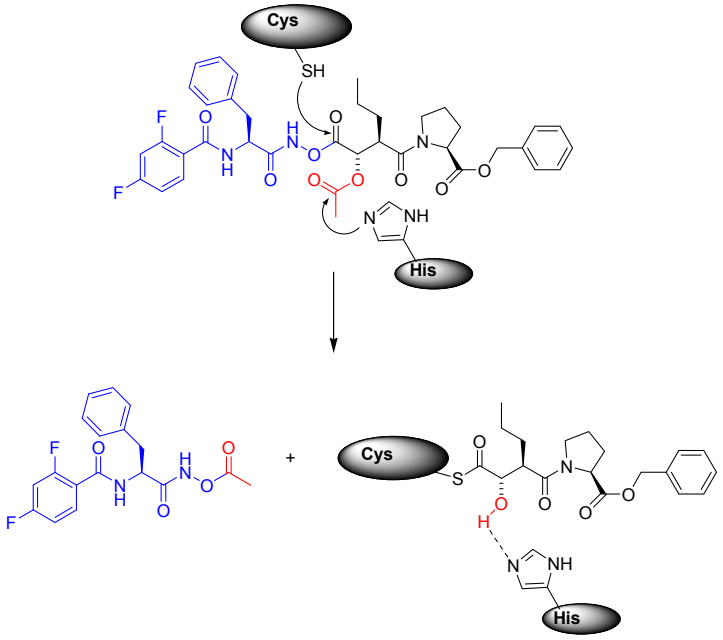

Fig. 6. Inhibition of cathepsin B by mechanism-base dual inhibitor.

2.4 Reversible inhibitors

The strategy in design of reversible inhibitors of cysteine proteinases is commonly the same as in the case of irreversible ones with the exception that the reaction between electrophilic warhead of the inhibitor and the enzyme is reversible. An aldehyde, a metyl ketone, a α-ketoamide or a nitrile groups usually act as the reactive electrophiles. Representative examples of such inhibitors are shown in Figure 7. Some of them are currently being profiled in animal models to further delineate the role of these enzymes in cancer disease processes.

A wide variety of these inhibitors were obtained using computer-aided design. For example, high-resolution X-ray crystallographic data and molecular modeling studies were used to find out one of the most potent inhibitors of cathepsin B (K_i=7nM) - dipeptide nitrile shown

in Figure 8 (Greenspan et al., 2001). In the Figure 8 also the mechanism of reversible binding of this inhibitors was outlined.

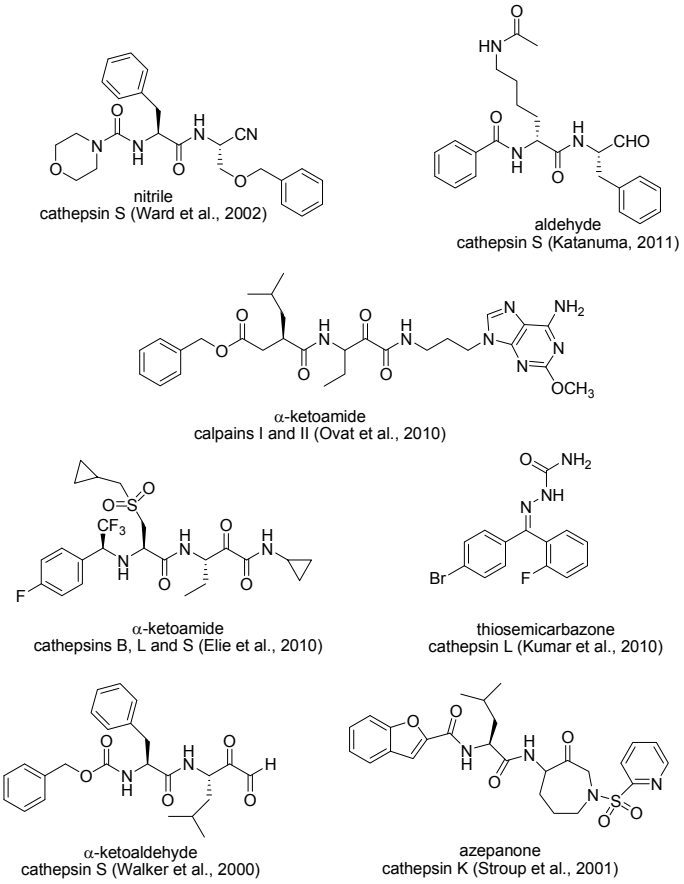

Fig. 7. Representative examples of reversible inhibitors of cysteine proteinases.

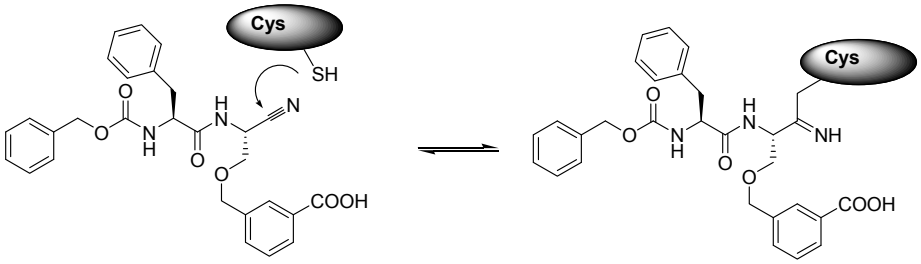

Fig. 8. Mechanism of inhibition of cathepsin B by dipeptidyl nitrile.

2.5 Metalloinhibitors

The field of metallodrugs is dominated by compounds, which interact with DNA and cause its direct damage. In recent years, however, it was well established that some of them exert cytotoxic activity affecting certain enzymes. Rhutenium (II)–arene derivatives exhibit remarkable selectivity towards solid tumors, most likely by inhibiting two vital enzymes for cancer development – thioredoxin reductase and cathepsin B. The most active inhibitor of cathepsin B is reversibly bound to the active site of the enzyme (Casini et al., 2008). Docking studies revealed that the most important interactions responsible for its activity are those with the residues flanking the active site (Fig. 9).

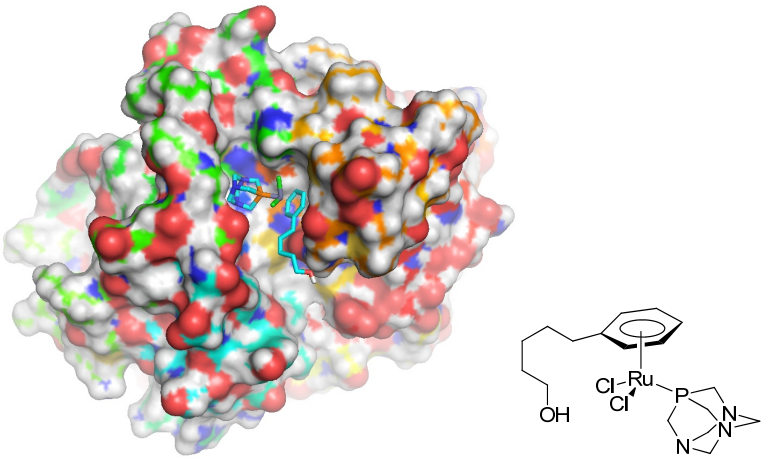

Fig. 9. The most active organorhutenium inhibitor of cathepsin B and its mode of binding in the active site of the enzyme as modeled by docking approach.

Quite contrary, newly synthesized series of organotelluranes appeared to be potent, irreversible inhibitors of cathepsins V and S (Piovan et al., 2011). Tellurium atom is an electrophilic center, which undergoes nucleophilic attack of cysteine thiol at the active site of the enzyme. In this reaction tellurium-halogen bond is broken and new tellurium sulfur bond is formed (Fig. 10). Considering the electrophilicity of the chalcogen, it is known that tellurium is less electronegative than selenium and, due to its greater capacity to stabilize the negative charge, bromide is a better leaving group than the chloride, we can explain the highest reactivity of the dibromo-organotelluranes toward cysteine cathepsins.

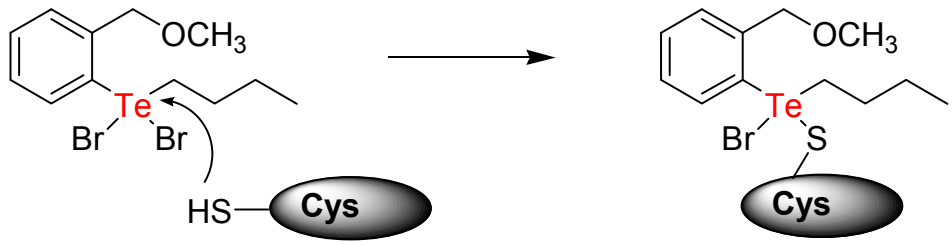

Fig. 10. Mechanism of irreversible inhibition of cathepsins by organotellurane.

3. Serine proteinases

Serine proteinases emerged during evolution as the most abundant and functionally diverse group of proteolytic enzymes - over one third of them belong to this class. They typically contain a catalytic triad of serine, histidine and aspartic acid residues in their catalytic active sites, which are commonly referred to as the charge relay system. This implies common mechanism of peptide bond hydrolysis. It goes through two-step hydrolytic process, which allows acylation of the serine residue by peptide substrate followed by hydrolysis of this adduct and regeneration of the enzyme.

Several serine proteinases have been implicated as important regulators of cancer development. This family includes enzymes involved in mediating of plasminogen (urokinase-type and tissue-type plasminogen activators), as well as serine proteinases stored in secretory lysosomes of various leukocytes, namely mast cell chymase, mast cell tryptase, and neutrophil elastase. Although most secreted serine proteinases emanate from host stromal cells, recent studies implicate a superfamily of cell-surface associated serine proteases, also known as Type II Transmembrane Serine Proteinases (TTSP), such as matriptase and hepsin, as important regulators of cancer development.

Plasmin proteolytic cascade is functionally contributing to neoplastic progression, including acquisition of a migratory and invasive phenotype by tumor cells, as well as remodeling of extracellular matrix components via activation of matrix metalloproteinases. Urokinase-type and tissue-type plasminogen activators (uPA and tPA respectively) regulate enzymatic activity of plasmin. uPA plays a crucial role in tissue remodeling, while tPA is important in vascular fibrinolysis (Naffara et al., 2009).

Mast cell-derived chymases and tryptases are stored in secretory granules. Their release into the extracellular milieu triggers a proinflammatory response as well as induces a cascade of protease activations, culminating in activation of matrix metalloproteinase 9. As a result neoplastic progression is observed (Fiorucci & Ascoli, 2004; Takai et el., 2004).

Neutrophil elastase, a serine protease abundantly present in neutrophil azurophilic (primary) granules, is transcriptionally activated during early myeloid development. Little is known about the role of this proteinase in cancer progression, however, it has the ability to cleave almost every protein contained within the extracellular matrix including, but not limited to: elastin, collagen, fibronectin, laminin, and proteoglycans. Interest in neutrophil elastase during neoplastic processes stems from recent clinical reports that correlate elevated expression of this enzyme with poor survival rates in patients with primary breast cancer and non-small cell lung cancer. It also has recently been found to initiate development of acute promyelocytic leukemia (Naffra et al., 2009; Sato et al., 2006)

Most serine proteinases are expressed by supporting tumor stromal cells, whereas membrane-anchored serine protease appear to be largely expressed by tumor cells at the cell surface and are thus ideally located to regulate cell–cell and cell–matrix interactions. Increasing evidence demonstrates that aberrant expression of enzymes such as matriptase and hepsin is a hallmark of several cancers and recent studies have defined molecular mechanisms underlying TTSP-promoted tumorigenesis, a processes causing carcinomas of skin, breast, and prostate (Choi et al., 2009). Similar association with cancer has led to great interest in kallikreins (Di Cera, 2009), a large family better known for its role in regulation of blood pressure through the kinin system. Prostate-specific antigen (PSA), a serine protease also belonging to the human kallikrein family, is best known as a prostate cancer biomarker since its expression is highly restricted to normal and malignant prostate epithelial cells.

3.1 Proteinous inhibitors

Typically serine proteinases have active site clefts that are relatively exposed to solvent. This permits the access to polypeptide loops of substrates and endogenous inhibitors. By forming strong proteinase-inhibitor complexes the latter ones regulate the activity of proteolytic enzymes and play important physiological roles in all organisms. Therefore, it is not surprising that they are considered as potential anticancer drugs and are already being tested in clinics.

Proteinous serine proteinase inhibitors were the first used against cancer and are so far the most intensively studied (Castro-Guillén et al., 2010; Otlewski et al., 2005). A small metalloprotein, Birk-Bowman inhibitor, isolated from soybeans as far as in 1946, is 8kDa polypeptide of the documented activity in a variety of tumors. Other members of this family have also proved their anti cancer activity, with field bean protease inhibitor being strongly active against skin and lung tumors, and tepary bean inhibitor affecting proliferation and metastasis of fibroblast (Castro-Guillén et al., 2010; Joanitti et al., 2010; Sakuhari et al., 2008). Another classes of similar inhibitors of serine proteinases also exhibit promising anti-cancer properties, to mention only: Kunitz-type inhibitors (Sierko et el., 2007; Wang et al., 2010), serpins (Catanzaro et al., 2011; Li et al., 2006), antileukoprotease (Xuan et el., 2008), nexin (Candia et el., 2006) and lunasin (Dia & de Meija, 2010; Hsieh et al., 2010).

Paradoxically, the action of proteinase inhibitors in some cases results in poorer prognosis and promotion of the cancer development (Fayard et al, 2009; Ozaki et al., 2009). This is contrary to what would be expected from proteinase inhibitor and shows that more detailed studies are required in order to understand their action. These observations also indicate the need for development of inhibitors of different types. Examination of crystal structures of inhibitors bound by various proteinases is a useful tool to study architecture and requirements of serine proteinase binding sites. This is because 3-5 amino acid residues of proteinaceus inhibitor, properly spatially located with respect to each other, interact with small binding region of the enzyme. The binding modes of Bowman-Birk inhibitor from *Vigna unguinocula* with β-chymotrypsin (Barbosa et. al., 2007), and structure of textilinin-1 from the venom of Australian *Pseudonaja textilis* snake complexed with trypsin (Millers et al., 2009) are shown in Figure 11 as representative examples.

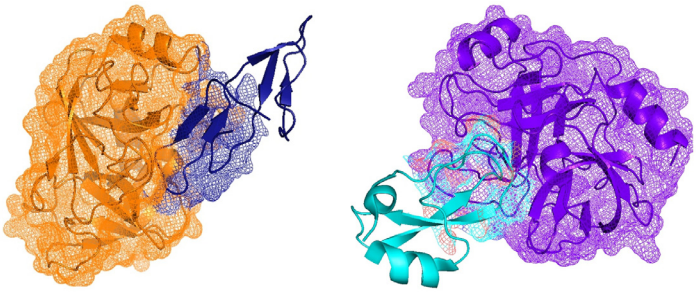

Fig. 11. The binding modes of Bowman-Birk inhibitor with β-chymotrypsin (left-hand side) and textilinin with trypsin

Mutation of the already known protein inhibitors is one of the means to construct highly specific inhibitors of chosen proteinase. Such strategy was applied to obtain specific and potent inhibitors of human kallikrein 14. A human serpin, named α-1-antichymotrypsin,

was used to change its specificity by modifying five amino acids of its reactive center loop, a region involved in inhibitor–protease interaction. This region was replaced by two pentapeptides, previously selected by kallikrein 14 using phage-display technology. In this manner inhibitors with high reactivity towards the enzyme were generated (Fleber et al., 2006).

Sensing the binding site of chosen proteinase by studying structure of bound regions of its inhibitors and substrates is a classical tool for the design of new inhibitors of these enzymes. This concept is well illustrated by the discovery of cyclic peptides mimicking binding fragment of plasminogen activator (uPA) to its cell surface associated receptor (uPAR). The minimal portion of uPa able to bind effectively to uPAR was selected by systematic deletions of peptidyl fragments from the N- and C-terminus of the starting protein inhibitor. Cyclization of the minimal effective structure results in introduction of constrains that limit the conformational freedom of the molecule and ensure proper spatial arrangement of the amino acid residues interacting with the receptor. In that manner cyclic peptides, mimetics of uPA, (the most effective one is shown in Figure 12) were synthesized and found to effectively compete with uPA binding (Schmiedeberg et al., 2002).

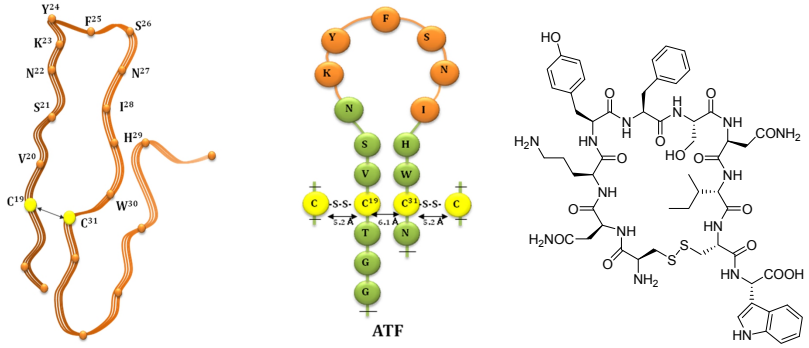

Fig. 12. Fragment of uPA selected as a scaffold for the preparation of cyclic peptidomimetics and the structure of the most effective of them

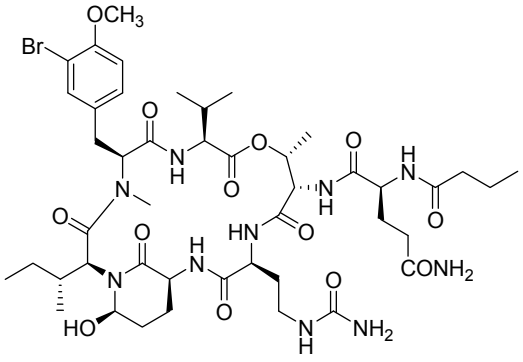

Fig. 13. Structure of Symplocamine A

Similar cyclic peptides, inhibitors of various proteinases, were also isolated from natural sources. For example, out of more than 100 compounds of this class isolated from cyanobacteria about half have been reported to inhibit trypsin or chymotrypsin. Recently isolated Symplocamine A (Figure 13), molecule of strong serine protease inhibitory activity, appeared to exert high level of cytotoxicity against variety of cancer cells *in vitro* thus being a potential agent against cancers (Linington et el., 2008).

3.2 Irreversible inhibitors

Similarly as in the case of cysteine proteinases irreversible inhibitors of serine proteinases are prominent class of their inactivators. They usually bind covalently to one of the nucleophilic moieties of amino acids present in an active site of the enzyme (most likely hydroxylic group of serine) using an electrophilic warhead. Although there are many classes of irreversible inhibitors of serine proteinases available today (Powers, et al., 2002) only limited examples entered clinical studies as anticancer agents. Therefore, new low-molecular inhibitors of enzymes involved in cancer development and metastasis are still strongly desirable. Recent studies are concentrated on the synthesis of inhibitors containing non-typical warheads (representative examples are shown in Figure 14).

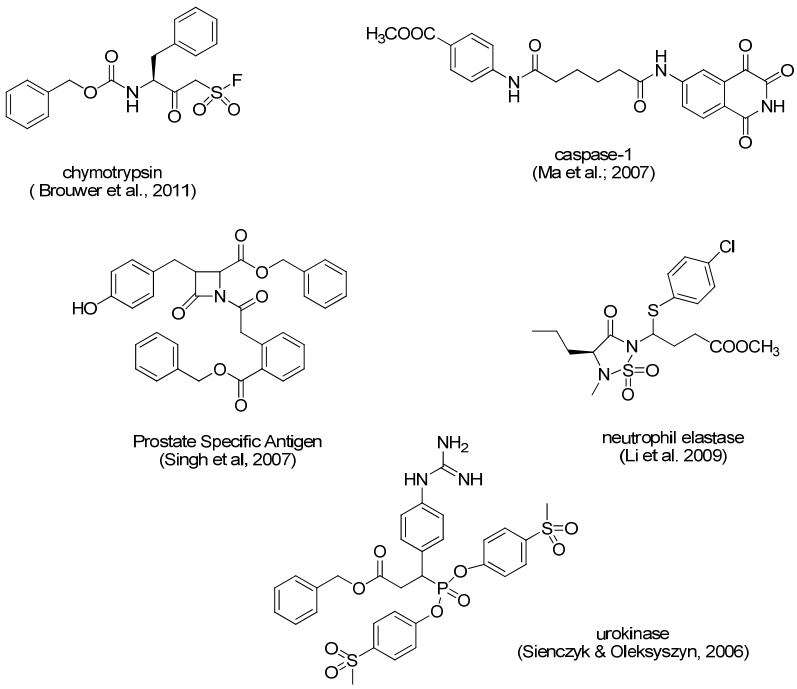

Fig. 14. Representative examples of irreversible inhibitors of serine proteinases.

Diphenyl phosphonates seem to be the most promising and general group of these inhibitors. They may be also classified as competitive transition-state analogues. On a molecular level they phosphonylate specifically active-site serine residue thus blocking the catalytic triad of serine, histidine and aspartic acids responsible for the formation of enzyme-substrate acyl

intermediate and its further hydrolysis (Fig. 15). Anyway, the mode of action of phosphonates towards serine proteinases is not yet fully elucidated and minor variations were observed, depending on the targeted enzyme and conditions (Grzywa et al. 2007; Joossens et al., 2006; Sieńczyk et al., 2011; Sieńczyk & Oleksyszyn 2006; Sieńczyk & Oleksyszyn, 2009).

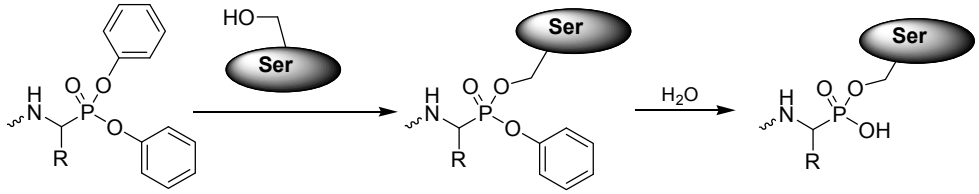

Fig. 15. Schematic illustration of the mechanism of action of the diphenyl α-aminophosphonate inhibitors of serine proteinases

3.3 Reversible inhibitors

Inhibitors of urokinase (also called urokinase-type plasminogen activator, uPA) are the biggest family of reversible serine protease inhibitors. Development of small molecule uPA inhibitors has began with aryl guanidines, aryl amidines, and acyl guanidines, molecules that contain positively charged guanidine, amidine, or simple amines as anchors able to interact with the negatively charged site chain of Asp189 (Lee et el., 2004). Although they exhibited moderate potency and poor selectivity they constituted a good starting point for the development of new effective generations of uPA inhibitors. Intensive studies using various approaches resulted in many inhibitors, which quite frequently revealed *in vitro* anticancer properties. Determination of three-dimensional structure of this enzyme either in native state or complexed with various inhibitors is vital for the design of new effectors of urokinase (Huai et al. 2008; Klinghofer et al. 2001; Sperl et. al. 2000).

For, example, an extremely simple inhibitor UK 122 (Fig. 16) was designed in a stepwise process. The first step was a selection of moderate inhibitors of uPA by screening a library of 16,000 synthetic compounds. This resulted in four promising inhibitors of the enzyme sharing very similar chemical structures. They were further optimized by using crystal structure of the enzyme-Amiloride complex and by applying molecular modeling methods. As a result UK 122 was found (Zhu et al., 2007). This compound significantly inhibited the migration and invasion of pancreatic cancer cell line.

Another example may be the use of three-dimensional quantitative structure-activity relationship (3D QSAR) studies to elucidate structural features required for uPA inhibition and to obtain predictive three-dimensional template for the design of new inhibitors. 3D QSAR was performed on five reported classes of the urokinase inhibitors by employing widely used CoMFA (Comparative Molecular Field Analysis) and CoMSIA (Comparative Molecular Shape Indices Analysis) methods (Bhongade & Gadad 2006). As a result the significance of various structural elements bound at different urokinase subsites was identified. These subsites may be combined to improve overall activity of newly designed inhibitors.

Inhibitors of other serine proteinases were studied as anticancer agents quite scarcely. Most of the obtained inhibitors were designed to affect with prostate specific antigen (PSA) and matriptase by adopting the procedures used for designing of other serine proteinase inhibitors. Some of them exhibit promising anticancer properties in cell culture systems. Representative examples of these inhibitors are shown in Figure 16.

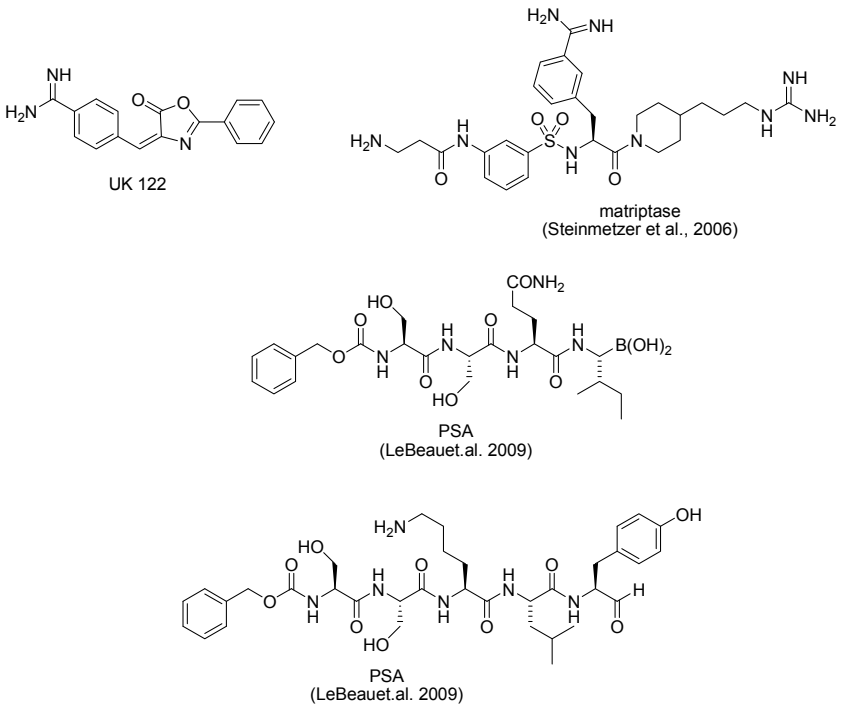

Fig. 16. Representative examples of reversible inhibitors of serine proteinases.

4. Threonine proteinases

The sequencing of human genome revealed that threonine proteinases account only for about 5% of the whole pool of proteinases. From these proteinases, only proteasome is considered as a target for potential anticancer agents. Since tightly ordered proteasomal degradation of proteins plays crucial role in the cell cycle control potential of proteasome inhibitors is currently under intensive investigations.

The proteasome is a highly conserved intracellular nonlysosomal multicatalytic protease complex, degrading proteins usually tagged with a polyubiquitin chain. The 26S proteasome is a 2,000 kDa multisubunit cylindrical protein comprised of a 20S core catalytic component (the 20S proteasome) capped at one or both ends by 19S regulatory components (Figure 17). Proteasome 20S has three major sites of different activities designed as "chymotrypsin-like", "trypsin-like" and "caspase-like". These three activities are responsible for the cleavage of protein after hydrophobic, basic, and acidic amino acid residues, respectively. Analysis of the proteasome catalytic mechanism has revealed the importance of the N-terminal threonine as catalytic nucleophile. Thus, proteolytic machinery of the proteasome is an important target for the design of anticancer drugs (Abbenante & Fairlie, 2005; Delcros et al., 2003; Goldberg, 2007). A wide variety of inhibitors of proteasome were developed and evaluated (Delcros et al., 2003). This process culminated in discovery of bortezomib (*Velcade*, Figure 17), which decreases proliferation, induces apoptosis and enhances sensitivity of tumor cells to radiation or chemotherapy (Adams, 2002; Goldberg, 2007).

The most significant step in development of proteasome inhibitors was the decision by A. L. Goldberg and colleagues to create in 1993 the company *MyoGenics*. The goal was to synthesize proteasome inhibitors that could prevent muscle atrophy that occur in various disease states, such as cancer cachexia. This led to the production of a series of inhibitors that were freely distributed to academic laboratories and contributed to the enormous leap forward in understanding the multiple roles of the proteasome in cells.

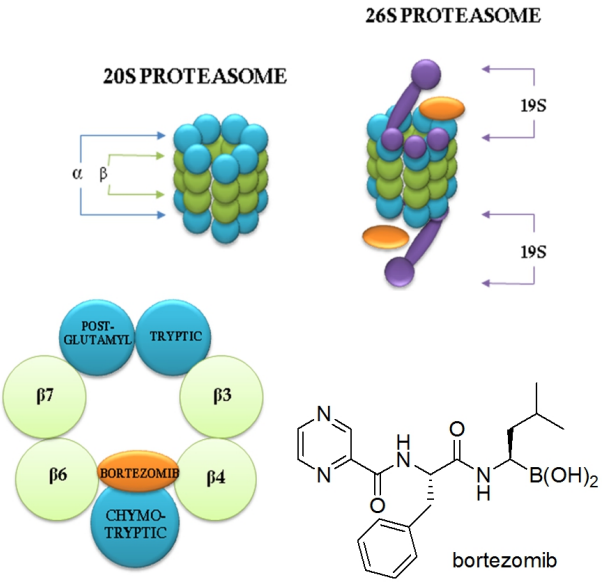

Fig. 17. Schematic structure of proteasome with indication of the binding site of bortezomib

4.1 Inhibitors from natural sources

The 20S proteasome is a tubular molecule with the proteolytic active sites on the inner surface. Thus, substrate molecules have to be translocated through the internal cavity to the catalytic sites. The X-ray crystallographic analysis has shown that the translocation channel is too narrow to allow passage of folded proteins. Protein substrates should be firstly unfolded and then degraded. Quite surprisingly, classical protein inhibitor of serine proteinases, bovine pancreatic trypsin inhibitor (BPTI) appeared to exert similar activity against proteaseome *in vitro* and *ex vivo* (Yabe & Koide, 2009). The molar ratio of BPTI to the proteasome 20S in the complex was estimated as approximately six to one, suggesting that two out of three proteinase activities of this complex were inhibited. This interesting finding has opened a new front in proteasome inhibition studies.

The majority of proteasome inhibitors have a structure of small cyclic and linear peptides built on scaffolds provided by natural substances. Lactacystin (Figure 18), produced by *Strepromyces* (Omura et. al., 1991), rearranges in neutral pH to highly reactive lactone-Omuralide, which irreversibly acylates proteasome active site threonine. Minute modification of the latter one led to the more potent inhibitor MNL-519 (Abbenante & Fairlie, 2005). Isolation of *Actinomycete* products – epoxomycin and eponemycin, and evaluation of their inhibitory activity (Hanada, et. al, 1992; Sugawara et al, 1990) has

stimulated studies on their analogues (representative structure is shown in Fig. 18). This resulted in several potent inhibitors, which display non-typical mechanism of action (Elofsson et e., 1999; Zhou et el., 2009). A hemiacetal is first formed between the ketone portion of the inhibitor and threonine hydroxyl, followed by epoxide ring opening by the free amine of the N-terminal threonine to give a stable morpholino adduct.

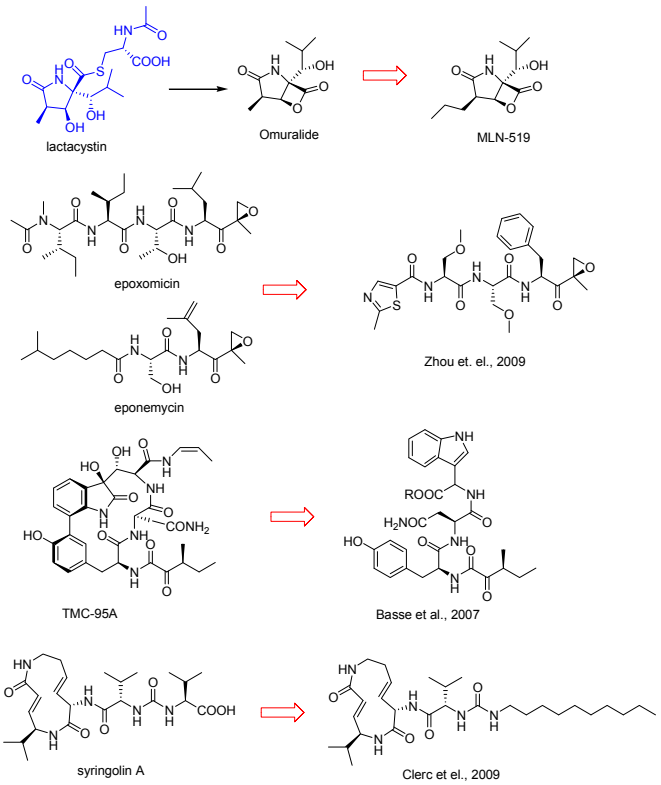

Fig. 18. Natural inhibitors of proteasome activity as scaffolds for synthesis improved ones.

Next example considers syringolines, reversible inhibitors of proteasome produced by *Pseudomonas siringae* (Coleman, et al. 2006). Elucidation of the crystal structure of syringolin B complexed with proteasome gave an insight into the structural requirements of good inhibitor. These findings were used successfully in the rational design and synthesis of a syringolin A-based lipophilic derivative, which proved to be one of the most potent proteasome inhibitors described so far (Clerc et el., 2009).

A limiting factor in the efficiency of peptidic inhibitors is that they are unstable in living organism because they are easily degraded by endogenous proteinases. This explains growing interest in non-peptidic inhibitors. Nature is an acknowledged source of such compounds and many inhibitors of proteasome were isolated and identified. These include such structurally diverse compounds as: ajoene isolated from garlic (Hassan, 2004), gliotoxin produced by *Aspergillus fumigatus* (Pahl et al., 1996), or triterpene-celastrol isolated from the root bark of medicinal plant *Tripterigium wolfordii* (Yang et al., 2006).

4.2 Synthetic inhibitors

The first inhibitors of proteasome were identified among the commercially available reversible tripeptide inhibitors of serine and cysteine proteinases. The easy access to the peptide aldehydes had lead to the development of a wide variety of inhibitors with an improved potency and selectivity. MG-132 (for its chemical structure see Figure 20) was one of the first synthetic inhibitors to be described and used in cell culture system (Adams & Stein, 1996). It exerts both, direct antiproliferative and cytotoxic effects towards tumor cells, and increases apoptosis induced by other agents. Recent studies have demonstrated the influence of absolute configuration of this tripeptide aldehyde on its cytotoxicity, with (*L*,*D*,*L*) isomer being the most active (Mroczkiewicz et al., 2010). Since a great number of tripeptide aldehydes contain side chains of non-coded amino acids but they usually correspond to natural *L*-amino acids this finding shed a new light on the importance of peptidyl absolute configuration.

Structurally related α-ketoaldehydes exert their action via mechanism similar to this described earlier for epoxyketones (Gräwert et al., 2011). This is a cyclization mechanism, which proceeds through formation of hemiketal with threonine hydroxyl followed by Schiff base formation between the nucleophilic *N*-terminal threonine and aldehyde moiety, which finally results in the reversible formation of a 5,6-dihydro-2*H*-1,4-oxazine ring (Figure 19). The examination of the binding mode of these inhibitors serves as a new lead for the development of anticancer drugs (Fig. 19).

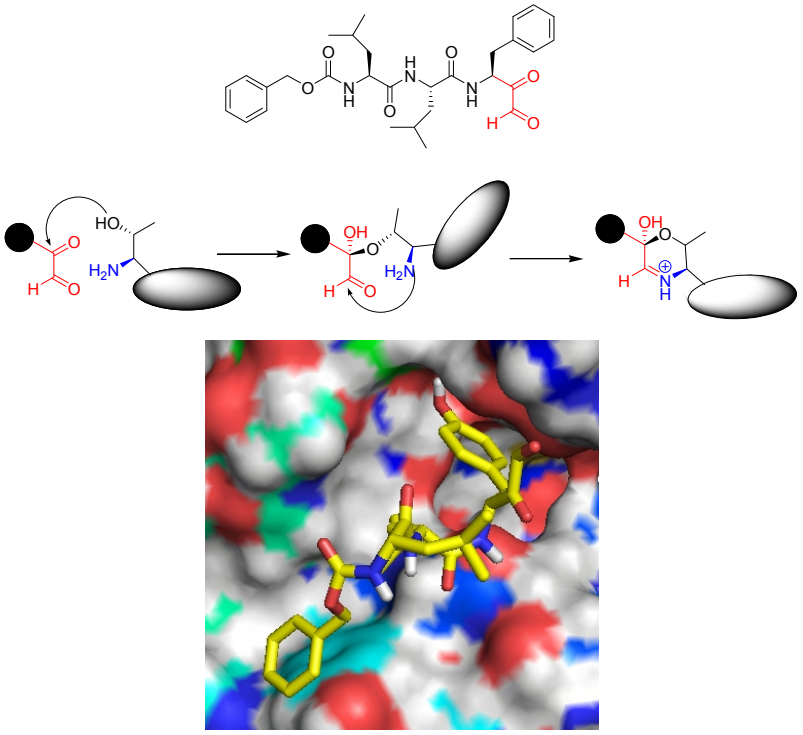

Fig. 19. Molecular mechanism of action of α–ketoaldehyde inhibitor of proteasome and the mode of its binding in the active site.

Searching for a new class of 20S proteasome inhibitors is a hot subject and to date a plethora of molecules that target the proteasome have been identified or designed (de Bettignies and Coux, 2010). Synthetic inhibitors possess a homogeneous structural profile - they are generally peptide-based compounds with a C-terminal pharmacophore function required for primary interaction with catalytic threonine of the enzyme. The peptide component seems to be important for determining specificity of the interactions with the enzymatic pockets. Essentially, most of these inhibitors act on the chymotrypsin-like activity of the proteasome although two remaining activities are also addressed.

Protection of the aldehyde moiety in a form of semicarbazone provides compounds that are more stable than counterpart aldehydes. They do not form adducts with cellular proteins and are irreversible inhibitors of proteasome requiring the action of this enzymatic complex to release inhibiting aldehyde. Thus, they may be classified as suicidal inhibitors. Recently two peptide semicarbazones, S-2209 and SC68896, were found to exert anti-melanoma and anti-glioma activities in preclinical studies (Baumann et el., 2009: Leban et al., 2008; Roth et el., 2009). For the latter one company was given an approval to start phase I/II clinical studies in 2011.

Structurally related *N*-acylpyrrole peptidyl derivatives were designed as irreversible inhibitors of proteasome. They appeared to possess unique biological profile and interact reversibly with β1 catalytic site of the proteasome also displaying good pharmacological properties (Baldisserotto et al., 2010). Molecular docking of the *N*-acylpyrrole molecule shown in Figure 20 enabled to rationalize the mode of their binding.

The vinyl sulfone group is less reactive than the aldehyde group but also binds irreversibly to the active sites. The advantage of vinylsulfone inhibitors is that they are easy to prepare. One of the most potent inhibitor - Ada(Ahx)$_3$-LLL-VFS, specifically and irreversibly inhibits both the constitutive and the induced proteasome by binding to their three active sites with approximately equal efficiencies (Kessler et al., 2001).

The screening of huge libraries of structurally variable compounds is a method for the identification of new cell-active inhibitors with novel chemical scaffolds. Such a procedure was also used in order to obtain new inhibitors of proteasome. Thus, a high-throughput screen of the Millennium Pharmaceuticals Inc. library (approximately 352,500 compounds) afforded 3015 hits, which were further optimized by applying X-ray crystallography and molecular modeling. In such manner 16 various structures were selected. They appear to exhibit high potency and selectivity towards β5 subunit of 20S proteasome. The crystal structures determined for the most active compounds (Fig. 20) enabled to determine the structural requirements of the inhibited subunit. Similar screening done on National Cancer Institute Diversity Set library composed of 1,992 compounds resulted in selection of four promising inhibitors of proteasome, with organocopper NCS 321206 (Fig.20) being the most active one (Lavelin et al., 2009).

Different approaches to the selection of new inhibitors of proteasome relayed on the use of computational tools, namely multistep structure-based virtual ligand screening strategy. First scoring engines were standardized using known inhibitors in order to obtain results similar to those found from crystallographic studies. It appeared that none of the presently developed scoring functions are fully reliable nor they fully correlate with experimental affinities. Therefore three protocols were used simultaneously - FRED, LigandFit and Surflex, to dock 300,000 compound collection (Chembridge). This enabled to select 200 molecules for further experimental testing, using MG-132 as a standard. Twenty of these molecules appeared to act as potent proteasome inhibitors showing variable profiles of

activity. Thus six of them inhibited all three activities of proteasome, eleven of them inhibited two types of enzymatic activities, whereas three inhibited only one type of activity (Basse et al., 2010). The most active and selective inhibitors against chymotrypsin-like and trypsin-like activities are shown in Figure 20.

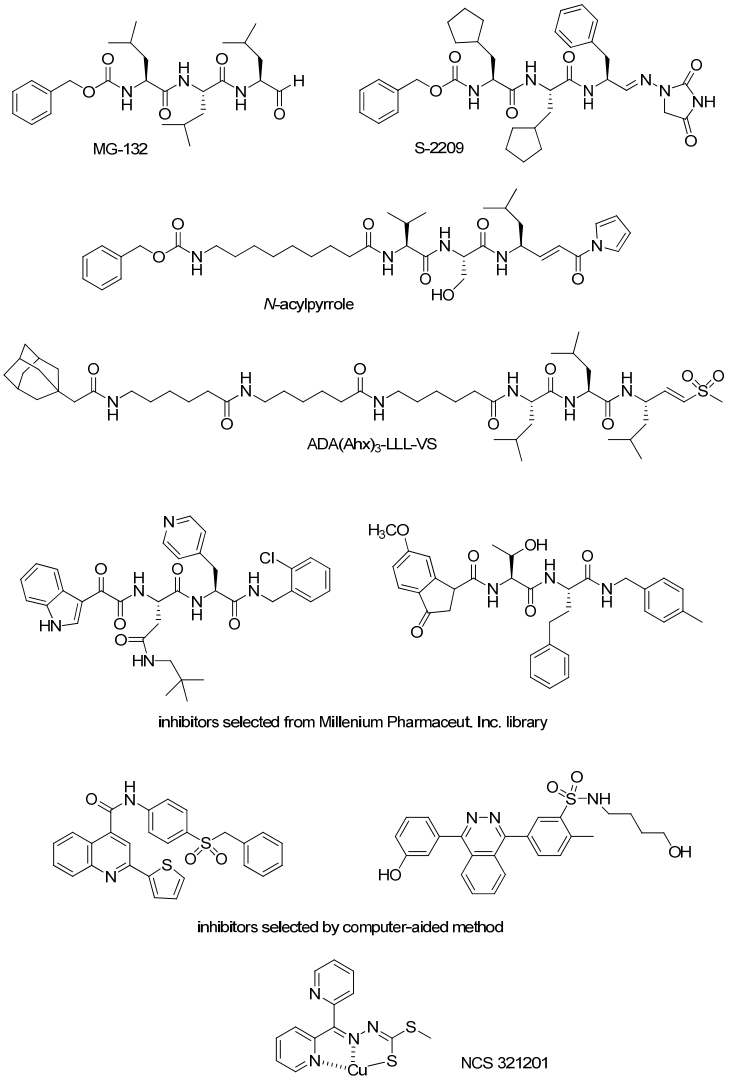

Fig. 20. Structurally diverse, synthetic inhibitors of proteasome.

The discovery of bortezomib was followed by intensive preclinical and clinical studies on many cancer models and cancer patients. This drug was approved in 2003 for treatment of multiple myeloma as a second line of the therapy. Today it is taken by approximately 50,000 patients worldwide (Goldberg, 2007) and is still being tested clinically against other forms of

cancer. Interestingly, recent studies have indicated that this drug is a multiple inhibitor and affects also serine proteinases in cell lysates (Arastu-Kapur et al., 2011). This finding may explain better the clinical profile of this drug. Alongside with physiologic studies synthesis and evaluation of inhibitory activity of its analogues have been carried out. Although in some cases inhibitors of similar potency were obtained (Aubin et al., 2005; Vivier et al., 2005; Zhu et al, 2010) none of them was found to be better than bortezomib.

5. Aspartic proteinases

This is the smallest family of proteinases, which accounts for only 3% of them and includes several physiologically important enzymes such as pepsin, chymosin, renin, gastricsin, cathepsin D and cathepsin E. Some members of this family, in particular cathepsins D and E, have been implicated in cancer progression. High cathepsin D expression is associated with shorter disease-free and overall survival in patients with breast cancer, whereas in patients with ovarian or endometrial cancer, cathepsin E expression has been reported to be associated with tumor aggressiveness.

Quite surprisingly, the aspartic protease napsin A, expressed in lung cells, where it is involved in the processing of surfactant protein B, suppressed tumor growth in HEK293 cells in a manner independent of its catalytic activity (Ueno et al., 2008). Further insight into mechanism involved may help in producing new drugs for renal cancer.

The most extensively investigated aspartic proteinase in the context of cancer is cathepsin D, with a particular emphasis on its role in breast cancer (Benes et al, 2008). In these studies several inhibitors of this enzyme are most commonly used including peptidomimetic pepstatin (Umezawa et al., 1970) and protein inhibitors from potato and tomato (Carter et al., 2002). Search for new inhibitors of this enzyme is practically limited to peptides containing non-typical amino acid – statine. Inhibitors of this type were obtained from both natural sources as well as were synthesized basing on the crystal structure of pepstatin A (Fig. 21) complexed by this enzyme. Statine, which is a component of pepstatin A, may be considered as an analogue of tetrahedral intermediate (or transition-state) of the enzymatic hydrolysis of *L*-leucylglycine (Fig. 21). Therefore it is not surprising that most of cathepsin D inhibitors contain this amino acid or its analogue within peptidic chain (Bi et al., 2000; McConnell et al., 2003). Of special interest are grassystatins (Fig. 21) isolated from cyanobacterium *Lyngbya* cf. *confervoides*. These peptidomimetics are equally active against cathepsins D and E (Kwan et al., 2009).

The new approach to the identification of inhibitors is appropriate selection of DNA aptamers strongly interacting with chosen enzyme. This methodology was used to identify the aptamer SF-6-3, which selectively and very strongly binds cathepsin E (Naimuddin et al., 2007).

6. Metalloproteinases

Metalloproteinases are the largest class of proteinases in human genome. They are a range of enzymes possessing metal ions in their active sites. Most of them are dependent on zinc ions, which play catalytic functions. Understanding their mechanism of action is of key importance to rational design of potent and specific inhibitors of these enzymes and, consequently, to obtain drugs of improved properties. Therefore, a substantial effort has been made to study the mode of binding of their substrates and inhibitors, as well as to elucidate the three dimensional structure of these enzymes and to define the detailed

mechanisms of catalyzed reactions. Despite extensive experimental and theoretical studies the mechanism by which the catalytic center of metalloproteinases functions is still the subject of debate and several mechanism have been proposed (Mucha et al., 2010).

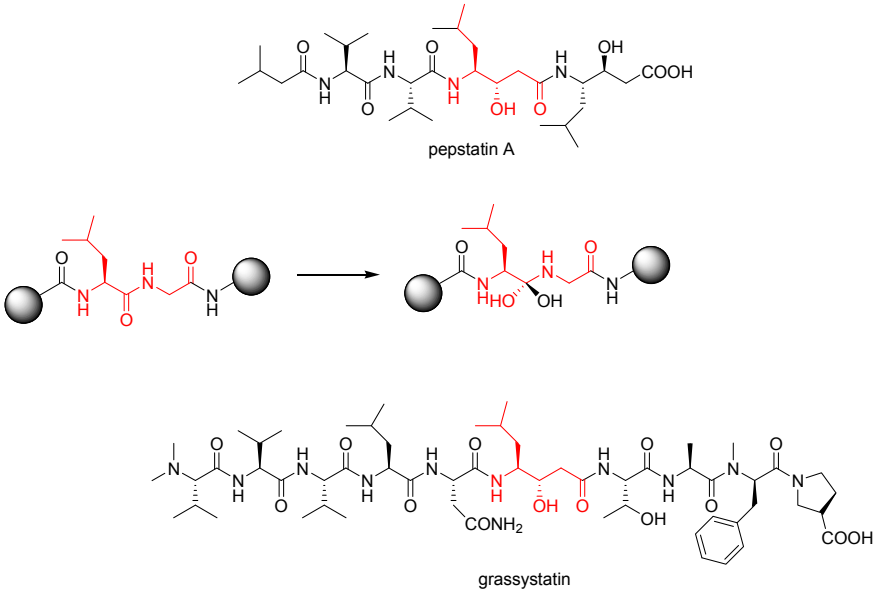

Fig. 21. Pepstatin A and grassystatin as transition-state analogues of peptide hydrolysis.

Matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs, adamalysins) and tissue inhibitors of metalloproteinases (TIMPs) together comprise an important set of proteins that are regulatory in matrix turnover and regulate growth factor bioavailability. There are 23 MMP, 32 ADAM and 4 TIMP proteins present in humans. This shows how complex system is involved in tumorigenesis and its regulation. For example, four tissue inhibitors of metalloproteinases (TIMP1, TIMP2, TIMP3 and TIMP4) are the main endogenous inhibitors for all the metallo-endopeptidases, of which there are more than 180.

6.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) consist of a multigene family of zinc dependent extracellular endopeptidases implicated in tumor growth and the multistep processes of invasion and metastasis, including proteolytic degradation of extracellular matrix, alteration of the cell–cell and cell–matrix interactions, cell migration and angiogenesis (Gialeli et al., 2011). These structurally and functionally related endoproteinases share common functional domains and activation mechanisms. The MMPs were the first proteinase targets seriously considered for combating cancer. After encouraging preclinical results in various cancer models several of the MMP inhibitors were tested in advanced clinical trials but all failed because of severe side effects or no major clinical benefit (Turk et el., 2006). These include: hydroxamate inhibitors batimastat, marimastat, and prinomastat and the non-hydroxamate ones such as neovastat (an extract from shark cartilage of a molecular mass up to 500kDa introduced by Aeterna) rebimastat and tanomastat (Fig. 22).

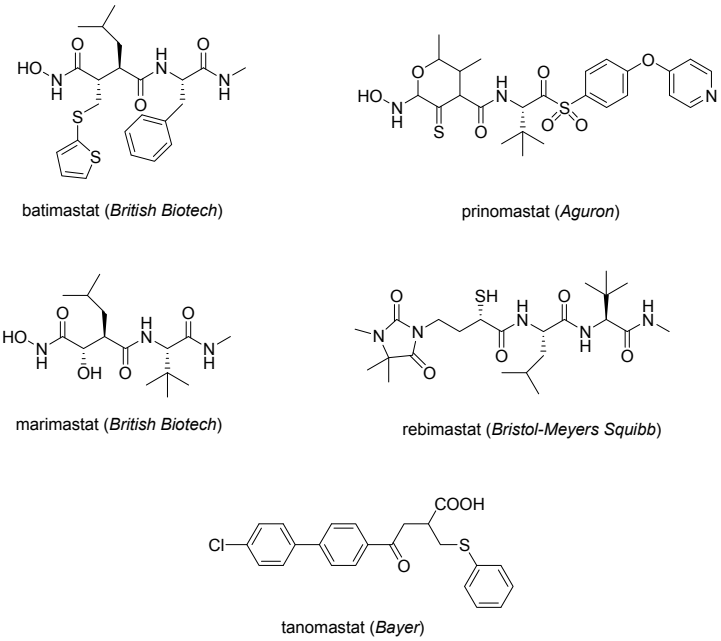

Fig. 22. Matrix metalloproteinase inhibitors, which failed in clinical studies. In parentheses companies, which introduced these compounds are given.

Clinical studies indicated that timeframe of targeting MMPs differs, depending on the stage of cancer, because the expression profile, as well as the activity of these enzymes, is not the same in the early stage compared to advanced cancer disease. As a consequence, the use of broad-spectrum inhibitors raises concerns that certain MMPs that exert anticancer effects are inhibited, which in turn may result in promotion of the disease (Gialeli et al., 2011). Thus, pharmacological targeting of cancer by the development of a new generation of effective and selective inhibitors to individual matrix metalloproteinases is an emerging and promising area of research (Devel et al., 2010; Manello, 2006). However, despite intense efforts, very few highly selective inhibitors of these metalloproteinases have been discovered up to now. This is because MMPs have catalytic domains composed of 160–170 amino acid residues that share a marked sequence similarity, with the percentage of identical residues being in the range of 33% to 90%. The three dimensional structure of the catalytic domains of 12 out of 23 human MMPs has been solved either by X-ray crystallography or NMR (Maskos, 2005), and the results supported that they are of significant similarity. The other cause of low specificity of most of MMP inhibitors is that their action relays on strong complexation of zinc ion present in the active sites of these enzymes. This is especially true in the case of hydoxamic acid-based inhibitors (Yiotakis & Dive, 2008), which are the most intensively studied so far (Attolino et al., 2010; Fisher & Mobashery, 2006; Nuti et al., 2010).

Among different zinc-binding groups, the phosphoryl moiety was thought to be the weakest binder. Indeed, it turns out that numerous peptide analogues with a phosphorus-containing moiety replacing the scissile amide bond have been found to regulate the activity of

metalloproteinases (Collinsová & Jiraček, 2000). The intense optimization of the phosphinic inhibitor structures, using parallel or combinatorial chemistry, is generally required to identify nanomolar inhibitors and to get selectivity (Dive et al., 2004). Without selective inhibitors, which are indispensable tools for studying the structure and the role of individual enzymes at different stages of complex tumorigenesis, anticancer strategies based on MMP inhibition are unlikely to provide important therapeutic benefits. Two representative inhibitors of this class of inhibitors are shown in Figure 23.
High-throughput screening of chemical libraries has also led to the discovery of unusual MMP inhibitors, selective against MMP-13. Among these, a new class of MMP inhibitors that do not possess a zinc-binding group and thus do not interact directly with the zinc active site ion is of special interest (Fig. 23) (Chen et al., 2000).

6.2 Aminopeptidases

Aminopeptidases are proteolytic enzymes that hydrolyze peptide bonds from the amino termini of polypeptide chains with the release of a single amino acid residue from polypeptide substrates. Although their involvement in tumorigenesis was well established the studies on their anticancer properties are far less developed than studies on MMPs. This may also result from the fact that physiologic role of these enzymes is far more complex.
A plethora of inhibitors of aminopeptidases have been synthesized and tested clinically against various pathological disorders, including cancers (Bauvois & Dauzonne, 2006; Mucha et al., 2010; Selvakumar et al., 2006; Wickström et al., 2011). Bestatin, a general inhibitor of aminopeptidases and aspartyl proteinases, has been the most intensively studied (Fig. 24). It was originally isolated from *Streptomyces olivoreticuli* more than 30 years ago (Umezawa et al., 1976). Bestatin studies in biological systems both *in vitro* and *in vivo*, resulted in discovery of several interesting properties of this compound such as ability to induce apoptosis in cancer cells, and anti-angiogenic, anti-malarial or immunomodulatory effects. Presently, bestatin (Ubenimex®) is on Japanese market where it is applied for treatment of cancer and bacterial infections. Examples of successful inhibition of aminopeptidases by bestatin include aminopeptidase N (CD13), leucine aminopeptidase (LAP) and aminopeptidase B. These aminopeptidases, as well as methionine aminopeptidase 2 are the most exploited targets to obtain new anticancer agents.
In contrast to MMPs selectivity of the inhibitor is not a required feature and in most cases general inhibitors of aminopeptidases are used in clinical studies. Such an example is tosedostat (Fig. 24) (Krige et. al, 2008; Moore et al., 2009), a hydroxamic acid inhibitor of M1 family of aminopeptidases (especially leucine aminopeptidase), which is now being introduced to the market by Chroma Therapeutics. In clinical studies tosedostat was well tolerated, given orally once a day, and it has produced encouraging response rates in difficult to treat patients with acute leukemia and a variety of blood related cancers. Tosedostat (CHR-2797) is a prodrug and exposure of cancer cells to this drug results in the generation of the active metabolite CHR-79888 (Fig. 24), which is poorly membrane-permeable, what limits its pharmacological activity. The use of prodrug results in intracellular accumulation of CHR-79888 and desirable physiological effect.

6.3 Carboxypeptidases

Carboxypeptidases cleave the peptide bond of amino acid residue at the carboxylic terminus of protein or peptide. Humans contain several types of carboxypeptidases, which have diverse functions ranging from catabolism to protein maturation. There is practically lack of

information about the role of carboxypeptidases in tumorigenesis. However, some of them were proposed as markers of individual tumors (Kemik et al., 2011; Lee et al., 2011). This indicates that they also might be considered as targets in anticancer therapy. Indeed, there are two reports on antitumor activity of two endogenous protein inhibitors of carboxypeptidases – latexin (Pallares et al., 2005) and retinoic acid-induced tumor suppressor retinoic acid receptor responder 1 (RARRES 1) (Sahab et al., 2011).

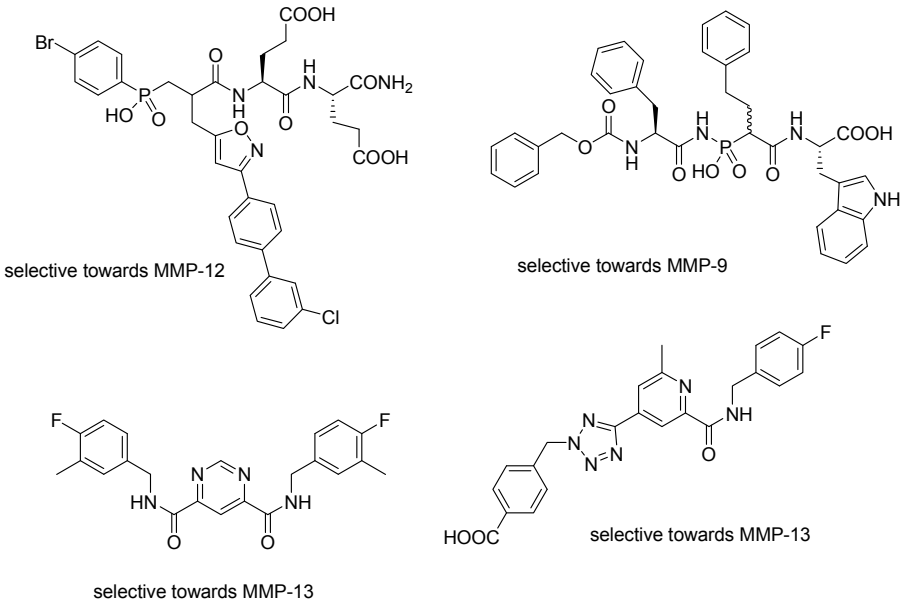

Fig. 23. Selective MMP inhibitors.

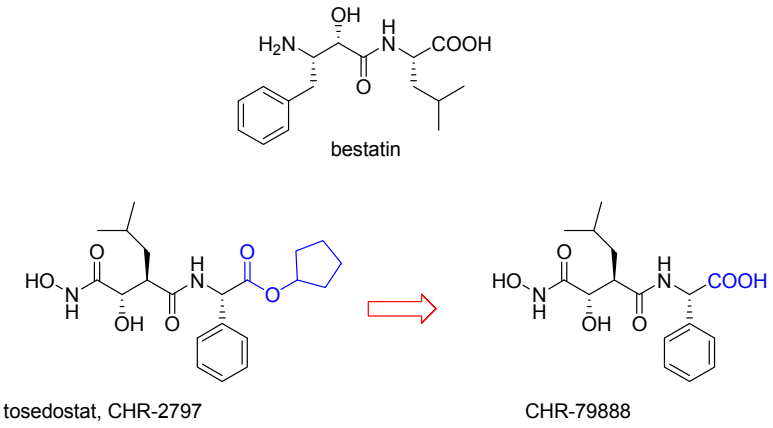

Fig. 24. Bestatin and tosedostat – general inhibitors of aminopeptidases and promising anticancer drugs.

7. Conclusion

Looking back at the progress made with anticancer therapies using inhibitors of various proteinases it is hard to consider it as particularly successful. Today the major successful areas in protease-targeted therapies are the cardiovascular, inflammatory and infectious diseases (mostly anti-HIV), however, the intensive studies on therapies of cancer and neurodegradative disorders are predicted. This is a good prognosis if taking into account that the annual spending for protease-directed drugs amounts close to US$ 10 billion annually (Turk, et al., 2006).

It is worth to note that past drug failures are not worthless. They provide not only invaluable lessons but are also a useful resource of data, which could still be used.

In order to achieve more satisfactory results, better understanding of the proteolytic network in tumor envinroment and increased knowledge in protease biology based on comprehensive analysis of protease activity in physiologically relevant conditions are required. The fact that tumor cells are only one part of the tumor environment and that extracellular matrix components and stromal cells are important contributors to the proteolytic activity of tumors should also be taken into consideration. For example, the use of transgenic animals may help in elucidation of the role of individual components of this complex networks.

Also the techniques of inhibitor design are developing significantly with *in silico* structure-based ligand design and various types of high-throughput screening being the major ones. Today's strategy in inhibitor design is to provide compounds complementary to active sites of the inhibited proteins, while the other concepts are used scarcely. One of the solutions is to design allosteric inhibitors altering proteinase activity by binding outside the enzyme active site, most likely in the cavity lacking any physiological role. The development of computer-aided methods for drug design (especially docking procedures) might be very helpful in this respect.

8. Acknowledgment

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4

Histamine Receptors as Potential Therapeutic Targets for Cancer Drug Development

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1. Introduction

Although research over the last decade has led to new and improved therapies for a variety of different diseases, anticancer drug therapy continues to have undesirable outcomes, including both poor response and severe toxicity. In addition to the critical need to discover new drugs, it is important to optimize existing therapies in order to minimize adverse reactions and maximize efficacy.

In the context of the complexity of cancer disease processes, future anticancer treatments will have to take into account the tumour microenvironment and aim to target the different cellular and molecular participants encompassed in a tumour, as well as their specific interactions.

In the present chapter we aimed to briefly summarize current knowledge on histamine and histamine receptors involvement in cancer, focusing on some recent evidence that points them out as a promising molecular targets and avenue for cancer drug development. On the basis of the role on immune system, it has been reported the efficiency of histamine as an adjuvant to tumour immunotherapy. In addition, we present here novel findings, suggesting the potential application of histamine and its ligands as adjuvants to tumour radiotherapy.

2. Histamine receptors

It is generally acknowledged that histamine is an important regulator of a plethora of (patho) physiological conditions and exerts its actions through the interaction with four histamine receptor subtypes. All these receptors belong to the family of heptahelical G-protein coupled receptors (GPCR) and they are the H_1, H_2, H_3 and H_4 histamine receptors (H_1R, H_2R, H_3R, H_4R). Based on the classical pharmacological analysis H_1R was proposed in 1966 by Ash and Schild (Ash & Schild, 1966) and H_2R was described in 1972 by Black et al. (Black et al., 1972). The third histamine receptor was discovered in 1983 by a traditional pharmacological approach, consisting of assessing the inhibitory effect of histamine on its own release from depolarized rat brain slices (Arrang et al., 1983). It was not until 2000-2001

that by using the H_3R DNA sequence, several independent research groups identified the novel H_4R highly expressed in immune cells (Coge et al. 2001b; Lui et al., 2001; Morse et al. 2001; Nakamura et al., 2000; Nguyen et al. 2001; Oda et al., 2000).
Recent studies employing human genetic variance and mice lacking specific receptors or the ability to generate histamine, have shown functions for the histamine pathway that extend well beyond the established roles. As a result, antihistamines may have wider applications in the future than previously predicted (Smuda & Bryce, 2011).

	Agonists	**Antagonists/ Inverse agonists**
H_1R	Histaprodifens, 2-(3-trifluoromethylphenyl) histamine	Mepyramine, cetirizine, terfenadine diphenhydramine, loratadine
H_2R	Amthamine, impromidine, arpromidine	Famotidine, ranitidine, cimetidine, roxatidine, zolantidine
H_3R	R-(α)-methylhistamine, imetit, immepip	Clobenpropit, thioperamide, iodoproxyfan
H_4R	Clobenpropit, VUF 8430, imetit, 4-methylhistamine, R-(α)-methylhistamine, OUP-16, clozapine	Thioperamide, JNJ7777120, VUF 6002, A-987306, A-940894

Table 1. Compounds most widely used in histamine receptor investigation

Like most other GPCR, histamine receptors exist as equilibrium between their inactive and active conformations. Constitutive activity has now been shown for all four types of histamine receptors, leading to the reclassification of some antagonists as inverse agonists. These members of the GPCR family may exist as homo- and hetero-oligomers at the cell surface, which could have different pharmacological and physiological effects (Bongers et al., 2007; Fukushima et al., 1997; Hancock et al., 2003; Leurs et al., 2002, 2009). Moreover, the affinity of histamine binding to different histamine receptors varies significantly. Thus, the effects of histamine and receptor ligands upon receptor stimulation are rather complex.
Pharmacologic agents are summarized in table 1.

2.1 Histamine H_1R

Since histamine is considered to be the most important mediator in allergies such as allergic rhinitis, conjunctivitis, atopic dermatitis, urticaria, asthma and anaphylaxis, the most commonly used drugs to treat these pathological disorders are antihistamines acting on the H_1R. In the lung, it mediates bronchoconstriction and increased vascular permeability. The H_1R is expressed in a wide variety of tissues, including airway and vascular smooth muscle, endothelia, gastrointestinal tract, liver, genitourinary and cardiovascular systems, central nervous system (CNS), adrenal medulla, chondrocytes and in various immune cells including neutrophils, monocytes, eosinophils, dendritic cells (DC), as well as T and B lymphocytes, in which it mediates the various biological manifestations of allergic responses. The coding sequence of the human H_1R is intronless and is located in the chromosome 3 (Bakker et al., 2001; Dy & Schneider, 2004; Leurs et al., 1995). The human H_1R

contains 487 amino acids and is a Gαq/11-coupled protein with a very large third intracellular loop and a relatively short C-terminal tail. The most important signal induced by ligand binding is the activation of phospholipase C (PLC)-generating inositol 1,4,5-triphosphate (Ins (1,4,5) P3) and 1,2-diacylglycerol leading to increased cytosolic calcium. In addition to the inositol signalling system, H_1R activation could lead to additional secondary signalling pathways. This rise in intracellular calcium levels seems to account for the various pharmacological activities promoted by the receptor, such as nitric oxide production, vasodilatation, liberation of arachidonic acid from phospholipids and increased cyclic guanosine-3′,5′-monophosphate (cGMP). Additionally, it was reported that H_1R can directly increase the cyclic adenosine-3′,5′-monophosphate (cAMP) levels (Davio et al., 1995). H_1R also activates NF-kB through Gαq11 and Gβγ upon agonist binding, while constitutive activation of NF-kB occurs only through the Gβγ (Bakker et al., 2001; Leurs et al., 1995; Smit et al., 1999). Recently, it was reported that the stimulation of H_1R induced H_1R gene expression through protein kinase C δ (PKCδ) activation, resulting in receptor upregulation (Mizuguchi et al., 2011).

2.2 Histamine H_2R

The H_2R principal action from a clinical point of view is its role in stimulating gastric acid secretion, thus H_2R antagonists are used in the relief of symptoms of gastro-oesophageal reflux disease treatment. The human H_2R intronless gene, encodes a protein of 359 amino acids and is located on chromosome 5. The H_2R has a ubiquitous expression as the H_1R. It is expressed in gastric parietal cells, heart, endothelial cells, nerve cells, airway and vascular smooth muscle, hepatocytes, chondrocytes and immune cells, such as neutrophils, monocytes, eosinophils, DC, and T and B lymphocytes (Black et al., 1972; Dy & Schneider, 2004; Leurs et al., 1995). The H_2R is coupled both to adenylate cyclase via a GTP-binding protein G_s, and phosphoinositide second messenger systems by separate GTP-dependent mechanisms. However, H_2R-dependent effects of histamine are predominantly mediated by cAMP that activates protein kinase A (PKA) enzymes phosphorylating a wide variety of proteins involved in regulatory processes. Activation of H_2R is also associated with other additional signal transduction pathways including activation of c-Fos, c-Jun, PKC and p70S6kinase (Davio et al., 1995; Fitzsimons et al., 2002; Fukushima et al., 1997).

2.3 Histamine H_3R

The H_3R has initially been identified in both central and peripheral nervous system as a presynaptic receptor controlling the release of histamine and other neurotransmitters (dopamine, serotonine, noradrenalin, γ-aminobutyric acid and acetylcholine) (Arrang et al., 1983; Bongers et al., 2007; Leurs et al., 2005; Lovenberg et al., 1999). The H_3R has gained pharmaceutical interest as a potential drug target for the treatment of various important disorders like obesity, myocardial ischemia, migraine, inflammatory diseases and several CNS disorders like Alzheimer's disease, attention-deficit hyperactivity disorder and schizophrenia. Pitolisant (BF2.649, 1-{3-[3-(4-chlorophenyl)propoxy]propyl} piperidine, hydrochloride) is the first H_3R inverse agonist to be introduced in the clinics. Its wake-promotion activity was evidenced in excessive diurnal sleepiness of patients with narcolepsy, Parkinson's disease or obstructive sleep apnea/hypopnea (Bongers et al., 2007; Lebois et al., 2011; Leurs et al., 2005; Schwartz, 2011). The human H_3R gene consists of either three exons and two introns, or four exons and three introns spanning 5.5 kb on

chromosome 20. Alternatively, the most 3' intron has been proposed to be a pseudo-intron as it is retained in the hH$_3$R(445) isoform, but deleted in the hH$_3$R(413) isoform. Overall similarity between the H$_3$R and the H$_1$R and H$_2$R amounts to only 22% and 20%, respectively (Bongers et al., 2007; Coge et al., 2001a; Dy & Schneider, 2004; Leurs et al., 2005; Tardivel-Lacombe et al., 2001; Wellendorph et al., 2002).

The cloning of the human H$_3$R has led to the discovery of many H$_3$R isoforms generated through alternative splicing of the H$_3$R mRNA. H$_3$R can activate several signal transduction pathways, including Gi/o-dependent inhibition of adenylate cyclase that leads to inhibition of cAMP formation, activation of mitogen activated protein kinase pathway (MAPK), phospholipase A2, and Akt/protein kinase B, as well as the inhibition of the Na+/H+ exchanger and inhibition of K+-induced Ca2+ mobilization (Bongers et al., 2007; Coge et al., 2001a; Leurs et al., 2005; Wellendorph et al., 2002). A negative coupling to phosphoinositide turnover in the human gastric cell line HGT has also been described (Cherifi et al., 1992). Moreover, at least 20 isoforms of the human H$_3$R have been described and they vary in the length of the third intracellular loop, their distinct CNS localization, differential signalling pathways and ligand binding affinity, which contribute to the heterogeneity of H$_3$R pharmacology (Bongers et al., 2007; Coge et al., 2001a; Hancock et al., 2003; Leurs et al., 2005).

2.4 Histamine H$_4$R

The identification by genomics-based approach of the human H$_4$R by several groups has helped refine our understanding of histamine roles. It appeared to have a selective expression pattern restricted to medullary and peripheral hematopoietic cells including eosinophils, mast cells, DC, T cells and monocytes. Therefore, growing attention is directed towards the therapeutic development of H$_4$R ligands for inflammation and immune disorders. Several lines of evidence suggest a role of the H$_4$R in chronic inflammatory skin disease and the H$_4$R might be a therapeutic target for diseases such as atopic dermatitis (Gutzmer et al., 2011). In addition, H$_4$R was reported to be present on other cell types including intestinal epithelium, spleen, lung, stomach, CNS, nerves of nasal mucosa, enteric neurons and interestingly in cancer cells (Cianchi et al., 2005; Coge et al. 2001b; Connelly et al., 2009; Leurs et al. 2009; Lui et al., 2001; Medina et al., 2006; Morse et al. 2001; Nakamura et al., 2000; Nguyen et al. 2001; Oda et al., 2000). The significance of the H$_4$R presence in various human tissues remains to be elucidated and therefore, new roles of H$_4$R are still unrevealed (Leurs et al., 2009; Zampeli & Tiligada, 2009). The H$_4$R cDNA was finally identified in the human genome database on the basis of its overall homology (37%, 58% in transmembrane regions) to the H$_3$R sequence and it has a similar genomic structure. On the other hand, the homology with H$_1$R and H$_2$R is of approximately 19%. The human H$_4$R gene that mapped to chromosome 18 is interrupted by two large introns and encodes a protein of 390 amino acids (Coge et al., 2001b; Leurs et al., 2009). H$_4$R is coupled to Gαi/o proteins, inhibiting forskolin-induced cAMP formation (Nakamura et al., 2000; Oda et al., 2000). Additionally, stimulation of H$_4$R leads to activation of MAPK and also increased calcium mobilization via pertussis toxin-sensitive pathway (Leurs et al., 2009; Morse et al., 2001).

Isoforms have been described for the H$_4$R which have different ligand binding and signalling characteristics. H$_4$R splice variants [H$_4$R (67) and H$_4$R (302)] have a dominant negative effect on H$_4$R (390) functionality, being able to retain it intracellularly and to inactivate a population of H$_4$R (390) presumably via hetero-oligomerization (Leurs et al.,

2009; van Rijn et al., 2008). In addition, H_4R dimeric structures that include homo- and hetero-oligomer formation and post-translational changes of the receptor might contribute to added pharmacological complexity for H_4R ligands (Leurs et al., 2009; van Rijn et al., 2006, 2008).

3. Histamine receptors in breast cancer

An estimated 1 million cases of breast cancer are diagnosed annually worldwide. Breast cancer is the most common neoplastic disease in women, and despite advances in early detection, about 30% of patients with early-stage breast cancer have recurrent disease, which is metastatic in most cases and whose cure is very limited showing a 5-year survival rate of 20% (Ferlay et al., 2010; Gonzalez-Angulo et al., 2007).

Histamine plays a critical role in the pathologic and physiologic aspects of the mammary gland, regulating cell growth, differentiation and functioning during development, pregnancy and lactation. Among monoamines, histamine demonstrates the greatest proliferative activity in breast cancer (Davio et al., 1994; Malinski et al., 1993; Wagner et al., 2003). Furthermore, histamine is increased in plasma and cancerous tissue derived from breast cancer patients compared to healthy group which is associated to an enhanced histidine decarboxylase (HDC) activity and a reduced diaminooxydase (DAO) activity that determine an imbalance between the synthesis and degradation of this monoamine. Histamine plasma level is dependent on the concentration of histamine in the tissues of ductal breast cancers, suggesting the participation of this monoamine in the development of this neoplasia (Reynolds et al., 1998; Sieja et al., 2005; von Mach-Szczypiński et al., 2009). A pilot study revealed that in samples of the same invasive ductal carcinoma patient, histamine peripheral blood levels tended to be reduced post-operatively (Kyriakidis et al., 2009). It was reported that in experimental mammary carcinomas, histamine becomes an autocrine growth factor capable of regulating cell proliferation via H_1R and H_2R, as one of the first steps responsible for the onset of malignant transformation. In this light, the *in vivo* treatment with H_2R antagonists produced the complete remission of 70% of experimental tumours (Cricco et al., 1994; Davio et al., 1995; Rivera et al., 2000). Many reports indicate the presence of H_1R and H_2R in normal and malignant tissues as well as in different cell lines derived from human mammary gland. H_2R produced an increase in cAMP levels while H_1R was coupled to PLC activation in benign lesions. On the other hand, H_1R was invariably linked to PLC pathway but H_2R stimulated both transductional pathways in carcinomas (Davio et al., 1993, 1996). However, the clinical trials with H_2R antagonists demonstrated controversial results for breast cancer (Bolton et al., 2000; Parshad et al., 2005).

Recently, it was demonstrated that H_3R and H_4R are expressed in cell lines derived from human mammary gland (Medina et al., 2006). Histamine is capable of modulating cell proliferation exclusively in malignant cells while no effect on proliferation or expression of oncogenes related to cell growth is observed in non-tumorigenic HBL-100 cells (Davio et al., 2002; Medina et al., 2006). Furthermore, histamine modulated the proliferation of MDA-MB-231 breast cancer cells in a dose-dependent manner producing a significant decrease at 10 $\mu mol.L^{-1}$ concentration whereas at lower concentrations increased proliferation moderately. The negative effect on proliferation was associated to the induction of cell cycle arrest in G2/M phase, differentiation and a significant increase in the number of apoptotic cells (Medina et al., 2006; Medina & Rivera, 2010b). Accordingly, by using pharmacological tools, results demonstrated that histamine increased MDA-MB-231 cell proliferation and also

migration via H_3R. In contrast, clobenpropit and VUF8430 treatments significantly decreased proliferation. This outcome was associated to an induction of apoptosis determined by Annexin-V staining and TdT-mediated UTP-biotin Nick End labelling (TUNEL) assay, which was blocked by the specific H_4R antagonist JNJ7777120. Also H_4R agonists exerted a 2.5-fold increase in the cell senescence while reduced migration (Medina et al., 2008, 2010c, 2011b). Furthermore, histamine differentially regulates expression and activity of matrix metalloproteinases, cell migration and invasiveness through H_2R and H_4R in MDA-MB-231 cells modulating H_2O_2 intracellular levels (Cricco et al., 2011).
In addition, histamine at all doses tested, decreased the proliferation of a more differentiated breast cancer cell line, MCF-7, through the stimulation of the four histamine receptor subtypes exhibiting a higher effect through the H_4R. Treatment of MCF-7 cells with the H_4R agonists, inhibited cell proliferation and increased apoptosis and senescence (Medina et al., 2011b). These results represent the first report about the expression of H_3R and H_4R in human breast cells and interestingly show that the H_4R is involved in the regulation of breast cancer cell proliferation, apoptosis, senescence, migration and invasion.
Recent results obtained with the orthotopic xenograft tumours of the highly invasive human breast cancer line MDA-MB-231 in immune deficient nude mice indicate that the H_4R was the major histamine receptor expressed in the tumour. Remarkably, *in vivo* JNJ7777120 treatment (10 $mg.kg^{-1}$, *p.o.*, daily administration) significantly decreased lung metastases, indicating that H_4R may be involved in the metastatic process (Medina & Rivera, 2010b). In addition, *in vivo* clozapine treatment (1 $mg.kg^{-1}$, *s.c.*, daily administration) significantly decreased tumour growth while enhanced survival of bearing tumour mice (Martinel Lamas et al., unpublished data).
Recent data indicate that H_3R and H_4R are expressed in human biopsies of benign lesions and breast carcinomas being the level of their expression significantly higher in carcinomas, confirming that H_3R and H_4R are present not only in cell lines but also in the human breast tissue. Furthermore, the expression of H_3R is highly correlated with proliferation and histamine production in malignant lesions while the 50% of malignant lesions expressed H_4R, all of them corresponding to metastases or high invasive tumours (Medina et al., 2008).
The identification of histamine receptor subtypes and the elucidation of their role in the development and growth of human mammary carcinomas may represent an essential clue for advances in breast cancer treatment. The presented evidences contribute to the identification of molecules involved in breast carcinogenesis and confirm the role of H_4R in the regulation of breast cancer growth and progression representing a novel molecular target for new therapeutic approach.

4. Histamine receptors in lymphomas and leukaemia

There is increasing evidence that histamine plays a role in cell differentiation and proliferation in several of normal tissues and in a wide range of tumours, including haematological neoplasias.
After an initial work in the late 1970s showing that histamine is able to induce haematopoietic stem cell proliferation via H_2R (Byron, 1977), a real rush broke out in searching for further effects of histamine in haematopoiesis and haematological neoplasias. The histamine levels were determined in lymph nodes of patients with malignant lymphomas, Hodking´s disease (HD) or non-Hodking lymphomas (NHL), and in all cases the values were higher than in controls. In patients with NHL, these levels showed

dependence on the grade of malignancy as they found to be significantly higher in those classified as high-grade malignant (Belcheva & Mishkova, 1995). Immunostaining and ELISA method also confirmed the presence of histamine in the cytoplasm of acute lymphocytic leukaemia (ALL) cells, and H_1R antihistamines inhibited their clonogenic growth. There was no correlation between the clonogenic growth of ALL cells and their histamine content, suggesting that while histamine may be important for the clonogenic growth of ALL cells; other factors also affect their clonogenity (Malaviya et al., 1996). Furthermore, leukaemia cell lines such as U937, expressed histamine receptors and a switch of histamine receptor expression from H_2R to H_1R during differentiation of monocytes into macrophages is observed (Wang et al., 2000).

Most patients with acute myeloid leukaemia (AML) achieve complete remission after induction chemotherapy. Despite ensuing courses of consolidation chemotherapy, a large fraction of patients will experience relapses with poor prospects of long-term survival. Interleukin-2 (IL-2) and interferon-alpha (IFN-alpha) are effective activators of lymphocytes with anti-neoplastic properties, such as T-cells or natural killer (NK) cells, constituting the basis for their widespread used as immunotherapeutic agents in human neoplastic disease. The functions of intratumoural lymphocytes in many human malignant tumours are inhibited by reactive oxygen species (ROS), generated by adjacent monocytes/macrophages. *In vitro* data suggest that those immunotherapeutic cytokines only weakly activate T cells or NK cells in a reconstituted environment of oxidative stress and inhibitors of ROS formation or ROS scavengers synergize with IL-2 and IFN-alpha to activate T cells and NK cells. Recently, IL-2 therapy for solid neoplastic diseases and haematopoietic cancers has been supplemented with histamine dihydrochloride (Ceplene), a synthetic derivative of histamine, with the aim of counteracting immunosuppressive signals from monocytes/macrophages. Histamine dihydrochloride inhibits the formation of ROS that suppress the activation of T cells and NK cells by suppressing the activity of NADPH oxidase via H_2R. When administered in addition to IL-2, histamine dihydrochloride enables the activation of these lymphocytes by the cytokine, resulting in tumour cell killing. This combination was recently approved within the EU as a remission maintenance immunotherapy in AML, as histamine dihydrochloride reduces myeloid cell-derived suppression of anti-leukemic lymphocytes, improving NK and T-cell activation. Further research in this area will shed light on the role of histamine with the aim to improve cancer immunotherapy efficacy (Hellstrand et al., 2000; Martner et al., 2010; Yang & Perry, 2011).

5. Histamine receptors in gynaecologic cancers

Gynaecologic cancers encompass a remarkably heterogeneous group of tumours: cervical, ovarian, uterine, vaginal, and vulvar cancer. It has been postulated that histamine plays a critical role in proliferation of normal and cancer tissues, including the mammary gland, ovarian and endometrium.

In the murine uterus, the rapidly dividing epithelial cells of the endometrium can be defined as the major sources of histamine. In these cells the level of HDC expression is controlled mainly by progesterone-mediated signals which, interestingly, induce maximal level of HDC expression on the day of implantation (Pós et al., 2004).

In vitro studies showed that histamine may play an important role in follicular development and ovulation via H_1R and H_2R in women, acting as apoptosis inducer, taking part in the selection process of the dominant follicle and stimulating ovulation (Szukiewicz et al., 2007).

Interestingly, histamine content increased unequivocally in ovarian, cervical and endometrial carcinoma in comparison with their adjoining normal tissues, suggesting the participation of histamine in carcinogenesis. Besides, exogenous histamine, at micromolar concentration, stimulated proliferation of human ovarian cancer cell line SKOV-3 (Batra & Fadeel, 1994; Chanda & Ganguly, 1995). Preliminary results show that H_4R is expressed in primary and metastatic ovarian carcinoma and also in gallbladder cancer (Medina & Rivera, 2010b).

Histamine levels within ovarian tissue during the oestrus may correspond to cyclic changes of mast cells content and distribution in the ovary, suggesting an involvement of these cells in local regulation of ovarian function (Adyin et al., 1998; Nakamura et al., 1987). Interestingly, mast cells can typically be found in the peritumoural stroma of cervix carcinomas, as well as in many other cancers. Furthermore, high numbers of active, degranulated mast cells have been described in HPV infections and cervical intraepithelial neoplasias (Cabanillas-Saez et al., 2002; Demitsu et al., 2002). Hence, a functional relationship between mast cells and tumour cells has been proposed, where mast cells are involved in stimulating tumour growth and progression by enhancing angiogenesis, immunosuppression, mitogenesis, and metastasis (Chang et al., 2006). Mast cell activation leads to the release of inflammatory mediators, including histamine. Increased histamine levels have been described in the cervix lesions, where they have been associated with tumour growth and progression. Moreover, histamine receptors have been reported in different cell lines and tissues derived from experimental and human cervical neoplasias. The functional significance of immune cell infiltration of a tumour, specifically of mast cells located at the periphery of several neoplasias, is still a matter of controversy. Histamine acting via H_1R in cervical cancer cells could be pro-migratory, but when acting via H_4R could inhibit migration. On the other hand, other results also showed that cervical carcinoma cell mediators can activate mast cells to degranulate, demonstrating an active and dynamic cross-talk between tumour cells and infiltrating mast cells as shown in morphologic studies of neoplastic tissues (Rudolph et al., 2008).

In the light of these results, further investigations have to be done in order to elucidate the physiological role of histamine receptors on cell proliferation, as well as its implication in gynaecologic cancer progression with a potential interest for cancer treatment.

6. Histamine receptors in colorectal cancer

Colorectal cancer is one of the leading causes of cancer death among both men and women worldwide (Ferlay et al., 2010). It has been previously described that the histamine catabolising enzymes, DAO or histamine N-methyltransferase (HNMT), activities were significantly lower in adenoma tissue than in healthy mucosa in the same patients (Kuefner et al., 2008). Furthermore, HDC expression and its activity are increased in many human tumours including colorectal cancer (Cianchi et al., 2005; Masini et al., 2005; Reynolds et al., 1997). The levels of histamine were elevated in colon carcinoma and this is directly related to an increase in HDC expression and a decrease in DAO activity (Chanda & Ganguly, 1987). Also, the distribution of histamine receptors in the normal intestinal tract was reported (Sander et al., 2006). It was showed the expression pattern of H_1R, H_2R and H_4R in intestinal tract, receptors that were over expressed in the colon of patients with irritable bowel syndrome and food allergies. Furthermore, the H_3R was not detected in intestinal tissue (Sander et al., 2006). This data was further confirmed by Boer K et al, that also demonstrated

a decreased of H_1R and H_4R protein levels in colorectal cancer while the levels of the H_2R were not modified compared to normal colon mucosa (Boer et al., 2008).
It was described that the H_1R antagonist, loratadine, inhibited proliferation and enhanced radiosensitivity in human colon cancer cells (Soule et al., 2010). Also the H_2R seems to be implicated in the proliferation of colon cancer. In 1994 Adams, showed that *in vivo* and in two human colonic adenocarcinoma cell lines, C170 and LIM2412, cell proliferation induced by histamine in a dose dependent manner was blocked by H_2R antagonist, cimetidine (Adams et al., 1994). Ranitidine, another H_2R antagonist, also showed to extend the survival of patients who were under surgery of colorectal cancer (Nielsen et al., 2002). It is well known the effects of histamine in the immune system, according to this it was demonstrated that patients receiving cimetidine or famotidine before curative resection augmented the probabilities of having tumour infiltrating lymphocytes in their tumours than control patients (Adams & Morris, 1996; Kapoor et al., 2005). Furthermore, earlier studies demonstrated that histamine induced *in vitro* and *in vivo* cell proliferation and this outcome was blocked by H_2R antagonists (Adams et al., 1994; Cianchi et al., 2005). This effect was associated with the attenuation of anti-tumour cytokine expression in the tumour microenvironment exerted by histamine, thus resulting in stimulated colorectal cancer growth (Takahashi et al., 2001; Tomita & Okabe, 2005). In addition, H_2R antagonist significantly suppressed the growth of tumour implants in mice by inhibiting angiogenesis via reducing VEGF expression (Tomita et al., 2003).
As it was described above, the expression of the H_4R seems to be suppressed in human colorectal cancer. It was also demonstrated that the levels of the H_4R are reduced in advanced colorectal cancer compared with those in an initiating state, which suggest that the H_4R expression is regulated during the progression of the disease (Fang et al., 2011). The stimulation *in vitro* of the H_4R by a specific agonist induced an augmented expression of the $p21^{Cip1}$ and $p27^{Kip1}$ proteins, producing an increase of arrested cells in the G1 phase. It has been proposed that prostaglandin E2 (PGE-2), the main product of the cyclooxygenase-2 activity, is implicated in colorectal cancer development. In this line, it has been demonstrated that histamine is fully implicated in the production of PGE-2 by its two receptors H_2R and H_4R in two human colon carcinoma cell lines (Cianchi et al., 2005). Histamine effect can be blocked by zolantidine, an H_2R antagonist, and also by JNJ7777120, an H_4R antagonist, whereas mepyramine, an H_1R antagonist, has no effect on the production of PGE-2. Furthermore, JNJ7777120 inhibited the cell growth induced by histamine in three different human colon cancer cell lines and also inhibited the histamine-mediated increase in VEGF in two cell lines. Combined treatment with zolantidine (an H_2R antagonist) and JNJ7777120 determined an additive effect on reducing the histamine-induced VEGF production and histamine-stimulated proliferation (Cianchi et al., 2005), suggesting the involvement of H_4R in colon carcinogenesis (Boer et al., 2008).

7. Histamine receptors in melanoma

Malignant melanoma arises from epidermal melanocytes and despite being the cause of less than 5% of skin cancers, it is responsible for the large majority of skin cancer deaths (Ferlay et al., 2010). Early detection is vital for long-term survival, given that there is a direct correlation between tumour thickness and mortality (Cummins et al., 2006).
Melanoma cells but not normal melanocytes contain large amounts of histamine that has been found to accelerate malignant growth (Pós et al., 2004). The absence of expression of

HDC in Mel-5 positive melanocytes isolated from skin samples of healthy persons, suggest that the level of HDC is strongly associated with malignancy in the skin (Haak-Frendscho et al., 2000). As a functional consequence of the inhibition of HDC protein synthesis, specific antisense oligonucleotide strongly (> 50%) decreased the proliferation rate of both WM938/B and HT168/91 human melanoma cells. Similar effects were found with other two melanoma cell lines WM35 and M1/15, suggesting that endogenous histamine may act as an autocrine growth factor (Hegyesi et al., 2000). On the other hand, overexpression of HDC markedly accelerated tumour growth and increased metastatic colony-forming potential along with rising levels of local histamine production that was correlated with tumour H_2R and rho-C expression in mouse melanoma (Pós Z et al., 2005).

It has been previously reported the expression of H_1R, H_2R and H_3R in melanoma cell lines (Hegyesi et al., 2005). In addition, it was described that in human melanoma cells, histamine acting through the H_1R decreases cell proliferation, whereas it enhances growth when acting through the H_2R (Lázar-Molnar et al., 2002). Furthermore, there is no evidence of mitogenic signalling through the H_3R in human melanoma (Hegyesi et al., 2005).

H_1R function is involved in chemotaxis via PLC activation, and its subsequent intracellular calcium mobilization. Proliferation assays showed that histamine exerted a concentration dependent dual effect on proliferation of the WM35 primary melanoma cell line. High concentrations of histamine (10^{-5} M) had an inhibitory effect while lower concentrations (10^{-7} M) increased colony formation. Similar results were achieved when using H_1R agonist 2-(3-fluoromethylphenyl)histamine and H_2R agonist arpromidine, respectively. The use of ranitidine, famotidine and cimetidine, all H_2R specific antagonists, abolished the stimulatory effect of histamine on cell proliferation, indicating the participation of H2R in this mitogenic role of histamine. Second messenger measurement indicated that H_2R are linked to cAMP production, thus suggesting an involvement of PKA in the mitogenic pathway triggered in this system, which is corroborated by the fact that forskolin and permeable cAMP analogues also produce a dose-dependent increase on cell proliferation (Lázar-Molnar et al., 2002).

Numerous *in vivo* studies employing animal models bearing syngenic or xenogenic melanoma grafts demonstrated that both endogenous and exogenous histamine have the ability to stimulate tumour growth while H_2R antagonists (e.g. cimetidine, famotidine, roxatidine) inhibited this effect (Pós et al., 2005; Szincsák et al., 2002; Tomita et al., 2005; Uçar, 1991). Additionally, H_2R antagonists stimulated melanogenesis and inhibited proliferation in B16-C3 mouse melanoma cells (Uçar, 1991). It was also found that melanoma tumour growth was not modulated by *in vivo* histamine treatment while treatment with terfenadine, an H_1R antagonist, *in vitro* induced melanoma cell death by apoptosis and *in vivo* significantly inhibited tumour growth in murine models (Blaya et al., 2010).

Differences between melanoma cells in their capacity to produce and degrade histamine could explain the different sensitivities of melanoma cell types to exogenous histamine treatment. Moreover, there is evidence that cytokines can influence HDC expression and activity. It has been shown that there is a regulation loop between interleukin 6 (IL-6) and histamine: histamine increased IL-6 expression and secretion in metastatic lines via the H_1R, and IL-6 treatment increased the HDC and histamine content in primary melanoma lines (Lázar-Molnar et al., 2002). Interferon-gamma (IFN-gamma) produced by surrounding immune cells decreases HDC expression, affecting melanoma growth and also impairs antitumour activity of the immune system, then contributing to the escape of melanoma cells from immunosurveillance (Horváth et al., 1999; Heninger et al., 2000). Furthermore,

mast cell activation initiates upon ultraviolet-B irradiation, which triggers histamine secretion acts as a cellular immunity suppressor (Chang et al., 2006).
Moreover, the role of histamine in local immune reactions was further supported by the results of Hellstrand et al., who found that histamine can inhibit the ROS formation of monocytes/macrophages in the tumour (Hellstrand et al., 2000). This may explain the clinical benefit demonstrated by histamine (Ceplene) as an adjuvant to immunotherapy with IL-2 in several phase II and III clinical trials in metastatic melanoma (Agarwala, 2002). The addition of histamine dihydrochloride to an outpatient regimen of IL-2 is safe and well tolerated and demonstrates a survival advantage over IL-2 alone (9.4 vs. 5.1 months) in melanoma patients with liver metastases (Agarwala, 2002). However, a second confirmatory phase III study failed to show any survival benefit for those patients (Naredi, 2002).
Besides, Medina et al. showed that exogenous histamine modulated the activity of the antioxidant enzymes, increasing superoxide dismutase while decreasing catalase activity in WM35 melanoma cells. Accordingly, histamine treatment markedly augmented the levels of hydrogen peroxide and diminished those of superoxide anion, indicating that the imbalance of antioxidant enzymes leads to the cell proliferation inhibition (Medina et al., 2009).
Furthermore, it was demonstrated that WM35 and M1/15 melanoma cells express H_4R at the mRNA and protein level. By using histamine agonists and antagonists it was shown that the inhibitory effect of histamine on proliferation was in part mediated through the stimulation of the H_4R. Treatment with a specific H_4R antagonist, JNJ7777120 and the use of siRNA specific for H_4R mRNA blocked the decrease in proliferation triggered by the H_4R agonists. Furthermore, the decrease in proliferation exerted by H_4R agonists was associated with a 2-fold induction of cell senescence and an increase in melanogenesis that is a differentiation marker on these cells (Massari et al., 2011). Current studies indicate that the H_4R is expressed in the 42% of human melanoma biopsies of different histopathological types, showing cytoplasmic localization and confirming that the H_4R is present not only in these cell lines but also in human melanoma tissue (Massari et al., 2011).
The *in vivo* subcutaneous daily 1 $mg.kg^{-1}$ histamine or 1 $mg.kg^{-1}$ clozapine (H_4R agonist) injections of M1/15 melanoma cell tumour bearing nude mice showed a survival increase vs. control group (treated with saline solution). Besides, results showed an antitumour effect of histamine and clozapine, including suppression of tumour growth (Massari et al., unpublished data). Further studies are needed to corroborate the H_4R importance as potential target for new drug development for the treatment of this disease.

8. Histamine as a potential adjuvant to radiotherapy

8.1 Radioprotectors

Radiotherapy is the most common modality for treating human cancers and relies on ionising radiation induced DNA damage to kill malignant cells. Eighty percent of cancer patients need radiotherapy at some time or other, either for curative or palliative purpose. To optimise results, a cautious balance between the total dose of radiotherapy delivered and the threshold limit of the surrounding normal critical tissues is required. In order to obtain better tumour control with a higher dose, the normal tissues should be protected against radiation damage. Therefore, the role of radioprotective compounds is of utmost importance in clinical radiotherapy (Hall & Giaccia, 2006; Mah et at., 2011). Ionising radiation causes damage to living tissues through a series of molecular events. DNA double-strand breaks (DSBs), which are exceptionally lethal lesions, can be formed either by direct energy

deposition or indirectly through the radiolysis of water molecules, which generate clusters of ROS that react with DNA molecules. Because human tissues contain 80% water, the major radiation damage produced by low linear transfer energy (LET) radiation is due to the aqueous free radicals. DSBs are essentially two single stranded nicks in opposing DNA strands that occur in close proximity, severely compromising genomic stability (Grdina, 2002; Hall & Giaccia, 2006; Mah et at., 2011). A series of complex pathways collectively known as the DNA damage response (DDR) is responsible for the recognition, signalling and repair of DSBs in cells, ultimately resulting in either cell survival or cell death (Mah et at., 2011). These free radicals react not only with DNA but also with other cellular macromolecules, such as RNA, proteins, membrane, *etc*, and cause cell dysfunction and mortality. Unfortunately, these reactions take place in tumour as well as normal cells when exposed to radiation. Therefore, to improve the efficacy of radiotherapy there is an intense interest in combining this modality with ionising radiation modifiers, such as radioprotectors. These compounds mitigate damage to surrounding non-malignant tissue (Brizel, 2007; Grdina, 2002; Hall & Giaccia, 2006; Hosseinimehr, 2007).

The most remarkable group of true radioprotectors is the sulfhydryl compounds. The simplest is cysteine, a sulfhydryl compound containing a natural amino acid (Table 2). In 1948, Patt discovered that cysteine could protect mice from the effects of total-body exposure to X-rays if the drug was injected or ingested in large amounts before the radiation exposure. At about the same time, in Europe independently discovered that cysteamine could also protect animals from total-body irradiation (Table 2). However, cysteine is toxic and induces nausea and vomiting at the dose levels required for radioprotection. A developmental program was initiated in 1959 and conducted at the Walter Reed Institute of Research to identify and synthesize drugs capable of conferring protection to individuals in a radiation environment by the U.S. Army. Over 4.000 compounds were synthesized and tested and it was discovered that the covering of the sulfhydryl group by a phosphate group reduced toxicity (Grdina, 2002; Hall & Giaccia, 2006; Nucifora et al., 1972).

The concept of the therapeutic ratio is central to understanding the rationale for using radioprotectors. It relates tumour control probabilities and normal tissue complication probabilities to one another. An ideal radioprotector will reduce the latter without compromising the former and should also be minimally toxic itself. Radioprotective strategies can be classified under the categories of protection, mitigation, and treatment. Protectors are administered before radiotherapy and are designed to prevent radiation-induced injury. Amifostine is the prototype drug (Table 2). Amifostine is the only radioprotective agent that is approved by FDA for preventing of xerostomia induced by gamma irradiation in patients under radiotherapy (Grdina et al., 2009; Hall & Giaccia, 2006; Hosseinimehr, 2007; Kouvaris et al., 2007, Wasserman & Brizel, 2001). Its selectivity for normal tissue is due to its preferential accumulation in normal tissue compared to the hypoxic environment of tumour tissues with low pH and low alkaline phosphatase, which is required to dephosphorylate and activate amifostine (Calabro-Jones et al., 1985; Grdina, 2002; Mah et at., 2011). The active metabolite, WR-1065 scavenges free radicals and is oxidised, causing anoxia or the rapid consumption of oxygen in tissues. This sulfhydryl compound is one of the most effective radioprotectors known nowadays, but there are two main problems of its using. The first one is their toxicity and the second is the short-ranged activity. Amifostine is also the unique radioprotector widely used in clinic on chemotherapy applications (Grdina et al., 2009; Hall & Giaccia, 2006; Hosseinimehr, 2007).

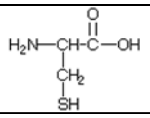
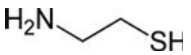
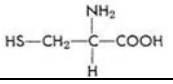
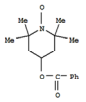
COMPOUND	SIDE EFFECTS	CHEMICAL STRUCTURE
Amifostine (WR-2721)	Drowsiness, feeling of coldness, flushing/feeling of warmth; hiccups, nausea, sneezing, vomiting	
Cysteamine	Depression, stomach or intestinal ulcer and bleeding, liver problems, skin condition, decreased calcification of bone, seizures, broken bone, decreased white blood cells	
Palifermin	Skin rash, flushing, unusual sensations in the mouth (tingling, tongue thickness)	C_{721}-H-$_{1142}$-N-$_{202}$-O_{204}-S_9
Cysteine	Toxic, nausea, vomiting	
Tempol	Constipation; diarrhoea, severe allergic reactions (rash; hives; itching; difficulty breathing; tightness in the chest; swelling of the mouth, face, lips, or tongue), loss of appetite, muscle weakness, nausea, slow reflexes, vomiting	

Table 2. Radioprotectors. Extracted and modified from http://www.wolframalpha.com/entities/chemicals/palifermin/hs/j8/6k/; http://www.drugs.com

Mitigants are administered after radiotherapy but before the phenotypic expression of injury and are intended to ameliorate injury. The keratinocyte growth factor (KGF), palifermin, has been approved as a new, targeted therapy for the prevention of severe oral mucositis in patients with head and neck cancer undergoing post-operative radiochemotherapy and can be considered as the prototype mitigant (Weigelt et at., 2011) (Table 2). Palifermin, like the natural KGF, helps maintain the normal structure of the skin and gastrointestinal surface (lining) by stimulating cells to divide, grow and develop (Le et at., 2011; Weigelt et at., 2011).

Treatment is a strategy that is predominantly palliative and supportive in nature. Pharmacologic radioprotective strategies should be integrated with physical strategies such as intensity-modulated radiotherapy to realize their maximum clinical potential (Hall & Giaccia, 2006; Le et al., 2011).

In addition, low-to-moderate doses of some agents such as nitroxides, adrenoceptor agonist, were found to have radioprotective activity in experiments but their application in clinic remains doubtful. Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidinyloxy) belongs to a class of water-soluble nitroxides which are membrane-permeable stable free radical compounds that confer protection against radiation-induced damage (Bennett et at., 1987; Mah et at., 2011; Muscoli et at., 2003) (Table 2). It is thought to elicit its effects through the oxidation of reduced transition metals, scavenging free radicals and mimicking superoxide dismutase activity (Jiang et al., 2007).

8.2 Histamine as a radioprotector

Despite many years of research there are surprisingly few radiation protectors in use today, whose clinical value is limited due to their toxicity; thus, the development of effective and nontoxic agents is yet a challenge for oncologists and radiobiologists (Hall & Giaccia, 2006).

The acute effects of irradiation result from the death of a large number of cells in tissues with a rapid rate of turnover. These include effects in the epidermal layer or skin, gastrointestinal epithelium, and haematopoietic system, in which the response is determined by a hierarchical cell lineage, composed of stem cells and their differentiating offspring. In clinical radiotherapy, the tolerance of normal tissues for radiation depends on the ability of clonogenic cells to maintain a sufficient number of mature cells suitably structured to preserve organ function (Hall & Giaccia, 2006). During radiotherapy for intra-abdominal and pelvic cancers, radiation seriously affects radiosensitive tissues such as small intestine and bone marrow (Erbyl et al., 2005; Hall & Giaccia, 2006). It was previously demonstrated that histamine treatment (daily subcutaneous injection, 0.1 $mg.kg^{-1}$) significantly protects mouse small intestine against radiation-induced toxicity ameliorating histological injury and improving trophism of enterocytes (Medina et al., 2007). Histamine completely prevented the decrease in the number of crypts evoked by whole body irradiation, which is vital for small intestine restoration since the intestinal crypt contains a hierarchy of stem cells that preserve the potential to regenerate the stem cell population and the tissue after cytotoxic exposure (Potten et al., 2002). Histamine radioprotective effect on small intestine was related to an increased rate of proliferation as evidenced by the enhanced proliferation markers immunoreactivity [5-bromo-2'-deoxyuridine (BrdU), and proliferating cell nuclear antigen (PCNA)]. Additionally, this outcome was accompanied by a reduction in the number of apoptotic cells per crypt and a modification of antioxidant enzyme levels that could lead to enhance the antioxidant capacity of intestinal cells (Medina et al., 2007). Histamine also protects rat small intestine against ionising radiation damage and this effect was principally associated to a decrease in intestinal cell crypt apoptosis (Medina & Rivera, 2010a).

The bone marrow pluripotent stem cells, such as erythroblast, are particularly radiosensitive and, after whole body irradiation, an important grade of aplasia is observed increasing the possibility of haemorrhage and/or infection occurrence that could be lethal. The survival of stem cells determines the subsequent repopulation of bone marrow after irradiation (Hall & Giaccia, 2006). Results demonstrated that histamine (0.1 $mg.kg^{-1}$) significantly reduced the grade of aplasia, ameliorating the oedema and vascular damage produced by ionising radiation while eliciting a significant conservation of the medullar progenies on bone marrow in mouse and rat species, increasing the number of megakaryocytes, myeloid, lymphoid and erythroid cells per mm^2. The histamine effect is mediated at least in part by an increase in the rate of proliferation, as evidenced by the enhanced PCNA protein expression and BrdU incorporation, and is associated with an enhanced HDC expression in irradiated bone marrow cells (Medina et al., 2010; Medina & Rivera, 2010a). In this line, it was reported that a faster bone marrow repopulation was observed in wild type in comparison with HDC-deficient mice and that intracellular HDC and histamine content in regenerating bone marrow populations is increased after total-body irradiation (Horvath et al., 2006).

Despite improvements in the technology for delivering therapeutic radiation, salivary glands are inevitably injured during head and neck cancer radiotherapy, causing devastating side-effects which results in salivary hypofunction and consequent xerostomia (Burlage et al., 2008; Hall & Giaccia, 2006; Nagler, 2002). Salivary glands of rat are quite similar to human salivary glands in which salivary flow is rapidly reduced after radiation exposure (Nagler, 2002). Recent results demonstrated that histamine markedly prevented radiation injury on submandibular gland, ameliorating the histological and morphological alterations. Radiation significantly decreased salivation by approximately 35-40%, which

was associated with a reduction of submandibular gland wet weight and an alteration of epithelial architecture, vacuolization of acinar cells and partial loss of eosinophilic secretor granular material. It is worth noting that histamine treatment (0.1 $mg.kg^{-1}$) completely reversed the reduced salivation induced by radiation, preserving glandular function and mass with normal structure organization of acini and ducts. Histamine prevented radiation-induced toxicity in submandibular gland essentially by suppressing apoptosis of ductal and acinar cells, reducing the number of apoptotic cells per field (Medina et al., 2011a).
To summarize, histamine treatment can selectively modulate cellular damage produced by ionising radiation, thus preventing radiation induced damage on small intestine, bone marrow and salivary glands. Furthermore, histamine *in vitro* enhances the radiosensitivity of breast cancer cells (Medina et al., 2006) while does not modify that of melanoma (Medina et al., 2007). Despite histamine may be proliferative in some cancer cell types, it may still be beneficial as radioprotector in view of the fact that it is only administered for a short period of time to reduce the radiation induced damage. It is important to highlight that histamine radioprotective effect was demonstrated in two different rodent species, which suggests that histamine could exert a radioprotective action in other mammals. Also, no local or systemic side effects were observed upon histamine administration in both species.
The presented evidences indicate that histamine is a potential candidate as a safe radioprotective agent that might increase the therapeutic index of radiotherapy for intra-abdominal, pelvic, and head and neck cancers, and enhance patient quality of life by protecting normal tissue from radiation injury. However, the efficacy of histamine needs to be carefully investigated in prospective clinical trials.

9. Conclusions

In this chapter, we have presented major findings of the most recent research in histamine cancer pharmacology. These data clearly indicate that histamine plays a key role as a mediator in most human tumours. Interestingly, histamine is not only involved in cancer cell proliferation, migration and invasion, but also the tumour microenvironment and immune system responses are tightly affected. In human neoplasias H_3R and H_4R seemed to be the main receptors involved in the control of the metabolic pathways responsible for tumour growth and progression, suggesting that H_3R and H_4R represent potential molecular targets for cancer drug development. Finally, a novel role for histamine as a selective radioprotector is highlighted, indicative of the potential application of histamine and its ligands as adjuvants to radiotherapy.

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5

Histone Deacetylase Inhibitors as Therapeutic Agents for Cancer Therapy: Drug Metabolism and Pharmacokinetic Properties

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1. Introduction

The processes of absorption (A), distribution (D), metabolism (M) and excretion (E) (collectively referred as ADME) determine the pharmacokinetics (PK) of a compound. Lack of optimum PK is one of the major reasons for compounds to fail in the clinic resulting in high attrition rates. In the beginning of 1990, 39% of the drugs failed in the clinic due to poor PK emphasizing its importance in drug development (Waterbeemd and Gifford, 2003). In 1988, a study of the pharmaceutical companies in UK showed that non-optimal PK was one of the major reasons (~40%) for termination of drugs in development (Prentis et al., 1988). In the last two decades this number dropped to ~ 10% (Yengi et al., 2007). The main reasons for this significant drop in the number of compounds failing for PK reasons can be attributed to the following: a) application of concepts of drug metabolism and PK to design compounds in medicinal chemistry programs (Smith et al., 1996); b) development of *in vitro* ADME assays that are predictive of *in vivo* behavior (PK) of drugs (Obach et al., 1997; Venkatakrishnan et al.,2003; Pelkonen and Raunio, 2005; Thompson,2000; Fagerholm, 2007); c) use of the Lipinski rule of 5 to design oral drugs (Lipinski, 2000); d) development of computer programs to predict the human PK parameters and profiles based on *in vitro* ADME properties of drugs (Jamei et al., 2009); e) PK/PD correlation studies in preclinical setting and f) high throughput screening of ADME properties in *in vitro* and *in vivo* assays for hundreds of compounds in the lead identification to lead optimization stages of drug discovery. The consequence of all the above mentioned developments in ADME have resulted in the frontloading of non-drug like compounds early in drug discovery and ultimately reducing the attrition rates of compounds in the clinic.

Histone acetylases (HATs) and Histone deacetylases (HDACs) are enzymes that carry out acetylation and deacetylation, respectively, of histone proteins (Minucci and Pelicci, 2006). Histone proteins form a complex with DNA called as nucleosomes, which are the structural units of chromatin. The interplay of HATs and HDACs activities regulate the structure of chromatin and control gene expression. The aberrant expression of HDACs has been linked to the pathogenesis of cancer (Minucci and Pelicci, 2006). Histone deacetylase inhibitors

(HDACi) are an emerging class of therapeutic agents that induce tumor cell cytostasis, differentiation and apoptosis in various hematologic and solid malignancies (Mercurio et al., 2010; Stimson et al., 2009). They are known to exert their anti-tumor activity by inhibiting the HDACs, which play an important role in controlling gene expression by chromatin remodeling, that affect cell cycle and survival pathways (Stimson et al., 2009). Inhibitors of histone deacetylases (HDACi) also show promising anti-inflammatory properties as demonstrated in a number of animal and cellular models of inflammatory diseases and for diabetes (Christensen et al., 2011). The HDACi Zolinza (Vorinostat/ Suberolyanilide hydroxamic acid [SAHA]) and Romidepsin (FK228) have been approved by the FDA (United States Food and Drug Administration) for the treatment of cutaneous T cell Lymphoma (CTCL) (Mann et al., 2007, Grant et al.,2010) and for peripheral T cell lymphoma (PTCL)(http://www.accessdata.fda.gov/drugsatfda_docs/appletter/2011/022393s004ltr.pdf) as such demonstrating clinical "proof-of-principle" for this class of compounds.

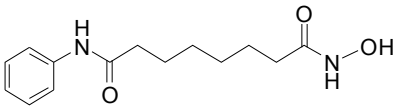
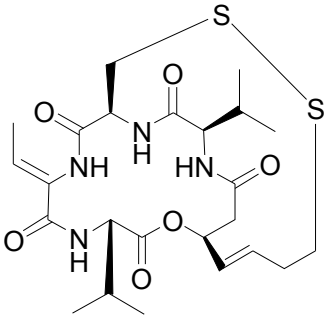
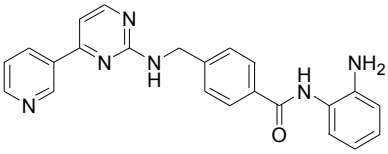
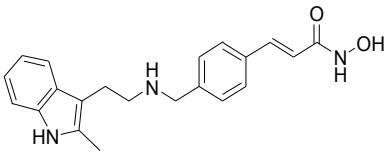
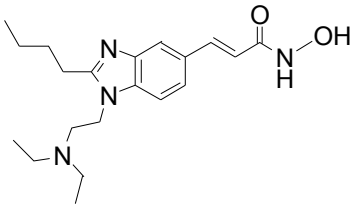
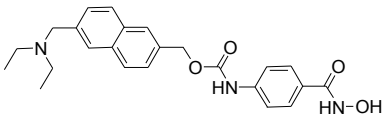
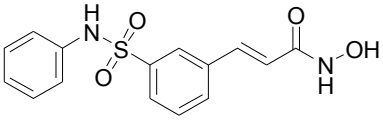
Four groups of HDAC inhibitors have been characterized: (i) short chain fatty acids (e.g., Sodium butyrate and phenylbutyrate), (ii) cyclic tetrapeptides (e.g., Depsipeptide and Trapoxin), (iii) benzamides (e.g. MGCD0103 (Mocetinostat), Cl-994 and MS-275 (Entinostat)), and (iv) hydroxamic acids (e.g., SAHA [Vorinostat/Zolinza]), LBH589 (Panabinostat), SB939 (Pracinostat), ITF2357 (Givinostat), PXD101 etc). Table 1 shows compounds that are currently in different stages of clinical development.

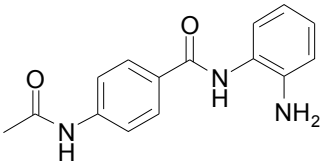
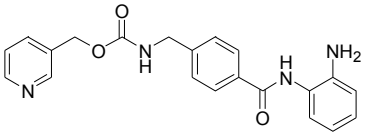
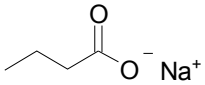
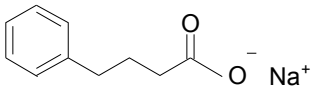
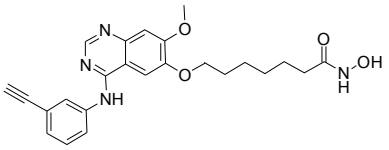
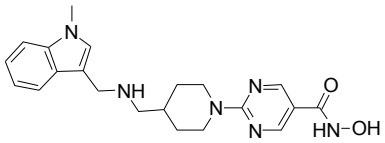
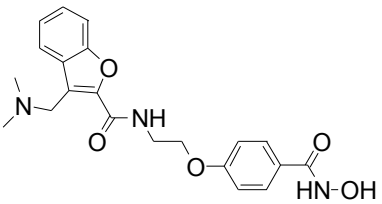
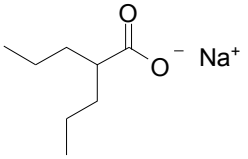
The clinical progress that has been made by hydroxamic acid derivatives as HDAC inhibitors is of particular interest because they are usually considered as non-druggable and are down-prioritized in lead identification campaigns attributing to their poor physicochemical and ADME properties. SB939 (Pracinostat) is a potent HDACi that was discovered and developed at S*BIO (Wang et al., 2011; Novotny-Diermayr et al, 2011) to overcome some of the ADME and PK/PD (Pharmacokinetic/Pharmacodynamic) limitations of the current HDACi. The pharmacokinetics and drug metabolism aspects of the four classes of HDACi have not been reviewed extensively. In this article, we review the pharmacokinetic and drug metabolism properties of SB939 and the preclinical and clinical ADME aspects of other HDAC inhibitors in the clinic.

2. Short chain fatty acids

2.1 Sodium butyrate (SB)

Sodium butyrate is a short chain fatty acid inhibitor of HDAC enzymes that is in phase 2 clinical trials. The PK of SB in preclinical species was characterized by poor bioavailability, short $t_{1/2}$ (< 5 min in mice and rabbits), leading to challenges in oral administration (Coradini et al, 1999; Daniel P et al, 1989). Butyrate was found to be transported by via a carrier mediated transport system MCT1 in Caco-2 cells suggesting that the absorption of SB might be saturable (Stein et al., 2000). SB has been reported to significantly increase the cytochrome P450 3A4 (CYP3A4) activity in Caco-2 cells transfected with CYP3A4 (Cummins et al; 2001) and induce P glycoprotein (PgP) *in vivo* (Machavaram et al., 2000). Due to its low potency very high doses were required to achieve pharmacological concentrations in animals and humans (Kim and Bae, 2011). In PK studies in mice and rats, SB showed rapid clearance (CL) with non-linear PK resulting from the high doses (up to 5 g/kg in mice), based on which the authors indicated that high doses would be problematic in humans (Egorin et al., 1999). In a clinical pharmacology study in leukemia patients, where SB was administered as continuous intravenous (IV) infusions (at a dose of 500 mg/kg/day) over a

Compound name	Structure	Class	Stage of clinical development*
Vorinostat (ZOLINZA™)		Hydroxamic Acid	Approved (2006)
Romidepsin (Istodax)		Cyclic peptide	Approved (2009)
MGCD0103 (Mocetinostat)		Benzamide	Phase 2
LBH589 (Panabinostat)		Hydroxamic Acid	Phase 2
SB939 (Pracinostat)		Hydroxamic Acid	Phase 2
ITF2357 (Givinostat)		Hydroxamic Acid	Phase 2
PXD101 (Belinostat)		Hydroxamic Acid	Phase 2

Compound name	Structure	Class	Stage of clinical development*
CI994 (Tacedinaline)		Benzamide	Phase 2
MS-275 (Entinostat)		Benzamide	Phase 2
Sodium Butyrate		Short chain fatty acid	Phase 2
Sodium Phenylbutyrate		Short chain fatty acid	Phase 2
CUDC-101		Hydroxamic acid	Phase 1
JNJ-26481585		Hydroxamic acid	Phase 1
CRA 24781 (PCI-24781)		Hydroxamic acid	Phase 1
Sodium Valproate		Short chain fatty acid	Phase 2

*Reference from http://www.fda.gov

Table 1. HDAC inhibitors in clinical development

10 day period, SB declined rapidly post infusion with a very short $t_{1/2}$ (~ 6 min), with high systemic clearance (CL~5 L/h/kg) and low volume of distribution (V_d =0.74 L/kg) (Miller et al., 1987). The amount of unchanged SB in urine was minimal suggesting that SB's clearance was primarily by metabolism. The authors concluded that the lack of efficacy of SB in the leukemic patients was due to its low plasma levels and very short $t_{1/2}$ (Miller et al., 1987).

2.2 Sodium phenyl butyrate (PB)

Sodium phenyl butyrate (PB) is an aromatic fatty acid HDACi, with low potency of 0.5 mM that is in phase 2 trials for cancer. PB (Buphenyl) has already been approved by the FDA for patients with hyperammonemia (Gilbert et al., 2001).

In a phase 1 study in patients with solid tumors, the PK of PB was characterized by rapid absorption (time of peak concentration [t_{max}] ~1.8 h), dose proportional increase in oral exposures between doses of 9 and 36 g/day, a short $t_{1/2}$ of 1 h, with mean absolute oral bioavailability (F) of 78% (Gilbert et al., 2001). In the same study, the major circulating metabolites of PB were phenylacetate (PA) and phenyacetylglutamine (PG), the exposures of which were 46-66% and 70-100% respectively of PB, suggesting extensive metabolic clearance of PB in humans. The highest percentage of patients that showed stable disease was from the 36 g/day cohort, in which the time above 0.5 mM was ~ 4.0 h (Gilbert et al., 2001). In another phase 1 study in patients with myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML), where PB was dosed as IV infusions, PB showed non-linear PK between 125 and 500 mg/kg/day, with PA and PG being formed as major metabolites (Gore et al., 2001). The low potency of PB requires very high doses in humans, leading to non-linear kinetics, thus making it a less attractive chemotherapeutic agent. In another phase 1 study, where PB was evaluated as continuous IV infusions (120 h) in solid tumors, the PK of PB was best described by saturable elimination, and PG was the major metabolite found in urine which was indicative of extensive metabolic clearance of PB in humans (Carducci et al., 2001). In the same study the plasma clearance (CL) of PB increased during the infusion period in some patients at higher dose levels. In a dose escalation oral study of PB in patients with glioma, who also received anticonvulsants concomitantly, the mean CL of PB was significantly higher than in solid tumor patients, and the possible reason was attributed to the induction of cytochrome P450 (CYP450) enzymes by anticonvulsants (Phuphanich et al., 2005). Thus it appears that the CYP450 metabolism might play a significant role in clearance of PB in humans.

2.3 Sodium valproate

Sodium valproate is a short chain fatty acid that is currently in phase 1 and 2 clinical trials in patients with solid tumors and hematological malignancies (Federico and Bagella, 2011). Sodium valproate (Depakote) has been previously approved for use in epilepsy patients and is in medical use for the last 3 decades (Federico and Bagella, 2011). It is a moderately potent inhibitor of class 1 HDAC enzymes with promising antitumor effects *in vitro* and *in vivo*. The human ADME of sodium valproate is characterized by a) high plasma protein binding (PPB) of 90 % with concentration dependent PPB; b) weak inhibitor of some CYP450, epoxide hydrolase and glucoronosyl transferases; c) entirely metabolized by the liver via glucoronidation and β-oxidation pathways with less than 3% of unchanged parent drug found in the urine; d) minimum drug-drug interaction (DDI) potential with CYP450 inhibitors as CYP450 mediated oxidation is a minor pathway ; e) high absolute oral bioavailability (90%); f) mean terminal half-life of 9-16 h (Depakote prescribing information, http://www.accessdata.fda.gov/drugsatfda_docs/label).

3. Cyclic tetrapeptides

3.1 Romidepsin (FK228, depsipeptide, ISTODAX™)

Romidepsin is a bicyclic peptide that was isolated as a secondary metabolite from a naturally occurring soil bacterium, and found to be a potent anti-tumor agent *in vitro* and *in vivo* (Ueda et al., 1994) and subsequently found to be a potent HDACi. It was approved by the FDA for treatment of patients with refractory CTCL (Mercurio et al., 2010). Romidepsin is a high molecular weight drug (Mw ~ 541), highly lipophilic, and insoluble in water, necessitating intraperitoneal and subcutaneous administrations in pharmacology studies (Ueda et al., 1994). The *in vitro* PPB of Romidepsin to human plasma was 92-94 % over a concentration of 50-1000 ng/mL, indicating high binding (http://www.accessdata.fda). Romidepsin is a substrate of PgP and MRP1 (Xiao et al., 2005). Depsipeptide was extensively metabolized by human liver microsomes, leading to the formation of at least 10 different metabolites, and was found to be primarily metabolized by CYP3A4 *in vitro* (Shiraga et al., 2005). Among the metabolites formed, mono-oxidation, di-oxidation, reduction of disulfide metabolites and two unidentified metabolites were the major metabolites in humans (http://www.accessdata.fda). It did not seem to inhibit any of the major human CYP450 enzymes *in vitro*, and there are no reports on its effect on the induction of human CYP450s (http://www.accessdata.fda). The preclinical PK of depsipeptide was characterized by high systemic CL and long $t_{1/2}$ (~ 6.0 h) in mice (Graham et al., 2006). In rats, the volume of distribution at steady state (V_{ss}) was very high (100 L/kg) and systemic CL was high (~ 49 L/h/kg), $t_{1/2}$ was short (18 min), and had poor oral bioavailability (F= ~ 2-11%) (Li and Chan, 2000). The low F in rats may be could be due to high first-pass effect, poor solubility and PgP efflux. Systemic CL (~1.8 L/h/kg) and $t_{1/2}$ (205 min) were moderate in nonhuman primates (Berg et al., 2004). In a radiolabelled mass-balance study in rats with FK228, approximately 98% of the dose was recovered in excreta with ~ 79% of the dose in the feces, and biliary clearance appeared to be the main clearance mechanism (http://www.accessdata.fda; Shiraga et al., 2005). Unchanged FK228 accounted for 3% of the dose, with > 30 metabolites detected in bile, indicating extensive metabolism of FK228 (Shiraga et al., 2005). The clinical PK of Romidepsin was characterized by low V_{ss} (54 L), low CL (20 L/h), and a short $t_{1/2}$ (~ 3.5 h) (http://www.accessdata.fda; Woo et al., 2009). The intra-patient variability was moderate to high (30-80%) and the inter-patient variability was high (50-70%) (http://www.accessdata.fda;). Despite the high inter-patient variability the AUC and C_{max} increased dose proportionally (http://www.accessdata.fda).

Romidepsin is the only HDACi that seems to be a PgP substrate. Romidepsin induced PgP expression in the HCT15 tumor cell line and conferred resistance to its action (Xiao et al., 2005). A possibility of correlation between PgP induction and the poor response rate of Romidepsin in cancer patients has been proposed (Xiao et al., 2005).

4. Benzamides

4.1 Mocetinostat (MGCD0103)

Mocetinostat (MGCD0103), an aminophenyl benzamide, is a potent inhibitor of HDAC 1, 2, and 3 enzymes and has recently completed Phase 2 clinical trials (Mercurio et al., 2010). It is a small molecule (Mw~396) and moderately lipophilic (LogP=2.6). There is no information available on its permeability, microsomal stability, metabolism, plasma protein binding, CYP450 inhibition and induction. In preclinical PK studies in mice, rat and dog,

Mocetinostat showed moderate V_{ss} (0.35 -0.91 L/kg), moderate to high CL (1.7 to 4.3 L/h/kg), short $t_{1/2}$ (0.6-1.3 h), with F ranging between low (mice =12%), moderate (rat=47%) and low-high (dogs=1-92%) (Zhou et al., 2008). In preclinical PK and PD studies, where the dihydrobromo salt of Mocetinostat was used, the dosing formulations required acidification and cosolvent addition indicating solubility issues (Zhou et al, 2008).
In a phase 1 study in patients with leukemia, the oral PK of Mocetinostat was characterized by rapid absorption (t_{max} = 0.5-1.2 h), mean elimination $t_{1/2}$ of 7-11 h, and a dose related increase in peak plasma concentration (C_{max}) and area under the concentration-time curve (AUC) between 20 and 60 mg/m^2 and tended to plateau at higher doses (Garcia-Manero et al., 2011). Based on the lack of accumulation upon repeated dosing, it was suggested that induction or inhibition of drug elimination was unlikely in humans (Le Tourneau and Siu, 2008).

4.2 CI994 (N-acetyldinaline)

CI994 (N-acetlydinaline), belonging to the benzamide class, is a HDACi with promising antitumor activities in preclinical xenograft models, and subsequently progressed to phase 1 2 clinical trials (Richards et al., 2006). CI994, a small molecule (MW=269.3) and with poor aqueous solubility, was developed as an acetylated analogue of Dinaline (GOE-1734), which, also showed equivalent antitumor activity (LoRusso et al., 1996). CI994 was eventually identified as an active metabolite of Dinaline (LoRusso et al., 1996). Limited data is available on its *in vitro* ADME. It showed low PPB in mice (20%) (Foster et al., 1997). In an oral PK and metabolism study in mice, where CI-994 was dosed once daily at 50 mg/kg for 14 days, it showed moderately rapid absorption (t_{max}= 30-45 min), 2 compartment disposition with a terminal $t_{1/2}$ on day 1 (9.4 h) being longer than on day 14 (3.4 h), and oral CL ranging between 0.42 (Day 1) -0.52 (day 14) ml/min (Foster et al., 1997). High amounts of unchanged drug (42-58% of dose) were found in the urine with minimal amounts in fecal samples, suggesting that renal clearance was a major clearance pathway for CI-994. Low amounts of Dinaline were found in urine and feces indicating that *in vivo* conversion of CI-994 to Dinaline were not significant. In rhesus monkeys, the PK of CI-994 was characterized by low volume of distribution (V_d) (0.3 L/kg) and CL (0.05 L/h/kg), a moderate $t_{1/2}$ (7.4 h), and high brain penetration (Riva et al., 2000). The oral bioavailability of CI-994 in preclinical species was 100% (Riva et al., 2000). In a phase 1 study in cancer patients following oral dosing (5-15 mg/m^2), CI-994 showed rapid absorption (t_{max} 0.7-1.6 h), oral CL ranging between ~30-48 ml/min/m^2), dose proportional increases in C_{max} and AUC, and moderately long $t_{1/2}$ (7.4-14 h) (Prakash et al., 2001). In the same study, no food effects were observed on the oral PK of CI-994.

4.3 Entinostat (MS-275)

Entinostat (MS-275) is a small molecule, synthetic benzamide that is currently in phase 2 trials (Mercurio et al., 2010). It is moderately lipophilic (LogD= 1.79), with moderate plasma protein binding (fraction unbound [f_u] ranged between 0.375 to 0.439 in preclinical species, and 0.188 in humans) (Hooker et al., 2010; Acharya et al., 2006). In preclinical pharmacology studies, the t_{max} of Entinostat ranged between 30-40 minutes with a $t_{1/2}$ of ~ 1 h in rats, mice and dogs, and the oral bioavailability was high (F~ 85%) (Ryan et al., 2005). In a radiolabeled tissue distribution and brain penetration study in baboons, radioactivity was cleared both by renal and biliary systems, and showed poor brain penetration (Hooker et al,

2010). The authors concluded that PgP mediated efflux was probably not the main mechanism for the poor brain penetration.

The clinical PK of Entinostat, in cancer patients, was characterized by variable absorption rates (t_{max} ranged between 0.5 to 60 h), a mean terminal elimination half-life of ~ 52 h, low oral clearance (CL/F=17.4 L/h/m^2), nearly dose proportional increase in exposures with dose (range 2-12 mg/m^2), and with substantial interpatient variability (Ryan et al., 2005). The nearly 50 fold longer $t_{1/2}$ in humans was not predicted based on the preclinical PK (Ryan et al., 2005). The possible reasons for the extended $t_{1/2}$ in humans were attributed to entero-hepatic recirculation and higher binding to human plasma proteins to some extent (Ryan et al., 2005). In an *in vitro* study, no metabolites could be detected after incubation of MS-275 in human liver microsomes, indicating that hepatic metabolism was a minor pathway of elimination in humans (Acharya et al., 2006).

5. Hydroxamic acids

5.1 Vorinostat (suberoylanilide hydroxamic acid [SAHA], ZOLINZA™)

Vorinostat (SAHA, ZOLINZA™), belonging to the hydroxamic acid class, was the first HDACi to be clinically approved for the treatment of refractory cutaneous T-cell lymphoma (Mann et al., 2007). Vorinostat (M_w=264) is poorly soluble in aqueous solutions ~ 191 µg/mL [~0.7 mM] (Cai et al., 2010), has a pKa of 9.2 and a LogP ~1.0 (http://www.accessdata.fda). It was moderately permeable in Caco-2 cell permeability assays (~ 2 X 10^{-6} cm/sec), based on which, and its poor solubility, it was classified as a Biopharmaceutical Classification System (BCS) class 4 drug (http://www.accessdata.fda). It displayed low to moderate binding to plasma proteins, with mean PPB of 71.3, 62.5, 43.6, 32.4, and 31.1 % in human, rabbit, dog, rat and mouse plasma, respectively (http://www.accessdata.fda). The mean blood-to-plasma partition ratio was 1.2, 0.7, and 2.0 in rat, dog and human blood, respectively (http://www.accessdata.fda). In *in vitro* metabolism studies, using S9 and liver microsomal fractions from rat, dog and humans, the major metabolic pathway was *O*-glucoronidation of Vorinostat in all the 3 species, and a minor pathway was the hydrolysis of parent to 8-anilino-8-oxooctanoic acid (8-AOO) (http://www.accessdata.fda). In metabolism studies with hepatocytes from rat, dog and humans, the major metabolites formed in all the 3 species were 4-anilino-4-oxobutanoic acid (4-AOB, β-oxidation product) and 8-AOO (hydrolysis). In dog hepatocytes, the *O*-glucoronide was also a major metabolite, with human hepatocytes generating small amounts of it (http://www.accessdata.fda). The CYP450 enzymes were not responsible for the biotransformation of Vorinostat (http://www.accessdata.fda).

In preclinical studies in rats and dogs (Sandhu et al., 2007), the PK of Vorinostat was characterized by high systemic CL (7.8 and 3.3 L/h/kg in dog (> liver blood flow of ~ 1.9 L/h/kg) and rat (=liver blood flow of 3.3 L/h/kg), respectively), low to moderate V_{ss} (1.6 and 0.6 L/kg in dog and rat respectively), short half-lives (12 min in dog and rat), and poor oral bioavailability (11 % and ~ 2% in dog and rat, respectively). The *O*-glucoronide and 4-AOB metabolites of Vorinostat were detected in significant levels in both the species following oral dosing (AUC ratio of *O*-glucoronide to Vorinostat was ~ 1.0 and 2.3 in dog and rat, respectively; and the AUC ratio of 4-AOB to Vorinostat was 10 and 23 in dog and rat, respectively). In excretion studies with radiolabeled Vorinostat, 89-91% and 68-81% of the total dose was recovered in urine of rat and dog, respectively. The major metabolites in rat urine (over a period of 24 h) were acetaminophen-O-sulfate (~16-19%), 4-AOB (47-48%),

6-anilino-oxohexanoic acid (6-AOB) (~10-14%), O-glucoronide in trace amounts, and the parent accounting for 0.7- 5%. In dog urine, the major metabolites found were 4-AOB (31-34%), ortho-hydroxyaniline O-sulfate (17-21%), with minor amounts of the O-glucoronide and carnitine esters of 6-AOH and 8-AOO. Thus, Vorinostat was primarily cleared by metabolism and renally excreted in rat and dog. The data suggest that the low bioavailability of Vorinostat in rat and dog was due to a high first-pass effect and not due to absorption since the > 90% of the dose was recovered in urine, indicative of high intestinal absorption (fraction of dose absorbed [F_a]=0.8-1.0) (Sandhu et al., 2007).

Vorinostat did not inhibit any of the major human CYP450 enzymes (http://www.accessdata.fda). It did not significantly induce CYP1A2, 2B6, 2C9, 2C19 and 3A4 in freshly cultured human hepatocytes, although the induction activity of 2C9 and 2C19 were suppressed at the highest concentration (http://www.accessdata.fda).

In the first clinical trial in cancer patients Vorinostat was administered intravenously as a 2 h infusion (Kelly et al., 2003). The intravenous route was chosen due to predictions of poor oral bioavailability based on its preclinical ADME properties (Kelly et al., 2003). In a subsequent phase 1 trial, Vorinostat was dosed orally in patients with advanced cancer in which the oral PK was also characterized (Kelly et al., 2005). Vorinostat showed dose proportional increase in C_{max} and AUC following single oral doses of 100, 400 and 600 mg, with the average terminal $t_{1/2}$ ranging between ~ 92 to 127 minutes, median t_{max} ranging between 53 to 150 minutes, and an absolute oral bioavailability of 43%. No apparent changes were observed in PK following multiple oral dosing. The $t_{1/2}$ following oral dosing was longer than the $t_{1/2}$ observed after i.v. dosing (range of ~35-42 min), suggesting that the elimination of Vorinostat was absorption rate limited (Kelly et al., 2005). In another study investigating the PK of Vorinostat, at 400 mg, and its major metabolites in cancer patients, the mean serum exposures of the O-glucoronide and 4-AOB were 3-4 fold and 10-to-13 fold higher, respectively, than that of Vorinostat (Rubin et al., 2006). In the same study, up to 18% and 36% of the O-glucoronide and 4-AOB, respectively, were recovered in urine, with the parent accounting for < 1 % of the total dose, clearly indicating that Vorinostat was cleared primarily by metabolism in humans, and that the O-glucoronide and 4-AOB were the major metabolites. The main enzymes responsible for the formation of the O-glucoronide were identified as the UDP-glucoronosyltransferases (UGTs), such as the UGTs 2B17 and 1A9, which are expressed in the liver, and the extrahepatic UGTs 1A8 and 1A10 (Balliet et al.,2009). UGT2B17 was one of the major enzymes contributing to the formation of the O-glucoronide of Vorinostat in humans (Balliet et al., 2009). Since UGTs are known to show extensive polymorphism, including UGT2B17, they have been associated with the variable PK and response of Vorinostat in patients (Balliet et al., 2009).

There have been no reports on allometric scaling or the predictions of human PK based on preclinical ADME data so far.

5.2 Panabinostat (LBH589)

Panabinostat (LBH589) is a cinnamic hydroxamic acid and a potent pan HDAC inhibitor that is currently in phase 2 clinical trials (Mercurio et al., 2010). Very little information is available on its preclinical ADME characteristics. It showed poor oral bioavailability in rodents (F=6% in rats) and moderate F in dogs (33-50%) (Konsoula et al, 2009).

Like SAHA, Panabinostat was first tried as an intravenous formulation in the phase 1 clinical trials (Giles et al., 2006). In that study, LBH589 showed dose proportional increase in

C_{max} and AUC between 4.8 and 14 mg/m^2, with the terminal half-life ranging between 8-16 h. The V_{ss} and CL were not reported. The oral PK of Panabinostat was characterized by rapid absorption (t_{max} =1-1.5 h), linear increase in dose between 20 and 80 mg and the terminal $t_{1/2}$ ranged between 16-17 h (Prince et al, 2009). In an oral mass-balance study in patients with advanced cancer, following a single oral dose of 20 mg of ^{14}C radioactively labeled Panabinostat, 87% of the administered dose was recovered in the excreta, with unchanged drug accounting for <3% of the administered dose in the feces, suggesting good oral absorption and extensive metabolism (Clive et al, 2006). The major circulating metabolites were glucoronidation products of Panabinostat, in addition to hydrolysis and reduction products. Thus, it appears that there is no single major metabolic pathway for the elimination of Panabinostat in humans. CYP3A4 does not significantly contribute to the elimination of Panabinostat in humans (DeJonge et al, 2009). Human PK data suggest that Panabinostat is a permeable drug and the poor bioavailability in preclinical rodents could be due high first-pass and poor solubility.

5.3 Givinostat (ITF2357)

Givinostat (ITF2357) is a pan HDAC inhibitor, belonging to the hydroxamic acid class that is currently in phase 2 trials for many hematological malignancies (Mercurio et al., 2010). Preclinical ADME information is either limited or qualitative for Givinostat. Metabolism was the primary clearance mechanism in preclinical species like rats, dogs, rabbits and monkeys, with excretion being biliary or renal (Furlan et al, 2011). In a phase 1 study in healthy volunteers, the oral PK of Givinostat was characterized by rapid absorption, dose proportional increases in C_{max} and AUC upon single and multiple oral dosing, and the terminal half-life ranged between 5-7 h (Furlan et al, 2011). Two major circulating metabolites of Givinostat, a carboxylate and an amide formed due to oxidation and reduction of the hydroxamic acid group, were detected at significant levels in plasma.

5.4 Belinostat (PXD101)

Belinostat (PXD101) is a hydroxamic acid class potent pan HDAC inhibitor that is currently in phase 2 clinical trials (Mercurio et al., 2010). It is a small molecule (Mw 318) and sparingly soluble in aqueous solutions (Urbinati et al., 2010). Preclinical ADME information on Belinostat is limited. Preclinical pharmacodynamic studies in mice (Plumb et al., 2003) and PK studies in non-human primates (Warren et al 2008) have been performed using IV administrations, suggesting that Belinostat may have poor solubility and bioavailability issues. However, in dogs an oral bioavailability of 30-35% was reported (Steele et al, 2011). In rhesus monkeys, clearance was rapid (425 mL/min/m^2) with a $t_{1/2}$ of 1.0 h (Warren et al 2008). In a PK/PD study in mice following IV dosing at 200 mg/kg, Belinostat declined rapidly in plasma (ca $t_{1/2}$ ~ 0.4 h), suggesting high systemic clearance (Marquard et al 2008). In the same study a correlation was observed between tumor concentrations and histone 4 acetylation levels indicating that Belinostat penetrated solid tumors.

In a phase 1 clinical study in patients with solid tumors, where Belinostat was administered as a 30 min IV infusion, its PK was characterized by dose proportional increase in AUC and C_{max}, and a short $t_{1/2}$ (0.45 to 0.79 h) (Steele et al., 2008). The oral PK of Belinostat following a 1000 mg/m^2 dose in patients with solid tumors, was characterized by mean t_{max} of 1.9 h (although the oral concentration-time profile showed a flat absorption phase), with a mean

$t_{1/2}$ of 1.5 h (Steele et al, 2011). High variability was observed in oral clearance (39-71%) due to which dose proportionality analysis was not attempted. The oral $t_{1/2}$ was longer than that of the IV, which was attributed to a slow absorption rate (Steele et al., 2011). Oral bioavailability ranged between low to moderate (20-50%) in patients with advanced solid tumors (Kelly et al., 2007). Although a correlation between H4 acetylation and concentrations was observed following oral dosing at 1000 mg/m^2 (Steele et al., 2011), recent phase 2 trials have employed IV dosing of Belinostat (Cashen et al., 2011). In another Phase 1 study, where the metabolism of Belinostat was studied in patients with hepatocellular carcinoma, five metabolites were identified (Wang et al, 2010). Glucoronidation was the most significant pathway of metabolism, and the methylated and amide (reduction of hydroxamic acid) products were also detected. The acid and N-glucoside forms of Belinostat were found as minor metabolites. In an *in vitro* assay using 12 isoforms forms of human UGTs, Belinostat was mainly cleared by UGT1A1 (Wang et al., 2010). The data taken together suggest that Belinostat was primarily cleared by phase 2 metabolism, involving UGT1A1, in humans.

5.5 CUDC-101

CUDC-101 is a small molecule (Mw 434.5) hydroxamic acid HDACi, synthesized by incorporating the hydroxamic acid group into the epidermal growth factor receptor (EGFR) pharmacophore, that exhibited antiproliferative effects *in vitro* and *in vivo* (Cai et al., 2010; Lai et al, 2010). The preclinical ADME of CUDC-101 is not available (Cai et al., 2010). The fact that CUDC-101 was dosed IV in the preclinical efficacy studies suggests that it may have had poor oral bioavailability (Cai et al., 2010). CUDC-101 is currently in phase 1 trials (Cai et al., 2010)

5.6 JNJ-26481585

JNJ-26481585 is a second-generation, small molecule hydroxamic acid based potent pan-HDACi that is currently in phase 1 trials (Mercurio et al., 2010). The preclinical ADME information for this compound is minimal. JNJ-26481585 has been shown to undergo extensive first-pass metabolism resulting in poor oral bioavailability in rodents, due to which it had to be dosed intraperitoneally (IP) in xenograft models (Arts et al., 2009). In a phase 1 oral PK/PD study in solid tumor patients, the exposures of JNJ-26481585 (dosed *q.d.* in 3 weekly cycles) increased dose proportionally between 2 and 12 mg (Postel-Vinay et al., 2009). In the same study promising antitumor activity was observed indicating orally active exposures were achieved in humans.

5.7 CRA-024781(PCI-24781)

CRA-024781(PCI-24781) is a small molecule, hydroxamic based pan HDACi that is currently in phase 1 trials (Mercurio et al., 2010). In preclinical murine models of efficacy, its PK was characterized by a very short $t_{1/2}$ (~ 7 min), very high CL (~ 18 L/h/kg) and high V_{ss} (~ 9 l/kg) (Buggy et al., 2006). It was administered intravenously at high doses of up to 200 mg/kg in the efficacy models, most probably owing to poor oral bioavailability and high CL (Buggy et al, 2006). In a phase 1 study in patients with solid tumors, where PCI-24781was dosed as a 2 h IV infusion, the mean elimination $t_{1/2}$ was ~ 6 h, high CL and moderately high V_{ss}, low oral bioavailability of 28%, with the carboxylic acid and amide metabolites formed at ~ 60 % of the parent (Undevia et al., 2008).

5.8 Pracinostat (SB939)

Pracinostat (SB939) is a hydroxamic acid based potent HDACi that is in multiple phase 2 clinical trials (http://clinicaltrials.gov/ct2/results?term=Sb939) in patients with solid tumors and hematological malignancies. Since the clinically advanced hydroxamic acid HDACi (Zolinza, Panabinostat and Belinostat) had ADME liabilities, such as poor solubility and oral bioavailability, we sought to identify a candidate that would achieve pharmacologically active exposures in humans when dosed orally. Pracinostat is a small molecule (Mw 359) moderately lipophilic base ($LogD_{7.4}$ =2.1) with high aqueous solubility (>100 mg/mL in water for the HCl salt of SB939) and high permeability with low efflux which indicated that Pracinostat would show high intestinal absorption *in vivo* (Wang et al., 2011). Based on its solubility and permeability Pracinostat was categorized as a BCS class 1 compound (S*BIO Data files). In preclinical PK studies Pracinostat showed higher oral bioavailability in mice (F=34%) and dogs (F=65%), than Zolinza, Panabinostat and Belinostat (table 2). The superior efficacy of Pracinostat, over Zolinza and Belinostat, when dosed orally in murine xenograft models was consistent its improved PK profile (Novotny-Diermayr et al., 2011). Pracinostat was found to selectively accumulate in tumors which correlated well with increased and prolonged acetylation levels in tumor which, in turn correlated with high tumor growth inhibition in mice (Novotny-Diermayr et al., 2011).

Preclinical ADME of Pracinostat was characterized by: a) in *in vitro* liver microsomal stability studies, Pracinostat was most stable in human and dog, moderate in mouse, and least stable in rat; b) uniform PPB of 84-94% in preclinical species and humans; c) was metabolized mainly by human CYP3A4 and 1A2; d) did not inhibit the major human CYPs except moderate inhibition of 2C19 (~ 6 μM); e) lack of significant induction of human CYP3A4 and 1A2 *in vitro*; f) metabolite identification studies using liver microsomes showed the formation of *N*-deethylation and bis-*N*-deethylation as major metabolites in addition to minor oxidative products; g) a glucoronidation product of SB939 was found as the major metabolite in rat urine following oral dosing; h) PK: high systemic clearance of 9.2, 4.5 and 1.5 L/h/kg in mice, rat and dog, respectively and high volume of distribution (V_{ss} ranged between 1.7 to 4.2 L/kg) in preclinical species; i) moderate F in mice and dogs and poor in rats (Jayaraman et al., 2011). In PK/PD studies in HCT116 xenograft models, studying the relationship between tumor growth inhibition and the PK/PD indices such as $AUC/IC_{50,HCT116}$, $C_{max}/ IC_{50,HCT116}$, and time above $IC_{50,HCT116}$, Pracinostat was found to have the highest PK/PD ratios for all the three PK/PD parameters when compared to Vorinostat, Panabinostat and Belinostat (figure 1) (Jayaraman et al., 2009).

Pracinostat showed linear allometric relationships for V_{ss} and CL in preclinical species. Prediction of human PK parameters using allometry indicated oral exposures would be achieved in humans with an acceptable $t_{1/2}$ which, was subsequently found to be consistent with the observed data from cancer patients (Jayaraman et al., 2011). The human PK of Pracinostat was simulated with the Simcyp ADME simulator (Jamei et al., 2009) using the physico-chemical and *in vitro* ADME data. The simulated PK profiles were in good agreement with the observed mean data, and the mean oral clearance and AUCs were predicted reasonably well (within 2 fold of observed data) (Jayaraman et al., 2011). Furthermore, simulations of drug-drug interactions (DDI) of Pracinostat in humans with the potent CYP3A inhibitor and inducers, ketoconazole and rifampicin, respectively, and with omeprazole (substrate of 2C19) showed lack of potential DDI at the clinically relevant dose of 60 mg (Jayaraman et al., 2011).

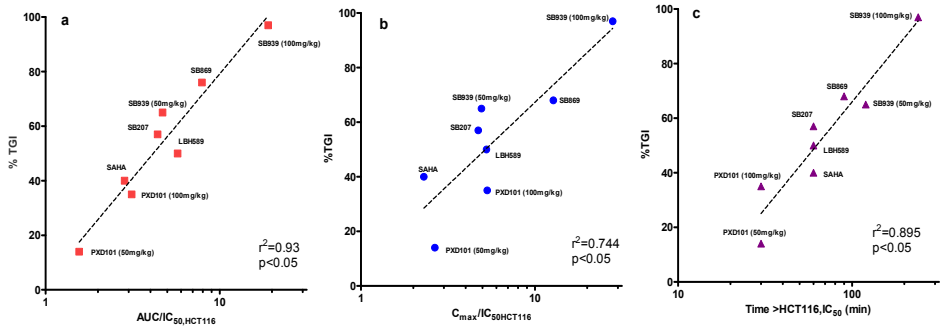


Fig. 1. The relationship between tumor growth inhibition (%TGI) and PK/PD parameters for HDACi in the murine HCT116 xenograft model (Jayaraman et al., 2009). a) AUC/$IC_{50, HCT116}$; b) C_{max}/ $IC_{50, HCT116}$; c) time above $IC_{50, HCT116}$.

Parameter	mice				dog	
	Pracinostat (SB939)	Vorinostat (SAHA)	Belinostat (PXD101)	Panabinostat (LBH589)	Pracinostat (SB939)	Vorinostat (SAHA)
C_{max}(ng/mL)	2632	501	489	116	1537	35
t_{max}(h)	0.17	0.5	0.17	0.17	0.8	0.7
$t_{1/2}$(h)	2.4	0.75	1.3	2.9	4.1	0.2
$AUC_{0\text{-inf}}$ (ng.h/mL)	1841	619	287	126	4481	55
F (%)	34	8.3	6.7	4.6	65	2

Table 2. Comparison of preclinical pharmacokinetics of Pracinostat with that of other advanced hydroxamic acid HDACi.

In the first phase 1 study in patients with solid tumors, Pracinostat showed rapid absorption (t_{max} = 0.9-2 h), dose proportional increase in C_{max} and AUC between 10 and 60 mg doses, a mean terminal $t_{1/2}$ of ~ 7 h, and lack of significant accumulation on repeated dosing (Yong et al, 2011). In the same study, pharmacologically active concentrations were achieved at the starting dose of 10 mg, and a dose dependent increase in histone acetylation was observed. At the 60 mg dose high acetylation levels was observed in all patients indicating sustained target inhibition, and two of the patients experienced prolonged disease stabilization. The clinical PK of Pracinostat was superior to the other hydroxamic acid HDACi in the clinic (table 3). The high aqueous solubility, permeability, good oral bioavailability and predictable human PK of Pracinostat contributed to obtaining active exposures in the clinic when dosed orally, which was in contrast to the intravenous dosing of Zolinza, Panabinostat and Belinostat in the initial clinical trials. The terminal $t_{1/2}$ of Pracinostat was longer than that of Zolinza and Belinostat, and shorter than Panabinostat.

In summary, the superior preclinical ADME of Pracinostat over Zolinza, Panabinostat and Belinostat was translated into the clinic.

Parameter	Pracinostat (SB939)*	Vorinostat (SAHA)#	Panabinostat (LBH589)%	Belinostat (PXD101)$
Dosage regimen	thrice weekly	once daily	thrice weekly	once daily
Recommended Dose (mg)	60	400	20	250
$t_{1/2}$(h)	7-9	0.8-3.9	16	1.5
$AUC_{0\text{-}inf}$(ng.h/mL)	1226(3.4μM)	1716 (6.5 μM)	183(0.54 μM)	2767 (8.7 μM)
Remarks	Orally active exposures achieved at FTIM&. Best-in-class profile.	FTIM dose was given IV due to poor F in preclinical species.	FTIM dose was given IV due to poor F in preclinical species. Limited exposure.	FTIM dose was given IV due to poor F in preclinical species. Poor PK/PD

* Yong et al., 2011
# Rubin et al., 2006
% Prince et al., 2009
$ Steele et al., 2011
& first time in Man

Table 3. Comparison of oral clinical pharmacokinetics of Pracinostat with hydroxamic acid HDAC inhibitors

6. Conclusions

The clinical use of the less potent short chain fatty acid HDACi (PB, SP and sodium valproate) in cancer patients was limited by the requirement of high doses and short half-life. The cyclic peptide drug Depsipeptide had to be administered intravenously because of poor solubility and oral bioavailability. The most clinically advanced hydroxamic acid HDACi such as Zolinza, Belinostat and Panabinostat were initially administered IV in patients owing to their poor solubility and oral bioavailability in preclinical species. Formulations were subsequently developed for oral administration. We succeeded in designing the hydroxamic acid pan HDACi Pracinostat (SB939) which had high solubility and permeability, with superior preclinical ADME and PK/PD properties when compared to the other hydroxamic acid HDACi, which subsequently helped to achieve pharmacologically active exposures upon oral dosing in cancer patients.

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Part 2

Anti-Infectives

6

Antimicrobial Peptides: New Frontiers in the Therapy of Infections

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1. Introduction

The discovery of antibiotics unquestionably represents a major achievement in the treatment of infectious diseases. However, the early optimistic expectations to definitely win the war against infections have not been met, mainly because of bacterial resistance, that has evolved to each antibiotic introduced into clinical practice and complicates infections in more vulnerable individuals, such as organ transplant receivers, AIDS, hemodialysis, and cancer patients. The development of resistance is inherent to the mechanism of action of classic antibiotics, that target specific bacterial enzymes, and could be overcome by new antibiotics with different targets, but in the last 40 years very few new antibiotics have reached the market. Indeed, the great majority of antibiotics presently in use for systemic infections derives by synthetic tailoring from a limited number of dated molecular scaffolds (Fischbach & Walsh, 2009). The wide-spread use of antibiotics for both medical and non-medical purposes prompted the emergence of a number of multi-resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci*, *Acinetobacter baumannii*, *Escherichia coli*, carbapenem-resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa, Clostridium difficile,* and *Mycobacterium tuberculosis*. Epidemic resistance to antibiotics has been described for a number of superbug pathogens, such as MRSA, *Streptococcus pneumoniae* and *Mycobacterium tuberculosis*, and multidrug- or pan-resistant gram-negative bacilli (Spellberg et al., 2008). It is estimated that infectious diseases, despite the availability of antibiotics, remain the second-leading cause of death worldwide (World Health Organization, 2004). On the other hand, the development of new drugs is considered no more fashionable by the pharmaceutical industry, due to the low probability to recover the huge investments required to license new antibacterials that will be used in a low number of selected infections. Indeed, this high cost-low revenue perspective made many large pharmaceutical companies to quit antibiotic discovery for more profitable therapeutics. That being said, let us approach the subject by reviewing the Pub-med literature regarding the up-to-date research on natural and synthetic antimicrobial molecules of proteinaceous nature, as alternatives to conventional antibiotics. Since the mid-1990s, bacterial genome sequencing was carried out with the aim to identify new bacterial targets. High-throughput target-based screening and combinatorial chemical libraries were developed, but after some time it was realized that the results were not up to the expectations, for at least three reasons: first, most enzymes essential for bacterial viability can not be easily affected by drugs; second, some of the structures that would be the best

antibacterial targets (e.g. ribosomes and nascent peptidoglycan) are not accessible to *in vitro* screening; and third, chemical libraries do not have the molecular complexity of naturally produced antibiotics (Baltz, 2008). To illustrate the difficulties relative to the new targets approach, scientists at GlaxoSmithKline reviewed the outcome of an extensive program of screening chemical libraries against multiple potential Gram-positive or broad spectrum targets over a period of seven years (Payne et al., 2007). Out of the 300 genes evaluated, 160 were found to be essential for viability. The screen against 67 target proteins covering a wide range of cellular metabolic activities yielded 16 hits, but only 5 yielded leads. Of these, only two (3%) were identified as potentially new targets. The authors noted that results were unsustainably poor in relation to the effort (Baltz, 2008). Because of these difficulties, the recent trend is to look for new antimicrobials by screening natural products, that are an inexhaustible source of bioactive compounds. Technical advances in genomics, bioinformatics, microbial ecology, synthetic biology, and systems biology offer new opportunities for multidisciplinary approaches to small molecule discovery (Davies, 2011; Walsh & Fischbach, 2010). The reserve of natural molecules produced by bacteria, fungi, plants, and vertebrates can offer both novel antibiotics that work in the classic way, and new antimicrobials that being based on different molecular scaffolds will more easily bypass resistance. Most of these natural substances are of peptidic nature and work by targeting conserved mechanisms, often shared by more than one pathogen. Antimicrobials of peptidic nature can be divided into two classes: the gene-encoded, ribosomally synthesized peptides, and the non-ribosomally synthesized peptide antibiotics, typically produced by bacteria and fungi. The latter are assembled by multi-enzyme complexes, contain d-amino acids and other non-proteinogenic amino acids, and often have a cyclic or branched structure (Wiesner & Vilcinskas, 2010). Some members of this class already on the market, such as bacitracin, gramicidin S, polymyxin B, the streptogramins, vancomycin and teicoplanin have limited clinical use, mostly because of toxicity, poor solubility, or limited spectrum of activity. The ribosomally synthesized antimicrobials (none of which is yet on the market) can be subdivided into two further classes depending on their source: the term "antimicrobial peptides" (AMPs) strictly speaking indicates peptides of eukaryotic origin, whereas peptides and proteins produced by bacteria are called bacteriocins. However, considering the similarities in terms of structure and mechanism of action, in this chapter the term AMPs will be used to globally indicate members of both classes. With this premise, we can say that AMPs are widely conserved, small amphiphilic antagonistic molecules produced by both prokaryotic and eukaryotic organisms. Bacteria secrete microcins and bacteriocins that inhibit bacterial food competitors present in the same environment, whilst plants and insects, that lack the adaptive immune response, rely on AMPs for protection against infections (Scott et al., 2008). In mammals, AMPs are present in neutrophils and on skin and mucosal surfaces, where they carry out direct antimicrobial activity and participate to the innate immune response (Maroti et al., 2011). The concept of developing AMPs as potent pharmaceuticals for human therapy dates back to the 1990s (Chopra, 1993), and most research articles since then published on this topic conclude by stating that AMPs represent promising therapeutic agents against bacterial, fungal, viral and parasitic diseases. Today the issue is more relevant than ever, due to the occurrence of two concomitant factors: the emergence of multi- or pan-drug-resistant bacterial strains, and the availability of new and sophisticated technical approaches to design, engineer and optimize AMPs for every specific application. The challenge is to design synthetic mimics that maintain the potency of natural AMPs, able to kill pathogens in the low micromolar concentration range, but lose some

flaws of natural molecules, such as low stability, immunogenicity, low bioavailability, and production cost (Sharma et al., 2009). According to their electrical charge, AMPs can be divided into anionic and cationic peptides. Anionic AMPs (AAMPs), found in vertebrates, invertebrates and plants, are active against bacteria, fungi, viruses, nematodes and insects. Their net negative charge ranges from -1 to -7, and their length from 5 to about 70 amino acid residues. In comparison with cationic AMPs (CAMPs), AAMPs have received little attention in the literature, and their mechanism of action so far has not been elucidated. For an outline of AAMP characteristics, the interested reader is referred to the exhaustive review by Harris et al. (2009). Vertebrate CAMPs can be also defined "host defense peptides" (HDPs), because beyond their direct antimicrobial activity, *in vivo* they often modulate the host immune response (Hölzl et al., 2008). By inducing chemokine and cytokine production, HDPs can recruit and activate immune cells, stimulate wound repair, and promote or inhibit angiogenesis (Wilmes et al., 2001). Moreover, certain CAMPs, such as amphibian temporins, neutralize bacterial endotoxins (Mangoni & Shai, 2009), and some of them, such as cecropin, buforin and magainin also exhibit selective direct cytotoxic activity against different types of human cancer cells (Schweizer, 2009). Typically, CAMPs are gene-encoded peptides derived from larger precursors by proteolytic processing, are 12-50 amino acid long with a net positive charge of +2 to +11, due to an excess of basic arginine and lysine residues, and have approximately 50% hydrophobic amino acids (Finlay & Hancock, 2004). Based on their molecular and conformational structure, CAMPs can be divided into four classes: cysteine-rich α-sheet structures stabilized by two to four disulphide bonds (human α- and β-defensins, plectasin, protegrins); linear α-helical peptides without disulphide bonds (cecropins, magainins and dermaseptins); loop-structured peptides with one disulphide bond (bactenecin, microcins from *Enterobacteriaceae*), and extended structures rich in glycine, tryptophan, proline, arginine and/or histidine (cathelicidins, indolicidin) (Hancock & Sahl, 2006). So far, more than fifteen hundreds AMPs have been identified; an updated AMP database is available on line at: http://aps.unmc.edu/AP/main.php (Wang G. et al., 2009). Positively charged CAMPs interact with the negatively charged microbial surface, and the interaction disrupts the membrane barrier function via pore-formation or unspecific membrane permeabilization. Different models, such as the toroidal pore (magainins) and barrel stave (alamethicin) models, which imply the formation of pores, or the carpet-like model (cecropins), in which the cell membrane is disintegrated and/or micellized, have been proposed to describe the structures formed between peptides and membrane phospholipids. The difference between the anionic charge of bacterial membranes and the neutral charge of mammalian cell membranes rich in zwitterionic phospholipids or cholesterol may help to explain the selectivity of action of many CAMPs (Wilmes et al., 2011). Bacterial killing is mediated by membrane disorganization, taking seconds to minutes, and/or by the binding to intracellular targets, that takes more time (3-5 hours). None mechanism is receptor-based, consistent with the finding that D-peptides are generally as active as L-peptides (Scott et al., 2008). AMPs show a highly conserved amphiphilic topology, with the hydrophilic and hydrophobic side chains segregated into distinct opposing regions or faces of the molecule. This topology is essential for insertion into and disruption of bacterial cytoplasmic membranes, and physicochemical properties, rather than any precise amino acid sequence, are responsible for AMP activity (Scott et al., 2008). Even non-peptidic compounds with amphiphilic structures, such as ceragenins, cholic acid derivatives, or polymers with phenylene ethynylene, polymethacrylate, β-lactam, or

polynorbornene backbones, are effective broad spectrum antibacterials (Chin et al., 2007; Lai XZ. et al., 2008). These compounds are not currently developed, but their low cost, ease of production and non-toxicity for mammalian cells make them suitable for sterile clothing and biocompatible medical materials, such as catheters, sutures and indwelling devices (Gabriel & Tew, 2008).

2. Mammalian defensins

Defensins are the prototypic mammalian HDPs. The presence inside neutrophil granules of proteins able to kill microorganisms with an oxygen-independent mechanism has been described in the 1980s (Ganz et al., 1986). Since then, defensins have been intensively investigated, and by now the family includes many structurally related peptides found in vertebrates, fungi, plants and insects. The discovery of defensin-like peptides produced by the myxobacteria *Anaeromyxobacter dehalogenans* and *Stigmatella aurantiaca*, demonstrated by *in silico* analysis, suggests that eukaryotic defensin genes are highly conserved (Zhu, 2007). Vertebrates express three defensin mature peptide subfamilies, defined α, β, and θ, with sequences of 29–35, 35–45, and 18 amino acids, respectively. Defensins are synthesized as 'prepropeptides' which are processed to various degrees depending on the expression site. The α- and β-defensins are products of distinct gene families evolved from an ancestral α-defensin gene that is expressed in species as ancient as venomous snakes (Selsted & Ouellette, 2005). Defensins α and β show a similar tertiary structure with triple-stranded β-sheet domains, but differ by the linear spacing and disulfide pairings of their six conserved cysteine residues. The θ-defensins, first observed in *Macaca mulatta* leukocytes, are the product of a post-translational head-to-tail ligation of two truncated α-defensins, resulting in cyclized octadecapeptides stabilized by three disulfide bonds. They are the only known cyclic polypeptides of mammalian origin, and are present in several species of Old World monkeys and in orangutans but not in humans or New World primates. Although humans express mRNA encoding θ-defensin orthologs, mutations that introduce stop codons into the otherwise open reading frame of the θ-defensin precursors abolish the peptide production (Penberthy et al., 2011). Defensins are directly active against a broad spectrum of bacteria, fungi, protozoa and enveloped viruses, and indirectly concur to host defense processes such as inflammation, angiogenesis and tissue healing (Penberthy et al., 2011).

Six α-defensins encoded by five genes have been identified in humans: HNP (human neutrophil peptide)-1 to -4, and HD (human defensin)-5 and -6. HNP-2 is a truncated form of HNP-1 or HNP-3 peptides (Wiesner, 2010). HNP 1-4 are produced by neutrophils, whereas HD-5 and HD-6 are synthesized and secreted by Paneth cells, a specialized form of epithelial cells that are found at the base of the crypts of Lieberkühn in the small intestine. On stimulation through Toll-like receptor-2, -3, and -5, neutrophils and Paneth cells release stored α-defensins to the extracellular milieu, where they exert their antimicrobial activity.

At least 33 human β-defensin genes have been discovered (Schutte et al., 2002), but so far only four human β-defensins (hBD-1 to -4) have been characterized in mucosal and epithelial cells. In addition to their antimicrobial properties, β-defensins recruit immature dendritic cells and T cells, and stimulate the maturation of antigen-presenting cells (McCormick & Weinberg, 2010). The expression of hBD-2 increases upon stimulation of numerous cell types with LPS or proinflammatory cytokines, whereas hBD-1 constitutive expression is not affected (Ryan at al., 2011). Human α- and β-defensins contribute to maintain a stable commensal microbiota in the intestinal tract, preventing bacterial

overgrowth. It is hypothesized that reduced defensin concentrations compromise host defense and predispose to ileal and colonic Crohn disease (CD) (Ramasundara et al., 2009). Patients with ileal CD are characterized by decreased expression of Paneth cell HD-5 and -6, whereas colonic CD is characterized by attenuated induction of β-defensins. On the contrary, in ulcerative colitis there is substantial evidence to support a significant up-regulation of the inducible β-defensins. Production difficulties, cell toxicity and concerns on the possible dysregulation of the tissue cytokine milieu have so far hindered the development of human defensins for therapeutic use (Chen H. et al., 2006; Kougias et al., 2005; Wencker & Brantly, 2005). Therefore, the defensin most promising for medical use is plectasin, a 40-amino acid peptide produced by the fungus *Pseudoplectania nigrella* (Mygind et al., 2005). Plectasin is active against *S. pneumoniae* and *S. aureus*, including penicillin-resistant strains, but weakly cytotoxic on mammalian cells. This selectivity is probably due to its recently clarified mechanism of action, that does not involve cell membrane disruption, but targets lipid II, a bacterial cell wall precursor (Schneider et al., 2010). Plectasin and one of its variants, the peptide NZ2114, are currently under development by Novozymes A/S as lead compounds to be used against vancomycin- and methicillin-resistant *S. aureus* (Brinch et al., 2010).

The presence of remarkably intact θ-defensin pseudogenes in humans, and the wide spectrum of antimicrobial activity of monkey θ-defensins, made these molecules the subject of extended research, that brought to the production of the human corresponding peptides, called ''retrocyclins'' (RCs), by using solid-phase synthetic approaches. RCs are synthetic, humanized θ-defensin cyclic octadecapeptides, active against HIV and herpes and influenza viruses, and able to neutralize anthrax toxin. RC-1, -2 and -3 prevent the entry of HIV-1 into target cells by blocking the virus envelope-cell membrane fusion. Studies are underway to develop RCs as local microbicides to prevent HIV-1 transmission. To this end, it has been observed that amino acid substitutions can be introduced into the RC backbone to improve the anti-HIV activity (Penberthy et al., 2011). RCs also have bactericidal activity, which makes them promising candidates for further development as topical microbicides to prevent bacterial sexually transmitted diseases. Following the observation that in eukaryotic cells aminoglycosides induce a low level of translational misreading, which suppresses the termination codon through the incorporation of an amino acid in its place, Venkataraman et al. utilized aminoglycosides to induce translational read-through of the θ-defensin pseudogene, which restored the expression of functional anti-HIV-1 retrocyclin peptides in human cervicovaginal tissue models (2009). These authors suggest that the topical application of aminoglycosides to induce the production of endogenous retrocyclins by the vaginal mucosa might soon become an effective method to combat HIV-1 sexual transmission. However, the field of RC applications is intended to widen, because it has been observed that Rhesus θ-defensin protects mice from SARS coronavirus pulmonary infection (Wohlford-Lenane et al., 2009), and that RC-2 protected both MDCK cells and chicken embryos from infection by the H5N1 avian influenza virus (Liang et al., 2010). The issue of the still prohibitive RC production cost is being addressed by Lee et al. by the use of chloroplasts as bioreactors. These authors developed a technique based on the use of chloroplast transformation vectors that allows the production of RC-101, a non-hemolytic and minimally cytotoxic RC-1 analogue with good anti-HIV-1 activity, and of protegrin-1 (a 18-residue AMP discovered in porcine leukocytes, that showed potent antimicrobial activity

against bacteria, fungi and yeasts) by tobacco chloroplasts (Lee et al., 2011). The process allows the production of adequate quantities of purified peptides to be used in preclinical studies. RCs share remarkable structural and functional similarity with other small hairpin peptides of 17–18 amino acids found in diverse species. Three groups of such peptides are gomesins, protegrins and tachyplesins/polyphemusins, which were isolated from spider hemocytes (Silva et al., 2000), porcine leukocytes (Storici & Zanetti, 1993), and horseshoe crab hemocytes (Nakamura et al., 1988), respectively. Gomesins, β-hairpin peptides consisting of 18 amino acids with two disulfide bridges, are active against fungi, bacteria, protozoan parasites and tumor cells (Moreira et al., 2007; Rodrigues et al., 2008). Protegrins are cysteine-rich, 18-residue β-hairpin peptides with 2 disulfide bridges, and show potent broad-spectrum activity that targets bacteria, filamentous fungi, yeast cells, and HIV-1 (Bulet et al., 2004; Cole & Waring, 2002). Tachyplesins are 17–18 amino acid peptides with a C-terminal alpha-amide group that forms a rigid 2-stranded anti-parallel β-sheet structure connected by a β-turn. Natural tachyplesins exhibit potent antibacterial and antifungal activity and modest anti-HIV activity, but unfortunately they lyse human erythrocytes (Penberthy et al., 2011).

3. Cathelicidins

Cathelicidin discovery can be traced back to the isolation of an antimicrobial disulfide-containing cyclic dodecapeptide from bovine neutrophils (Romeo et al., 1988), soon followed by the purification of two additional peptides, designated bactenecins (after the Latin words '*bacterium necare*'), and of conserved similar proteins in other species (Zanetti, 2005). The term 'cathelicidins' was proposed in 1995 to acknowledge the evolutionary relationship of the novel protein family to cathelin, a protein originally isolated from porcine neutrophils as an inhibitor of cathepsin L, and it is used to denote holoproteins containing a conserved N-terminal cathelin-like domain of 99-114 residues linked to a heterogenic C-terminal antimicrobial domain of 12-100 residues (Zhu, 2008). The C-terminal peptides exert direct and/or indirect antimicrobial activity following their cleavage from the holoprotein (Zanetti, 2005).

3.1 Mammalian cathelicidins

Cathelicidins have been found in every mammal examined, with substantial interspecies variation in the number of members. The only human cathelicidin so far identified, also defined human cationic antimicrobial peptide-18 (hCAP18), has been isolated from neutrophils in 1995, and its expression has been successively observed in skin, mucous epithelia, wound and blister fluid, and in seminal plasma (Zanetti, 2005). The coding gene is located on chromosome 3, and its expression is both constitutive (in sweat gland cells) and inducible by vitamin D3, LPS and butyric acid (in colonic epithelial cells) (White, 2010). Unlike neutrophil defensins, which are fully processed to mature peptides before storage in the azurophil granules, human cathelicidin is present as propeptide in the specific granules and is cleaved after secretion to generate the antimicrobial peptide LL-37, a cationic 37 amino acid AMP bearing tandem N-terminal leucine residues. There is evidence that within the same organism cathelicidins are processed by different proteases in different physiological contexts: in humans, the activation of neutrophil-derived hCAP18/LL-37 is carried out by the serine protease proteinase 3, whereas epididymal-derived hCAP18 in

seminal plasma is cleaved by the prostate-derived protease gastricsin (pepsin C) in the presence of vaginal fluid at low pH (Zanetti, 2005). LL-37 has a stable α-helical structure and kills bacteria by cell membrane disruption. Moreover, it binds LPS with high affinity, inhibiting LPS-induced cellular responses, and prevents macrophage activation by lipoteichoic acid and lipoarabinomannan (Scott et al., 2002). LL-37 also inhibits mycobacteria and induces a Toll-like receptor-mediated killing of *M. tuberculosis* by monocytes (Méndez-Samperio, 2010). It has been shown that LL-37 is expressed by human epithelial cells in inflammatory environments such as wound healing, but it is also present in significant amounts in sweat, thus providing an innate anti-microbial defense system to the skin surface under non-inflammatory conditions (Murakami et al., 2002). LL-37 is constitutively expressed in gut epithelium and in lung alveolar macrophages and neutrophils. However, native LL-37 is hemolytic and toxic to human leukocytes. *In vivo*, LL-37 cytotoxic effects are inhibited by its binding to plasma proteins, but the binding also lowers antimicrobial efficacy (Ciornei et al., 2005). Considering LL-37 multifunctional activity, further investigation is needed to better define its biological properties and its possible therapeutic applications in the fields of immunomodulation and bacterial control. The future of cathelicidins relies on the ability to design synthetic more effective and less toxic variants (Mookherjee & Hancock, 2007). Significant achievements in this field could be not too far, considering that a synthetic 13-amino acid peptide, IDR-1, conceptually based on LL-37, with no direct antimicrobial activity, protects against bacterial infections *in vivo* by inducing chemokine production and enhancing leukocyte recruitment (Scott et al., 2007). An IDR-1 derivative, IDR-1002, showed stronger protective activity *in vitro* and in mouse models of infection with *S. aureus* and *E. coli* (Nijnik et al., 2010). Another promising new molecule currently under investigation is novicidin, a linear cationic α-helical AMP derived from ovispirin, a cationic peptide originated from the ovine cathelicidin SMAP-29 (Dorosz et al., 2010). A bovine cathelicidin with broad-spectrum activity, termed indolicidin, was originally isolated from bovine neutrophils (Selsted et al., 1992). Indolicidin is a 13-residue cationic peptide rich in tryptophan and proline, with a significant leishmanicidal activity, mediated by the disruption of *L. donovani* promastigotes and induction of autophagic cell death (Bera et al., 2003). It is also active against bacteria, fungi, and HIV, but its cytotoxicity prevents its use for therapeutic purposes (Rokitskaya et al., 2011). Less toxic derivatives such as omiganan (MBI-226), a 12-residue, indolicidin-based peptide variant, are currently under development (Rubinchik et al., 2009). This molecule, active on a wide range of bacteria and fungi, is currently undergoing confirmatory Phase III clinical trials for the prevention of infections arising from short-term central venous catheters, surgical contaminated wounds, and for the treatment of acne and rosacea (Rubinchik et al., 2009).

3.2 Avian cathelicidins

The genes of five cathelicidins termed fowlicidin-1, -2, -3, (Xiao et al., 2005), cathelicidin-B1 (Goitsuka et al., 2007), and myeloid antimicrobial peptide 27 (van Dijk et al., 2005), have been discovered by screening the chicken genome with bioinformatic methods. Functional analyses indicate that the corresponding synthesized peptides are among the most efficacious cathelicidins ever identified, with antibacterial and LPS-neutralizing activities that make them attractive candidates as antimicrobial and anti-sepsis agents. Fowlidicin-1, -2 and -3, are highly active against both Gram-negative and Gram-positive bacteria including MRSA, even in the presence of salts, whilst many AMPs, as for example LL37, are inactivated by high salt concentrations. These features could be useful in the treatment of

cystic fibrosis and Crohn's disease, both of which are related with aberrant local expression or inactivation of antimicrobial peptides (Saravanan & Bhattacharjya, 2011). Starting from the consideration that fowlicidin-1 is active against bacteria in the 1 to 2 μM concentration range, has potent LPS-neutralizing activity, but is hemolytic and toxic to epithelial cells, a fowlicidin-1 analog denominated fowl-1(6-26), is currently being developed. It maintains the full-length peptide antibacterial and LPS-neutralizing efficacy, but following carboxyl-terminal amidation it is more stable in serum. The resulting peptide, fowl-1(6-26)-NH2, reduced bacterial titers in the peritoneal fluid and spleen and improved the survival of mice by 50% in MRSA-induced lethal infections, which makes it an excellent drug candidate against infections and sepsis induced by drug-resistant bacteria (Bommineni et al., 2010). In comparison with fowlicidin-1, fowlicidin-2 exhibits similar antibacterial efficacy but lower cell toxicity. To further reduce fowlicidin-2 toxicity several deletion analogs were designed and analyzed for their antibacterial, cytotoxic, membrane permeabilizing and LPS-neutralizing activities. This work brought to the identification of 2 short peptide analogs, fowlicidin-2(1–18) and fowlicidin-2(15–31), which maintained the antibacterial and LPS-neutralizing activities, but showed a significantly reduced cytotoxicity (Xiao et al., 2009).

3.3 Snake cathelicidins

Snake venoms are composed of active substances endowed with a wide array of neurotoxic, myotoxic, cardiotoxic, hemorrhagic, pro- and anticoagulant, antiparasitic and antibacterial effects. The whole venom of *Bothrops marajoensis*, a snake of the *Viperidae* family, as well as one of its purified components, i.e. L-amino acid oxidase, exhibits a strong inhibition on the growth of a wide range of microorganisms, such as *S. aureus, C. albicans, P. aeruginosa*, and *Leishmania* species (Costa Torres et al., 2009). The opportunities offered by the recent development of bioinformatic techniques have been exploited to identify the genes with AMP-related sequences expressed in venom gland tissues. Three cathelicidins from the elapid species *Naja atra* (Chinese cobra), *Bungarus fasciatus* (banded krait) and *Ophiophagus hannah* (king cobra) have been identified by molecular cloning (Zhao et al., 2008). Phylogenetic analysis suggests that snake cathelicidins are closely related to rodent neutrophilic granule proteins, avian fowlicidins and chicken myeloid antimicrobial peptide 27. Unlike the highly divergent mammalian cathelicidins, the nucleotide and deduced protein sequences of the three cloned elapid cathelicidins are remarkably conserved. Each of them has a 22 amino acid residue signal peptide, a conserved cathelin domain of 135 amino acids and a mature antimicrobial peptide of 34 amino acids. In order to explore the structure–function relationships relative to the bactericidal and hemolytic activities, king cobra cathelicidin (OH-CATH) has been used as a molecular template to develop shorter synthetic analogs. Among OH-CATH and its analogs, OH-CATH(5–34) has the lowest hemolytic activity but maintains a strong antimicrobial activity. To evaluate its potential clinic values, the biological activities of OH-CATH(5–34) have been compared with those of pexiganan, a well-known phase III AMP derived from magainin. The bactericidal activity of OH-CATH(5–34) against 5 different species of bacteria (*E. coli, P. aeruginosa, S. aureus, Enterobacter aerogenes* and *E. cloacae*) was 2–4 times stronger than that of pexiganan. Hemolytic activity of OH-CATH(5–34) against human erythrocytes was 0.69% while that of pexiganan was 16.5% at the dosage of 200 μg/ml. The intravenous LD_{50} value of OH-CATH(5–34) on mice was 7-fold higher than that of pexiganan (175 mg/kg vs 25 mg/kg). Taken together, these results suggest that OH-CATH(5–34) can be considered an excellent candidate for developing therapeutic drugs (Zhang et al., 2010).

4. Epithelial host defense peptides

Healthy intact skin controls microbial growth by the combined action of complementary systems. The stratum corneum layer, with its lipid-rich matrix and protective low pH, constitutes a physical and chemical barrier further supported by bacterial nutrient limitation and physical removal by desquamation. A wide array of bacteriostatic and bactericidal compounds belonging to the HDP family concurs to the prevention of skin infections by inhibiting potentially invading microorganisms and maintaining a balanced commensal flora on the skin. Once this biochemical barrier is disturbed, bacteria or bacterial factors have access to living epidermal keratinocytes and stimulate the innate immune response that goes under the name of inflammation (Meyer-Hoffert et al., 2011). In human skin HDPs are mainly produced by keratinocytes, neutrophils, sebocytes or sweat glands and are either expressed constitutively or following an inflammatory stimulus. The relevance of HDPs in the skin physiology and pathology is underlined by the fact that in several human skin diseases there is an inverse correlation between severity of the disease and the level of HDP production. Decreased HDP levels are associated with burns, chronic wounds, and atopic dermatitis. In contrast, in some cases HDP over-expression is believed to lead to increased protection against skin infections as seen in patients with psoriasis and rosacea, whose lesions rarely result in bacterial superinfection. In skin infections, such as *acne vulgaris*, increased levels of HDPs can be found in inflamed tissues, indicating a role of these peptides in the immune reaction to infection. The broad spectrum of antimicrobial activity, the low incidence of bacterial resistance and the immunomodulatory function are attractive features that suggest a high HDP potential as topical anti-infective drugs in several skin diseases (Schittek et al., 2008). The best studied among skin-related antibacterials are some members of the already treated defensin (hBD-1-3) and cathelicidin (LL-37) families, and a number of heterogeneous factors, such as psoriasin, dermcidin, RNase 7, and peptidoglycan recognition proteins.

4.1 Psoriasin

Psoriasin is a low molecular weight protein that owes its name to the fact of being intensely expressed by the keratinocytes of patients with psoriasis (Madsen et al., 1991). After cloning of the cDNA, the 11,457 kDa protein was classified in the S100 protein family, that so far includes 21 low molecular weight (9–13 kDa) calcium-binding proteins characterized by the solubility in 100 % ammonium sulphate, from which is derived the name of the family. S100 proteins regulate many epithelial cell functions such as intracellular Ca^{2+} signaling, differentiation, cell-cycle progression, cytoskeletal membrane interactions, and leukocyte chemotaxis (McCormick & Weinberg, 2010). Psoriasin antibacterial activity was discovered in 2005 (Glaser et al., 2005). The abundance of psoriasin on human skin together with its high antimicrobial activity against *E. coli* suggest that psoriasin may be an important factor in controlling *E. coli* growth on the skin surface. The physiological role of psoriasin in protecting the skin against *E. coli* colonization and infection has been confirmed by *in vivo* experiments with neutralizing antibodies. These experiments performed on the skin of various healthy people identified psoriasin as a principal *E. coli*–killing factor. Subsequent studies in cultured human keratinocytes identified flagellin, a Toll-like receptor-5 ligand, as the *E. coli* ''pathogen-associated molecular pattern'' responsible for the expression of psoriasin mRNA and protein. It has been observed that the production of psoriasin in

human skin can also be induced by *P. aeruginosa* flagellin and rhamnolipids (Meyer-Hoffert et al., 2011). Indeed, *P. aeruginosa* is another ubiquitous bacterium that is however not usually present on healthy skin. These studies suggest that human skin might control the microflora to prevent colonization of the skin surface by unwanted microbes. Psoriasin is a metal ion-binding protein with a Ca^{2+} and Zn^{2+} binding capacity. Experiments aimed to clarify the mechanism of its antibacterial action demonstrated that in low concentrations (0.5 μM) psoriasin kills 90 % of all exposed *E. coli*. In higher doses (> 30 μM) psoriasin is bactericidal against *P. aeruginosa* and *S. aureus*, also. It seems that the mechanism of antibacterial action is mediated by zinc deprivation, zinc being an essential trace element for bacterial metabolism. Correspondingly, mutation experiments with recombinant psoriasin confirmed that zinc but not calcium binding is of significance for antibacterial activity (Glaser et al., 2005). Further, it was recently reported that psoriasin at pH values under 6 can also induce the formation of pores in the bacterial membrane (Glaser et al., 2011). The production by normal human keratinocytes of antimicrobial peptides, such as β-defensin-2 and -3, RNase 7, and psoriasin may be induced by ultraviolet radiation (Glaser et al., 2009). It is known that exposure to UV rays, especially of the B-waveband (UV-B, 280–315 nm), may suppress both systemic and local immune responses to a variety of antigens, and to several microorganisms (Termorshuizen et al., 2002). These findings suggest that UV-B irradiation suppresses T-cell–mediated immune responses but up-regulates the innate immune response by inducing the release of antimicrobial peptides.

4.2 Dermcidin

Dermcidin (DCD) is a human HDP isolated from sweat in 2001 (Schittek et al., 2001). It participates in the defense of the cutaneous surface, being constitutively secreted by eccrine sweat glands, but its expression has not been observed in epidermal keratinocytes of healthy skin, and it is not inducible by skin injury or inflammation. To date, DCD gene and mature peptide have been identified in humans only, and they show no homology to other known AMPs. Full length DCD consists of 110 amino acid residues with an N-terminal 19 amino acids signal peptide that is the hallmark of secreted proteins. In the sweat fluid, the DCD protein is cleaved by means of a post-secretory cathepsin D-mediated proteolytic process, giving rise to anionic and cationic peptides, two of which are recognized as the real effectors of the antimicrobial activity. A C-terminal 47 amino acid peptide corresponding to aa 63-109 of the originally translated product, named DCD-1, and a related peptide, DCD-1L, consisting of DCD-1 plus the last leucine (L) residue of the original precursor protein, have been identified in the sweat (Lai YP. et al., 2005). These peptides possess a potent and wide-spectrum antimicrobial activity against *S. aureus, E. coli, Enterococcus faecalis, Listeria monocytogenes, Salmonellae, Pseudomonas* and *Candida albicans* (Hata & Gallo, 2008; Pathak et al., 2009). By means of nuclear magnetic resonance it has been established that DCD-1 has an α-helical structure with a helix-hinge-helix motif, which is a common molecular fold among antimicrobial peptides (Jung et al., 2010). It seems that the affinity with which DCD-1 binds to bacterial-mimetic membranes is primarily dependent on its amphipathic α-helical structure and its length (>30 residues), whereas its negative net charge and acidic isoelectric point have little effect on binding. These findings suggest that the DCD mode of action is similar to that of other membrane-targeting antimicrobial peptides, though the details of its antimicrobial action remain to be determined. Using immune electron microscopy, it has been shown that DCD-1 antimicrobial activity originates with its binding to the bacterial

membrane, and that the molecule effectively kills *S. epidermidis*. DCD-1L shows stronger antimicrobial activity than the parent peptide, and it is highly effective against drug-resistant *S. aureus*, as well as other Gram-positive and Gram-negative bacterial strains (Jung et al., 2010). The mechanisms by which DCD-derived peptides kill bacteria are still unclear. Bactericidal activity is time-dependent and induce bacterial membrane depolarization. However, these molecules do not induce pore formation in the membranes of Gram-negative and Gram-positive bacteria. Interestingly, LL-37, as well as DCD-derived peptides, inhibit bacterial macromolecular synthesis, especially RNA and protein synthesis, without binding to microbial DNA or RNA. Binding studies indicate that DCD-derived peptides bind to the bacterial envelope but show only a weak binding to lipopolysaccharide from Gram-negative bacteria or to peptidoglycan, lipoteichoic acid, and wall teichoic acid, isolated from *S. aureus*. In contrast, LL-37 binds strongly in a dose-dependent fashion to these components. These data indicate that the mode of action of DCD-derived peptides is different from that of the cathelicidin LL-37 and that components of the bacterial membranes play a role in the antimicrobial activity of DCD (Senyurek et al., 2009). Being a non-inducible factor, DCD contributes to the epithelial defense by modulating the surface colonization rather than by responding to injury and inflammation as is the case of the inducible peptides hBD-2 and -3, or LL-37 and psoriasin. Modulation and control of the skin-resident flora may be achieved by two types of effects: the non-specific prevention of microbial overgrowth on the skin surface, and the more specific prevention of skin colonization by pathogenic microorganisms, thereby establishing a host-friendly resident flora (Rieg et al., 2004). It appears that DCD could be the first of a new class of potential broad-spectrum antimicrobial drugs. In order to obtain large quantities of highly purified peptide for experimental use, it has been developed a method for the production of recombinant DCD-1L, in which it is expressed as a fusion protein, followed by enzymatic cleavage to release the active peptide. Recombinant DCD-1L is not cytotoxic against erythrocytes when assayed in PBS. This is supposed to be due to the presence of negatively charged sialic acid on the erythrocytes, that would electrostatically repel DCD-1L, which also has a net negative charge. This favorable condition suggests that it is of noteworthy potential as a therapeutic substance in clinical settings (Lai YP. et al., 2005).

4.3 Ribonuclease 7

RNase 7 was discovered as part of a broad screening protocol aimed at identifying antimicrobial agents in human skin (Harder & Schroder, 2002). Successively, it has been discovered that it is also expressed by various epithelial tissues, especially in the respiratory and genitourinary tract. It is a highly cationic protein that shares a potent antibacterial activity with another member of the human RNase A superfamily, the eosinophil-derived RNase denominated ECP (eosinophil cationic protein/RNase 3). ECP possesses bactericidal, antiviral and antiparasitic activities and inhibits mammalian cell growth. Its ribonucleolytic activity with common RNA substrates is low and does not appear to be necessary for the antibacterial capacity. The finding that RNase 7 exhibited both antimicrobial and ribonuclease activity gave rise to the speculation that the enzymatic activity is involved in microbial killing. However, although the mechanisms involved in the antimicrobial properties of RNase 7 are not completely understood, its bactericidal activity has been linked to its capacity to permeate and disrupt the bacterial membrane, independent of its RNase activity (Spencer, 2011). RNase 7 is currently considered a major component of the

antimicrobial protein and peptide group that constitutes the biochemical skin barrier (Boix & Nogués, 2007), being active in the low micromolar concentration range against *S. aureus*, *P. aeruginosa*, *Propionibacterium acnes*, and *C. albicans* (Spencer, 2011). The hypothesis that RNase 7 may play an important role in the cutaneous antimicrobial defense system is further supported by the observation that contact of keratinocytes with bacteria induces RNase 7 gene expression, a finding that is in agreement with what is known for other epithelial antimicrobial proteins like the human defensins hBD-2, hBD-3, and hBD-4. Of particular interest is RNase 7 activity against vancomycin-resistant *Enterococcus faecium*. This potent antimicrobial activity supports the idea that RNase 7 might be a useful agent to treat the emerging infections caused by this and other multiresistant bacteria.

4.4 Peptidoglycan recognition proteins

The definition "peptidoglycan recognition protein"(PGRP) was first introduced in 1996 to indicate a 19 kDa protein, present in the hemolymph and cuticle of a silkworm (*Bombyx mori*), that binds the peptidoglycan of Gram-positive bacteria and activates the prophenoloxidase cascade which generates melanin (Yoshida et al., 1996). Subsequently, many other similar molecules have been identified and added to the PGRP group, that includes conserved lectin-like proteins present in insects, mollusks, echinoderms, and vertebrates, but not in nematodes or plants. Mammals express a family of 4 PGRPs, which were initially named PGRP-S, PGRP-L, PGRP-I-α and PGRP-I-β (for short, long and intermediate-α and -β transcripts, respectively), analogous to insect PGRPs. These names were changed by the Human Genome Organization Gene Nomenclature Committee to PGLYRP-1, PGLYRP-2, PGLYRP-3, and PGLYRP-4, respectively. This terminology is also used for mouse PGRPs, and is now in use to selectively indicate vertebrate PGRPs. Both invertebrate and vertebrate PGRPs function as pattern recognition and effector molecules in innate immunity. All PGRP proteins have at least one C-terminal PGRP domain about 165 amino-acid residues long, that is homologous to the bacteriophage and bacterial type 2 amidases. This homology indicates that PGRPs and prokaryotic type 2 amidases might have evolved from a common primordial ancestor gene. Almost all PGRPs have two closely spaced, conserved Cys residues in the centre of the PGRP domain that form a disulphide bond, which is required for PGRP structural integrity and activity. Mammalian PGLYRPs are differentially expressed in various organs and tissues and perform both amidase and antibacterial activity. PGLYRP-1 is present in the granules of the polymorphonuclear leukocytes, and contributes to the killing of phagocytosed bacteria. PGLYRP-2, which is constitutively produced in the liver and secreted into the blood, is also induced in the skin and intestines. It is an *N*-acetylmuramoyl-L-alanine amidase that hydrolyzes peptidoglycan reducing its proinflammatory activity. PGLYRP-3 has direct bactericidal activity and is expressed in the skin, eyes, tongue, esophagus, stomach, and intestines. PGLYRP-4 and the PGLYRP-3:4 dimer also have direct bactericidal activity in the same tissues; PGLYRP-4 is also expressed in the salivary gland, mucus-secreting glands in the throat and in saliva (Dziarski & Gupta, 2006). It has been demonstrated that the bactericidal activity of human PGLYRP-1, PGLYRP-3, PGLYRP-4, and PGLYRP-3:4 against both Gram-positive and Gram-negative bacteria requires zinc (Wang M. et al., 2007). A previously unknown mechanism of bacterial killing by PGRPs has been recently elucidated by Kashyap et al., (2011). In Gram-positive bacteria, to perform their action PGRPs need to pass through the thick cell wall and bind

peptidoglycan near the cell membrane. This can be accomplished only at the separation sites of newly formed daughter cells during cell division. The binding induces the activation of the bacterial CssR-CssS two-component system, which triggers bacterial killing by inducing membrane depolarization and the production of toxic [OH]• in the cytoplasm. This process is accompanied by the cessation of all major intracellular biosynthetic reactions, probably because of the lack of membrane potential–dependent generation of energy. Due to the different external structure of Gram-negative bacteria, in which a thin peptidoglycan layer is covered by a lipopolysaccharide-containing outer membrane, the initial interaction of PGRPs with Gram-negative bacteria is different. The analysis of the localization of PGRPs in *E. coli* by immunofluorescence and confocal microscopy demonstrated that the entire *E. coli* outer membrane uniformly binds PGRPs at all stages of growth, in contrast to the described selective localization of PGRPs to cell separation sites in Gram-positive bacteria. Following the binding to the outer membrane, the CpxA-CpxR two-component system, functionally homologous to the CssR-CssS system of Gram-positive bacteria, is activated. The CssR-CssS and CpxA-CpxR systems are designed to detect and dispose of extracellular misfolded bacterial proteins at the cell membrane–cell wall interface, after these proteins have been exported from the cell, and in Gram-negative bacteria CpxA-CpxR also detects proteins in the outer membrane. Notwithstanding the difference between the initial interaction mechanism, the killing mechanism of PGRPs seems common for all bacteria and depends on the activation of the protein-sensing two-component systems. These two-component systems can therefore be considered appealing targets for the development of new antibacterial therapies. PGRPs could be used as a basis for the design of shorter molecules that would maintain the broad-spectrum bactericidal activity of natural factors, but would be more convenient in terms of immunogenicity and production costs.

5. Amphibian host defense peptides

The skin of amphibia Anura (frogs and toads) is one of the richest reservoir of biologically active peptides. These HDPs are produced by dermal glands, stored within granules and released on the skin surface upon stress, injury, or electrical or norepinephrine stimulation. Their synthesis is induced by contact with microorganisms and is transcriptionally regulated by the NF-jB/IjBa machinery (Mangoni & Shai, 2011). These peptides constitute a rich arsenal of broad-spectrum, cytolytic AMPs characterized by highly variable sequences (Vanhoye et al., 2003). It is estimated that there may be as many as 10^5 different peptides produced by the known 5000 species of anuran amphibians, and more than 400 have been already identified from South American *Hylidae* or European, Asian or North American *Ranidae* amphibians (Nicolas & El Amri, 2008). Therefore, the main work still concerns the screening and identification of the most useful factors. The structural characteristics of some amphibian peptides are interesting for their potential implications in the mechanism of antimicrobial activity. A class of structurally unique molecules, still in the characterization phase, contains an intermolecular disulphide bridge in the C-terminal portion of the peptide that creates a 7–9 residue macrocyclic region, sometimes referred to as the "Rana box". Many of these peptides originate from frogs belonging to the *Rana* genus and examples include esculentins, brevinins, ranacyclins, ranalexins, gaegurins, ranateurins and nigrocins (Haney et al., 2009). Here we focus on four families that include some of the better known and most representative peptides, namely magainins, dermaseptins, bombinins and temporins.

5.1 Magainins and magainin-related peptides

Magainin-1 and -2 are the first AMPs isolated from the skin of the African clawed frog *Xenopus laevis* (Zasloff et al., 1987). Because they were among the first identified antimicrobial peptides, there has been considerable research associated with magainin structure and mechanism of action. Magainins are 23 amino acid peptides with α-helical structure. Following the observation that magainin-2 possesses broad spectrum antibacterial and antifungal activity, many synthetic analogs have been developed in order to maximize the antimicrobial effects and minimize cytotoxicity. Magainin-A, a magainin-2 analog, underwent preclinical evaluation studies on *Macaca radiata* monkeys as local contraceptive, showing good spermicidal, antibacterial and antifungal activity (Clara et al., 2004), but has not been further developed. An extensive structure-activity investigation on magainin 2, performed by Zasloff and co-workers of Magainin Pharmaceuticals, resulted in the development of MSI-78 or pexiganan, a molecule that entered clinical trials for topical treatment of diabetic foot ulcers. In 1999 the FDA denied approval of pexiganan after completion of two phase III clinical trials, in which pexiganan resulted no more effective than already approved treatments for diabetic foot ulcers, and required addition clinical trials for consideration. Following successive acquisitions, pexiganan is by now being developed by Access Pharmaceuticals Inc. Improvements in clinical trial design, greater understanding of diabetic foot ulcers and topical anti-infective treatments, and advances in peptide manufacturing keep hopes alive regarding the potential FDA approval of pexiganan (Gottler & Ramamoorthy, 2009).

A new peptide that is not a magainin, but is often included in the magainin family, is peptidyl-glycine-leucine-carboxyamide (PGLa), whose existence was predicted through the screening of a c-DNA library for clones encoding the precursor of caerulein (Hoffmann et al., 1983), when searching in amphibian skin secretions for peptides closely related to mammalian hormones and neurotransmitters. In this study it was concluded that this peptide could form a membrane-active amphipathic helix similar to peptides with bacteriostatic, cytotoxic and/or lytic properties. The natural PGLa counterpart was isolated two years later from the skin secretions of *X. laevis* by the same group (Andreu et al., 1985). At neutral pH this peptide has a positive net charge of 5 because of the four lysine residues and the amino group at the N-terminal glycine. It also has an amidated C-terminus that provides good resistance to proteases (Lohner & Prossnigg, 2009). PGLa showed good *in vitro* activity against *E. coli*, *S. aureus*, and *S. pyogenes* in the concentration range of 10-50 μg/ml, and was also active, but at higher concentrations, against *P. aeruginosa* (200-500 μg/ml), *Saccharomyces cerevisiae* (100-200 μg/ml), and *C. albicans* (100-200 μg/ml) (Soravia et al., 1988). PGLa activity has been also tested *in vitro* against *Plasmodium falciparum*, at a concentration range of 20-60 μM (Boman et al., 1989). However, the peptide with the greatest potential for development into a therapeutically valuable anti-infective agent is by some considered the caerulein precursor fragment B1 (CPF-B1) (Mechkarska et al., 2010). Caerulein is a short peptide whose amino acid sequence shows a close resemblance to the mammalian gastrointestinal hormone gastrin. It was originally isolated from the skin of the Australian frog *Hyla caerulea* (De Caro et al., 1967). The structure of the caerulein precursor extracted from the *X. laevis* skin has been determined in 1985 (Wakabayashi et al., 1985). CPF-B1 is one of four CPF fragments (CPF-B1 -B4) that can be found in the skin secretions obtained from the norepinephrine-stimulated skin of the tetraploid frog *Xenopus borealis* (*Pipidae*). CPF-B1 is the most abundant fragment and is active against clinical isolates of the nosocomial pathogen MRSA and multidrug-resistant *Acinetobacter baumannii* with MIC

values in the range 4–8 μM. It is also active against *E. coli* (MIC=5 μM) and *C. albicans* (MIC=25 μM), and shows low hemolytic activity on human erythrocytes. The high potency of CPF-B1 against MRSA and multidrug-resistant *A. baumannii,* together with its low toxicity suggest that the peptide could be used for the topical treatment of skin infections caused by these pathogens and in therapeutic regimes to promote wound healing (Mechkarska et al., 2010).

5.2 Dermaseptins

Dermaseptins are genetically related α-helical amphipathic AMPs 28-34 amino acid-long, with 3-6 lysine residues, and a highly conserved tryptophan residue in the third position from the C-terminus residue (Zairi et al., 2007). Dermaseptins are present in the skin of *Hylidae* and *Ranidae* frogs. They show a remarkable identity in the signal sequences of their preproforms, but have clearly diverged to yield several families of microbicidal cationic peptides that are structurally distinct (Amiche & Galanth, 2011). Dermaseptin S1, the first member of the dermaseptin family to be discovered, was isolated from an extract of dried skin of *Phyllomedusa sauvagei* in the early 1990s (Mor et al., 1991). It was followed by the isolation of dermaseptin B2, also denominated adenoregulin for its ability to interact with the adenosine receptor, from the skin of the arboreal frog *P. bicolor* (Daly et al., 1992). Subsequently, dermaseptin B1, a 27-residue peptide, was also isolated from *P. bicolor* skin (Mor et al., 1994). These last 2 peptides were thought to be unrelated until attempts to clone their precursor polypeptides revealed the presence of a common preproregion and 5′- and 3′-UTRs (Amiche et al., 1993). Since then, additional dermaseptins were rapidly identified in various South American species and now constitute the dermaseptin super-family. Basic research on dermaseptins is of relevance because genetic studies on the evolution and diversity of frog skin AMPs may lead to the identification of new peptides with alternative targets. In addition, the discovery of new isoforms with novel structural and biochemical properties may also shed light on the exact roles of various parameters, such as net charge, percent of α-helical/β-sheet structure, amphipathy and conformational flexibility, on the ability of antimicrobial peptides to bind to and disrupt bacterial membranes (Nicolas & El Amri, 2008). Dermaseptin antimicrobial activity is currently being characterized and analogs are being developed. Dermaseptin S4 analogs are active against *Neisseria gonorrhoeae* (Zairi et al., 2007) and 15 analogs of dermaseptin S1, synthesized by our group, showed variable activity against *Trichomonas vaginalis*, *Herpes simplex virus-1* and human *Papillomavirus 16* (Savoia et al., 2008, 2010). These properties, coupled with the already demonstrated spermicidal activity of dermaseptins S, suggest that dermaseptins, as well as magainins, alone or even better in combination, could be used as topical contraceptives and microbicides to contemporarily prevent unwanted conceptions and sexually transmitted diseases.

5.2.1 Plasticins

Plasticins constitute a family of orthologous peptides with antimicrobial activity classified in the dermaseptin superfamily. They are quite similar as far as amino acid sequence, hydrophobicity, and amphipathicity are concerned, but differ markedly in their conformational plasticity and spectrum of activity (Vanhoye et al., 2003). The plasticins from phyllomedusid frogs of the *Hylidae* family may be divided into two classes on the basis of their cytolytic activities: the strongly cationic peptides plasticin-B1 (from *P. bicolor*) and -S1 (from *P. sauvagei*) that contain lysine residues and show potent, broad spectrum

antimicrobial activity and hemolytic activity; and the weakly cationic or neutral plasticins (plasticin-A1, from *Agalychnis annae,* plasticin -C1 and -C2 from *A. callidryas*, and plasticin-DA1 from *Pachymedusa danicolor*), that are hemolytic but devoid of antibacterial activity (Conlon et al., 2009). Plasticin-L1, more recently isolated from the South American frog *Leptodactylus laticeps*, falls into the second category and is devoid of cytolytic activity against Gram-positive and Gram-negative bacteria. However, in contrast to the other plasticins, it does not produce lysis of human erythrocytes at concentrations up to 500 μM (Conlon et al., 2009). The plasticin peptide family constitutes a good model to address the relationships between structural polymorphism, membrane-interacting property, and biological activity of antimicrobial, cell-penetrating, and viral fusion peptides (El Amri & Nicolas, 2008). Unlike amphipathic helical dermaseptins, plasticins display considerable conformational flexibility and polymorphism that modulate their ability to bind to and disrupt the bilayer membranes of prokaryotic and eukaryotic cells, and/or to reach intracellular targets (Nicolas & El Amri, 2008).

5.3 Bombinins and temporins

In the 1960s, Csordas and Michl published a series of papers that culminated in 1970 with a report on the characterization of a hemolytic and antibacterial peptide 24 amino acid long, isolated from the European toad *Bombina variegata* (Csordás & Michl, 1970). This peptide, called "bombinin," shares many of its general structural features with the larger group of magainins. A series of peptides from the skin of a closely related amphibian, *Bombina orientalis* (or Asian toad) has been subsequently isolated and characterized (Zangger et al., 2008). These peptides share considerable homology with bombinin and are called bombinin-like peptides, or BLPs, and were found to possess potent antibacterial activities but, unlike bombinin, lack any appreciable hemolytic activity. Other peptides, structurally unrelated with previously discovered bombinins and containing a D-amino acid as the second residue, are the bombinins H, endowed with both antibacterial and hemolytic properties (hence the final –H) that were isolated from *B. variegata* skin (Mignogna et al. 1993). The expression of the genes encoding the common precursor for bombinins and bombinin H has been shown to be induced by bacterial infection *in vivo* as well as *in vitro* (Miele et al., 1998). Remarkably, after processing of the precursor, bombinin H is further modified by a recently characterized peptidyl-aminoacyl-L/D-isomerase that catalyses the inversion of the stereochemistry of the second amino terminal residue (Jilek et al., 2011; Zangger et al., 2008).

The first molecule belonging to the temporin family was identified in methanol extracts of the skin of the Asian frog *Rana erythraea*. In the early 1990s, Simmaco et al. identified a family of similar peptides with antibacterial and antifungal properties from the skin secretion of the European red frog *Rana temporaria* and termed them temporins (Mahalka et al., 2009; Simmaco et al., 1996). Subsequently, many other temporins have been isolated from the secretions of other ranid frogs of both North American and Euroasian origin. At present, the temporin family includes more than 100 members, which share a number of unique properties, such as: i) a short amino acid sequence, that favors cost-efficient chemical synthesis: most temporins are 10–14 amino acid long, with a few 16–17 amino acids exceptions, and an ultrashort temporin of only eight amino acid residues, that represents the smallest naturally occurring linear AMP so far identified, has been isolated from the skin of the frog *Phelophylax saharica* (Abbassi et al., 2010); ii) a low positive charge ranging from +2 to +3 at neutral pH (in contrast with most AMPs belonging to other families, which usually

have a higher net positive charge); iii) high efficiency against a wide range of pathogens, that is retained in serum, and concomitant low or null toxicity to mammalian cells; iv) at least in some cases, immuno-modulatory and/or antiendotoxin activity (Mangoni & Shai, 2011). Temporins exhibit antibacterial, antifungal, antiviral and antiprotozoan activities. Their potent action against Gram-positive bacteria, including methicillin-resistant strains, is of particular interest, because a synergistic action of the temporin A-methicillin association has been observed in a rat model of infection with methicillin-resistant *S. epidermidis* (Ghiselli et al., 2002). Temporin-1Tl has a higher and broader spectrum of activity than the other isoforms, being active against fungi and Gram-negative bacteria such as *P. aeruginosa* and *E. coli*, but it disrupts human erythrocytes at microbicidal concentrations (Mangoni et al., 2011). Temporins-1Ta, Tb, and Tl have been shown to neutralize the toxic effect of LPS derived from various species of *E. coli*, by complexing with it and making it unavailable for interaction with macrophage receptors to stimulate the production of TNF-α, considered a primary mediator of endotoxemia (Mangoni & Shai, 2008). Owing to their characteristics, temporins are considered worth of further development. In this perspective, by studying the structure-activity relationship of a library of Tl derivatives, Mangoni and co-workers identified novel analogues with better properties that could be used for future developments (Mangoni et al., 2011).

6. Insect host defense peptides: Defensins and cecropins

Insects such as moths, flies and bees rely on a wide array of humoral peptidic factors to defend themselves against potential pathogens. A recently identified family of peptides isolated from the *Apis mellifera* royal jelly is represented by the jelleins. These are composed of 8–9 amino acids, are amidated at the *C*-terminus and bear a +2 charge (Romanelli et al., 2010). While these molecules are still in the characterization phase, the research on insect defensin went a little further. The core structure of invertebrate defensins is composed of an α-helical domain linked to a two-stranded antiparallel β-sheet with three or four disulphide bonds forming the so-called cysteine-stabilized α-helix β-sheet motif. Some antifungal peptides like drosomycin from *Drosophila melanogaster* contain an additional short N-terminal β-strand, so presenting a βαββ-scaffold that is similar to that of antifungal plant defensins (Wilmes et al., 2011). Royalisin, an insect defensin isolated from the royal jelly of *A. mellifera* (Fujiwara et al., 1990), consists of 51 amino acids, in which six cysteine residues form three disulfide bonds that give the molecule a compact globular structure. Royalisin inhibits the growth of Gram-positive bacteria and fungi and is particularly active against the honeybee pathogen *Paenibacillus larvae larvae*, that causes American foulbrood, a serious disease found in honeybee larvae (Bilikova et al., 2001). Recombinant and functionally active royalisin has been recently obtained with a yield of the final purified product in the range of 0.192 mg/L of bacterial cell culture. Considering that the substance shows antibacterial activity at the 1–27 μg/ml concentration range, this breakthrough makes it possible to proceed to an extensive characterization of royalisin for both beekeeping and human therapeutic purposes (Tseng et al., 2011).

6.1 Cecropins

Cecropins are lytic peptides that possess antibacterial activity *in vitro*, originally isolated from the hemolymph of the giant silk moth *Hyalophora cecropia* (Hultmark et al., 1980). The

killing is mediated by membrane permeabilization, with a detergent-like effect accompanied by pore formation (Bechinger & Lohner, 2006). Cecropin specificity of action relies upon the differences in the composition and physicochemical properties of germ and host cell membranes. Pore formation is easily achieved in bacterial membranes rich in anionic phospholipids, but not in animal cell membranes, rich in neutral phospholipids and further stabilized by cholesterol. Cecropins are considered worth of further development because they show a well demonstrated biological activity and consist of a single polypeptide chain well suited for economical production through recombinant DNA technology or peptide synthesis. Cecropin-like peptides are currently being developed following different strategies to improve antimicrobial and anticancer activity and diminish cytotoxicity (Plunkett et al., 2009; Wu et al., 2009). Based on the assumption that lysozyme is inactive on Gram-negative bacteria because it cannot reach the peptidoglycan layer, and that cecropin may disrupt the outer membrane of Gram-negatives, giving the enzyme access to peptidoglycan, a novel hybrid protein combining *Musca domestica* cecropin with human lysozyme has been expressed in *E. coli*. This chimeric protein showed an improvement of antibacterial activity and spectrum compared to its single original components (Lu et al., 2010). Another chimera, the cecropin AD peptide, composed by the first 11 residues of *H. cecropia* cecropin A and the last 26 residues of *H. cecropia* cecropin D, has been produced in a *Bacillus subtilis* expression system (Chen X. et al., 2009). The potent antimicrobial activity against *S. aureus* and *E. coli* of the recombinant product, and the low cost of the production process, with a yield of 30.6 mg of pure recombinant protein obtained from 1 liter of culture supernatant, make this molecule a suitable option for veterinary and medical applications. Cecropins have properties similar to those of melittin, a peptide that is the major component of the *A. mellifera* venom (Pandey et al., 2010). Some melittin analogues showed a drastic cytotoxicity reduction though maintaining comparable bactericidal activity. Two recombinant cecropin A- and cecropin B-melittin hybrid peptides CA(1-7)-M(4-11) and CB(1-7)-M(4-11) have been expressed in the yeast *Pichia pastoris*. Both chimeric peptides showed strong antibacterial activity against *E. coli, S. aureus, P. aeruginosa, Klebsiella pneumoniae, Bacillus subtilis, B. thuringiensis*, and *Salmonella derby* (Cao et al., 2010). The efficacy of a cecropin A-melittin hybrid peptide CA(1-8)M(1-18) and shorter derivatives against pan-resistant *Acinetobacter baumannii* has been tested both *in vitro* and in a mouse sepsis model. The peptide showed an *in vitro* good activity, that was not affected by the presence of capsule (Rodríguez-Hernández et al., 2006). However, *in vivo* the peptides showed bacteriostatic activity only, and PD_{50} was not achieved with non-toxic doses (López-Rojas et al., 2011). The antifungal and anti-inflammatory effects of a cecropin A(1-8)–magainin 2(1-12) hybrid peptide analog (P5) have been tested on *Malassezia furfur* and human keratinocytes. The minimal inhibitory concentration of P5 against *M. furfur* was 0.39 μM, making it 3–4 times more potent than commonly used antifungal agents such as ketoconazole (1.5 μM) or itraconazole (1.14 μM). P5 efficiently inhibited the expression of IL-8 and Toll-like receptor 2 in *M. furfur*-infected human keratinocytes without eukaryotic cytotoxicity at its fungicidal concentration. Moreover, P5 significantly down-regulated NF-kB activation and intracellular calcium fluctuation, which are closely related with enhanced responses of keratinocyte inflammation induced by *M. furfur* infection. Taken together, these observations suggest that P5 may be a potential therapeutic agent for *M. furfur*-associated human skin diseases because of its distinct antifungal and anti-inflammatory action (Ryu et al., 2011).

7. Bacterial antimicrobial peptides

A wide array of proteinaceous molecules is produced by Gram-positive and Gram-negative bacteria to counteract the proliferation of closely related microorganisms competing for limited resources within the same ecological niche (Héchard & Sahl, 2002). The first description of antagonistic interactions between different *Staphylococcus* strains was made in 1855 by Babes, who with Cornil co-authored the first text on bacteriology. The same phenomenon was described by Pasteur, who noted the inhibitory effect of common bacteria from urine on *Bacillus anthracis* (Pasteur & Joubert, 1877). However, the first clear documentation of an antibiotic agent produced by *E. coli* was provided by Gratia, who in 1925 demonstrated that in liquid media strain V (for virulence) produced a dialyzable and heat-stable substance (later referred to as colicin V) that even in high dilutions inhibited the growth of *E. coli* strain φ (Gratia, 1925). Subsequently, a number of colicins produced by *E. coli* and closely related members of the *Enterobacteriaceae* were discovered. Following the discovery that antibiotic substances of the colicin type are also produced by non-coliform bacteria, the more general term ''bacteriocin'' was coined (Jacob et al., 1953), to define proteinaceous antibiotics of the colicin type, characterized by predominant intra-specific killing activity, and adsorption to specific receptors on the surface of sensitive cells (Jack et al., 1995; Tagg et al., 1976). Bacteriocins produced by Gram-positive bacteria differ in many characteristics from those produced by Gram-negative bacteria: the former are initially produced as propeptides, which are subsequently separated from a leader peptide to form the biologically active molecule. In some cases, such as the lantibiotics, post-translational modifications are introduced into the propeptide region of the precursor molecule prior to cleavage of the leader component (Cotter et al., 2005a). In contrast, Gram-negative bacteriocins (colicins) are generally high-molecular-mass (29- to 90-kDa) proteins that contain characteristic domains specifying either attachment specificity, translocation, or killing activity. Similar domain constructs have been found in some of the pyocins produced by *P. aeruginosa* (Jack et al., 1995).

7.1 Gram-positive bacteriocins

Although there is not a definitive classification for bacteriocins from Gram-positive bacteria, it is generally accepted the division into class I, composed by post-translationally modified peptides containing lanthionine or methyl-lanthionine; class II, or heat-stable non-lanthionine-containing bacteriocins, which are small thermostable, non-modified proteins (with the exception of disulfide bridges linkage), with or without leader peptide; and class III, which includes secreted heat-labile, cell wall-degrading enzymes. A family of circular, post-translationally modified bacteriocins has been recently grouped to form a new class of bacteriocins, class IV, that encompasses globular, thermostable, helical, and post-translationally modified proteins, ranging between 35 and 70 amino acids, with the N- and C-termini linked by a peptide bond (Sanchez-Hidalgo et al., 2011).

7.1.1 Class I bacteriocins: Lantibiotics

Class I bacteriocins produced by lactic acid bacteria are the most widely investigated: they are small, heat-stable post-translationally modified peptides commonly called lantibiotics, that naturally occur in food and in the gastro-enteric tract of mammals. Some of them, such as nisin and lacticin, are widely used as antibacterial agents by the food and agricultural

industry of more than 50 countries (Chatterjee et al., 2005; Cotter et al., 2005b). Lantibiotics are ribosomally synthesized as precursor peptides, and post-translationally modified by the dehydration of serine and threonine residues and subsequent intramolecular addition of cysteine, resulting in the formation of (β-methyl) lanthionine thioether bridges, that characterizes the group (Abriouel et al., 2010). A N-terminal leader sequence is believed to keep the peptides inactive while inside the producing cell. To further protect themselves from the action of secreted lantibiotics, the lantibiotic-producing bacteria have evolved self-protection mechanisms that consist of individual immunity proteins (generically termed the LanI proteins), or of highly conserved ATP-binding cassette transporter (ABC-transporter) proteins, usually composed of two or three subunits, generically termed LanFE(G) (Draper et al., 2008). Many lantibiotics are extremely potent antibacterial agents with minimum inhibitory concentrations in the nanomolar range (Ross & Vederas, 2011). Lantibiotics are active against several very common food spoilage organisms (for example, *Listeria monocytogenes* and *Clostridium botulinum*) and show very promising activity against resistant *S. aureus* and enterococcal infections (Cotter et al., 2005a). In the last few years some bacteriocins have been considered for human health and medical purposes: nisin A, the prototype lantibiotic produced by *Lactococcus lactis,* is highly efficient against Gram-positive bacteria and has no human toxicity. It was discovered in 1928 and has been accepted by the Food and Drug Administration as a food additive in 1988. Its 34-amino acid residue structure contains five macrocyclic rings stabilized by thioether bonds (Turpin et al., 2010). Nisin inhibits the growth of vegetative Gram-positive bacteria by binding to lipid II, so disrupting cell wall biosynthesis and facilitating pore formation. Nisin also inhibits the outgrowth of bacterial spores, including *Bacillus anthracis* spores (Gut et al., 2011). However, natural nisin A is unsuitable for medical uses, being unstable and poorly soluble in neutral or basic conditions and easily inactivated by thiols such as cysteine and glutathione (Rollema et al., 1995). Nisin A derivatives obtained by amino acid substitution are being developed and evaluated as anti-mycobacterial drugs (Carroll et al., 2010). Lacticin 3147, another lantibiotic produced by lactic acid bacteria, is more stable than nisin and is active against MRSA and VRE at nanomolar concentrations (Piper et a., 2009). Lacticin 3147 consists of a 2-peptide (lacticin A1 and A2) system: lacticin A1 binds lipid II, and the complex binds lacticin A2, that induces pore formation in the bacterial membrane. To the class I bacteriocins also belongs thuricin CD, another 2-component peptide system produced by *Bacillus thuringiensis* and selectively active against *Clostridium difficile*. A problem inherent the current antibiotic treatment of *C. difficile*-associated bowel disease is that large-spectrum antibiotics can perturb the gut flora to the point to interfere with recovery and in same cases even to promote recurrences. These problems could be avoided by the use of thuricin CD that, according to extensive tests against a broad range of Gram-positive and Gram-negative bacteria, targets a restricted spectrum of spore-forming Gram-positive bacteria (Rea et al., 2010).

7.1.2 Class II bacteriocins

Class II bacteriocins include class IIa one-peptide pediocin-like bacteriocins and class-IIb, that are two-peptide bacteriocins (Nissen-Meyer et al., 2010). Pediocin PA-1 is a representative member of the class IIa bacteriocins, i.e. low molecular weight, plasmid-encoded peptides, with marked antilisterial activity, produced by *Pediococcus acidilactici*

(Devi & Halami, 2011). It is currently investigated as a useful tool to control *Listeria monocytogenes* in food (Hartmann et al., 2011). At least 15 two-peptide members of the class-IIb bacteriocins have been isolated and characterized since the first isolation of lactococcin G (Nissen-Meyer et al., 1992). Like lacticins, these bacteriocins consist of two different peptides, and optimal antibacterial activity requires the presence of both peptides in about equal amounts. The two peptides are synthesized as preforms that contain a 15–30 residue N-terminal leader sequence that apparently facilitates interaction with the dedicated ABC-transporter membrane protein and might possibly also function to keep the bacteriocin inactive until it has been secreted. The genes encoding the preforms of the two peptides are always found next to each other in the same operon along with the gene that encodes the immunity protein. Class IIb bacteriocins are still in the characterization phase, in order to develop variants useful for medical and biotechnological applications, such as infection treatment and food and animal feed preservation (Nissen-Meyer et al., 2010). Class III bacteriocins, like enterolysin A, are large antibacterial proteins with enzymatic activity (Nilsen et al., 2003), that for their structure are not considered suitable for drug development. On the contrary, low-molecular weight, circular class IV bacteriocins possess some interesting features: the joining of the ends protects from degradation by exopeptidase enzymes, increasing stability and making the molecules highly resistant to a wide range of pH and temperatures. A representative member of class IV bacteriocins is AS-48, that is the first reported circular bacteriocin and whose structure and genetic regulation have been elucidated. It can be considered a good starting point to develop analogs with new and/or improved features for practical chemical, pharmaceutical and agricultural applications. (Sanchez-Hidalgo et al., 2011).

7.2 Gram-negative bacteriocins

Enterobacteria can secrete colicins and microcins, both encoded by gene clusters that codify for their production, export and self-immunity. To date, all colicins and microcins found are plasmid-encoded, except class IIb microcins, that are chromosome-encoded. Colicin gene clusters are highly conserved, but simpler than microcin gene clusters. In contrast to microcins, the production of colicins is mainly induced *via* the DNA repair network, called the SOS response, that can be activated by an environmental stress, such as UV irradiation, exposure to DNA damaging agents, or cell starvation. The major differences between microcins and colicins, besides the molecular mass, lie in their structure and in the fact that contrary to most microcins, colicins are not post-translationally modified.

7.2.1 Colicins

Colicins are large proteins organized in three functional domains: a central receptor binding domain, an N-terminal translocation domain and a C-terminal catalytic domain. These domains, which are common to all colicins, ensure every common step of the colicin mechanisms of action, *i.e.* i) recognition by a specific receptor at the outer membrane, ii) translocation across the outer membrane and iii) lethal interaction with a specific cellular target. The ability of an *E. coli* strain to kill neighboring strains by releasing colicins into the surroundings has been known since the 1920s (Gratia, 1925). However, the mechanism by which colicins reach the target cell cytoplasm, crossing the outer membrane, the peptidoglycan layer and the inner membrane, has only recently begun to be unraveled at the molecular level. It seems that the penetration mechanism is similar, even though different

colicins parasitize different protein systems and kill cells by different mechanisms (Cascales et al., 2007). Colicins are not considered for development into suitable antibacterial drugs, mainly because of their high molecular weight, but their study has significantly contributed to the progress of basic research in a number of fields, such as the bacterial outer membrane protein receptors, and the proteins of the translocation machinery.

7.2.2 Microcins

The name microcin was introduced to distinguish the class of antibacterial peptides with molecular mass below 10 kDa, from the higher molecular mass colicins (Asensio et al., 1976). Whereas many antimicrobial peptides of bacterial origin are produced by large multi-domain enzyme complexes termed peptide synthetases, microcins are typically produced as ribosomally synthesized precursors, similar to the bacteriocins from Gram-positive bacteria. Microcins are encoded by plasmid- or chromosome-located gene clusters, which typically include open reading frames encoding the microcin precursor, self-immunity factors, secretion proteins and modification enzymes, giving rise to an amazing diversity of microcin structures and mechanisms of action (Duquesne et al., 2007). Microcins are secreted by enterobacteria (mostly *E. coli*) under conditions of nutrient depletion, and are involved in the regulation of microbial competition within the intestinal microbiota. They are generally hydrophobic, highly resistant to heat, extreme pH and proteases, and exert potent antibacterial activity in nanomolar concentrations, usually against a narrow spectrum of closely related species. Their mechanism of action has been defined as a "Trojan horse" behavior: they are recognized as siderophores by the outer membrane receptors of susceptible bacteria, and as such internalized; once inside they bind essential enzymes or interact with the inner membrane killing the bacterium (Duquesne et al., 2007). At present microcins are still into the characterization phase, and despite their potent antibacterial activity, they are not being developed as antibacterials (Corsini et al., 2010). Microcin E492, a pore-forming molecule produced by *Klebsiella pneumoniae*, beyond exerting antibacterial activity on related strains, has been shown to induce apoptosis of malignant human cell lines (Lagos et al., 2009). Microcin B17, produced by various *E. coli* strains harboring the 70-kb single-copy, conjugative pMccB17 plasmid, is a potent inhibitor of DNA gyrase, whereas microcin J25, the best-studied member of the lasso peptides, inhibits RNA polymerase (Oman & van der Donk, 2010).

8. Lipopeptides and lipoglycopeptides

Antimicrobial lipopeptides (LiPs) are non-ribosomally produced by bacteria and fungi during cultivation on various carbon sources. They are a class of antibiotics highly active against multidrug-resistant bacteria. Most native LiPs consist of a short (six to seven amino acids) linear or cyclic peptide sequence, either positively or negatively charged, with a fatty acid moiety covalently attached to the N-terminus. (Mangoni & Shai, 2011). Both the composition of the peptide moiety and the type of the lipophilic part are sensitive to modifications. In general, native LiPs are non-cell-selective and therefore quite toxic to mammalian cells. Despite toxicity, in 2003 a member of this family, daptomycin, which is active only toward Gram-positive bacteria, has been approved by the FDA in an injection formulation for the treatment of complicated skin and skin structure infections (SSSI) caused by susceptible strains of the following species: *S. aureus* (including methicillin-resistant

strains), *Streptococcus pyogenes*, *S. agalactiae*, *S. dysgalactiae* subspecies *equisimilis,* and *Enterococcus faecalis* (vancomycin-susceptible strains only). This example confirms the growing opinion that peptide-based antibiotics will be among the next generation of anti-infective therapy (Mangoni & Shai, 2011). Dalbavancin, oritavancin and telavancin are semisynthetic lipoglycopeptides active against multidrug-resistant Gram-positive pathogens (Zhanel et al., 2010). These molecules share a heptapeptide core that affects cell wall synthesis by inhibiting transglycosilation and transpeptidation, and contain lipophilic side chains that facilitate binding to cell membranes and increase antibacterial activity. Lipophilic residues also prolong *in vivo* half life, that is of 147-258 h for dalbavancin, of 393 h for oritavancin and of 12-24 h for telavancin. These drugs must be administered i.v. and are indicated for patients with complicated SSSI resistant to vancomycin. Telavancin has been approved for SSSI therapy by the FDA in September 2009. Dalbavancin, a teicoplanin derivative, has a long half life that allows for once weekly dosing. In published clinical trials, a dose on day 1 and 8 of treatment provided 14 days of antimicrobial activity, and demonstrated non-inferiority as measured by safety and efficacy for the treatment of uncomplicated SSSI, catheter-related bloodstream infections, and complicated SSSI (Welte & Pletz, 2010).

9. Bacteriophage endolysins

Since the pioneer work of d'Herelle, several studies demonstrated that bacteriophages can be successfully used in the therapy of animal and human bacterial infections (Harper & Enright, 2011; Verma et al., 2009). Phages are already used in the agricultural, food-processing and fishery industries, and for the treatment of human bacterial infections in Georgia and Eastern Europe (Housby & Mann, 2009). Recent experiments performed by Fu et al. on the efficacy of a bacteriophage cocktail to prevent the formation of *P. aeruginosa* biofilms on catheters in an *in vitro* model showed a 99.9% reduction of the number of bacteria (Fu et al., 2010). The human use of phages in Western countries has been hindered so far by cost, safety concerns about phage injection into the bloodstream, and by the sometimes inconsistent outcome of the treatments, due to the poor characterization of bacteriophage preparations. Moreover, the *in vivo* pharmacokinetics of phages are complex, being influenced by the host immune system-mediated phage clearance rate and by the possible insurgence of bacterial resistance due to lysogeny or mutations concerning metabolic steps or surface receptors (Payne &Jansen, 2003). However, phage therapy is considered a potential treatment for some selected infections, such as multidrug resistant *P. aeruginosa* lung infection in cystic fibrosis patients (Morello et al., 2011), and chronic otitis (Wright et al., 2009). A different approach overcoming some of the above-mentioned problems involves the use of purified phage products as anti-infective agents. Double-stranded DNA phages naturally produce endolysins, i.e. mureine-degrading enzymes, that have been originally studied and developed to control mucous membrane infections (Borysowski et al., 2006), and are also denominated "enzybiotics" (Briers et al., 2011). They only work on Gram-positive bacteria because the outer membrane of Gram-negative bacteria prevents direct lysin-peptidoglycan interaction (Fischetti, 2010). To this end, the paper from Briers et al. reports that the use of endolysins in conjunction with outer membrane permeabilizers resulted in strong lytic activity against *P. aeruginosa*, with a reduction of more than four log units of viable bacteria in 30 min. Endolysins, some of which have been found active against *B. anthracis* (Schuch et al., 2002), *S. pneumoniae* (Jado et

al., 2003) and *S. agalactiae* (Cheng et al., 2005), alone or in combination with conventional antibiotics or lysozyme, have a short half-life (15-20 min), but their action is so rapid that nanogram quantities kill sensitive Gram-positive bacteria in seconds after contact (Loeffler et al., 2001). Moreover, they are *per se* non toxic and, unexpectedly, not easily inactivated by antibodies (Fischetti, 2008). Considering that the endolysin target, peptidoglycan, is not present in eukaryotic cells, it can be anticipated that they will also be well tolerated by humans. Experiments performed on a murine model of pneumococcal pneumonia showed that an endolysin with muramidase activity, Cpl-1, protected 100% of mice when administered by intraperitoneal injections starting 24 hours after pulmonary infection (Witzenrath et al., 2009). These results suggest that Cpl-1 and related molecules could provide a new therapeutic option for pneumococcal pneumonia. The issue of the possible toxic effect due to the massive release of pro-inflammatory molecules by lysed bacteria has also been addressed. Circulating endotoxin, teichoic and lipoteichoic acids, and peptidoglycan could result in septic shock and multiple organ failure, but so far no side-effects related to lysin-induced bacteriolysis have been reported (Borysowski et al., 2006). According to experiments performed on a murine model, lysins may also cure already established infections (Witzenrath et al., 2009). More predictable endolysin applications include the elimination of bacteria from mucous membranes, the treatment of bacterial infections, and the biocontrol of bacteria in food.

10. Conclusions

Modern antibiotics are or derive from natural molecules isolated during the "golden age" of antibiotic discovery, i.e. the period between the1940s and the 1970s. Even those currently under development are nothing more than new, improved versions of these old natural products, because the chemical modification of existing molecules remains the most cost-efficient way to develop novel drugs active against resistant strains. As examples, we can cite the cephalosporin ceftaroline (Corey et al., 2010), the tetracycline amadacycline (PTK0796), the streptogramin NXL-103 (Politano & Sawyer, 2010) and the macrolide CEM-101 (Woosley et al., 2010). However, the perspective that these agents, new but based on old molecular scaffolds, will in their turn face the development of bacterial resistance, prompted both the academic community and the biotech/pharmaceutical companies to look for alternative strategies. In this scenario, the low molecular weight, broad-spectrum activity and rapid mode of action of AMPs make them promising drug candidates. Among potential advantages of some AMPs we can add the endotoxin-neutralizing ability and the capacity to modulate the host immune response. Moreover, they are usually unaffected by classic antibiotic resistance mechanisms (Zasloff, 2002). In this regard, however, concerns have been raised by the finding that some microorganisms are able to thwart AMP effects: *C. albicans* is sensitive to histatin-5, the most potent antifungal peptide present in human saliva (Edgerton & Koshlukova, 2000), but it produces aspartyl proteases that target this molecule, and in the presence of low histatin levels, as those occurring in AIDS patients, the yeast turns from a harmless commensal to a disease-causing pathogen (Meiller et al., 2009). Moreover, taking for granted that acquired resistance to AMPs is less likely to occur as compared to the traditional antibiotics, it has been observed that some Gram-negative bacteria can utilize various enzymes to reduce their surface net negative charge, so evading the action of cationic peptides (Roy et al., 2009). Several other bacterial strategies have been described that can result in decreased susceptibility to AMPs, such as secretion of

inactivating proteins or exportation via efflux pumps. Therefore, the onset of resistance in microbial populations consistently exposed to AMPs cannot be excluded (Peters et al., 2010), and a theoretical concern about the pharmacological use of AMPs closely related to human ones is that long term selection could generate organisms with unpredictable virulence potential (Fernebro, 2011). In addition to these (for now) theoretical concerns, we must recognize that some more practical AMP flaws, such as the high production cost and the susceptibility to proteolytic degradation, have until now effectively prevented AMPs from entering the market. Most AMPs today in preclinical and clinical trials have been developed for topical applications (Hancock & Sahl, 2006). Examples of indications are catheter site infections, cystic fibrosis, acne and wound healing. For complicated wounds and ulcers caused by multidrug-resistant bacteria, phage therapy, although the available literature is in many ways unsatisfactory, could be an option. In the EU, it has been proposed that specific sections concerning phage therapy should be included in the Advanced Therapy Medicinal Product Regulation to make it easier to get approval for clinical trials involving such therapy (Verbeken et al., 2007). For practical and economical reasons it could be easier to market purified lysins for which, so far, resistance development has not been observed. However, most lysins are endowed with short *in vivo* half-life (Loeffler et al., 2003), an issue that has to be solved before they enter clinical use. Another relevant issue is the possibility to use narrow spectrum AMPs, such as microcins. Conceptually, a broad spectrum antibiotic is not always the best choice, especially when considering its effects on the commensal flora and the risk of inducing opportunistic infections. However, the use of narrow spectrum antibiotics should be supported by diagnostics faster and more accurate than those in use today. The development of new antimicrobials is a formidable challenge, and out of this concern, in 2009 the U.S. and European Community presidencies established a Transatlantic Task Force, and the Infectious Diseases Society of America called for a global commitment to develop 10 novel antimicrobials by 2020 (Gwinn et al., 2010). In our view, the achievement of badly needed good results relies on a balanced interaction between well funded academic laboratories and the lead discovery departments of private companies, to make the most of existent and future techniques.

11. References

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7

microRNAs as Therapeutic Targets to Combat Diverse Human Diseases

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1. Introduction

For decades, control of cellular behavior was thought to be the exclusive purview of protein-based regulators. However, the recent discovery of small RNAs (sRNAs) as a universal class of powerful RNA-based regulatory biomolecules has the potential to revolutionize our understanding of gene regulation in practically all biological functions, as sRNAs have been found in diverse organisms from bacteria to plants to man. A class of sRNAs in eukaryotes, termed microRNAs (miRNAs), has been found to modulate a wide variety of cellular functions, including cell growth, cell differentiation, and apoptosis. miRNAs function as regulators by base-pairing with trans-encoded mRNAs to prevent translation of mRNA into protein at the post-transcriptional level. By modulating the expression levels of target genes, miRNAs enable rapid adaptation of cellular physiology in response to specific environmental changes. It is estimated that at least ~30% of the human genome is regulated by miRNAs. (Lewis, et al., 2005) In this review, we will discuss recent discoveries that implicate miRNA function in host immunity, including specific miRNA expression in immune cells and their regulation of immune cell development, miRNA regulation of innate and acquired immune response, and viral-encoded miRNAs. These recent advances have strong potential to translate fundamental research in miRNA function into clinical applications. We will also describe the challenges in bringing another RNA interference (RNAi) methodology, small interfering RNA (siRNA), to clinical trials. This analysis will serve as a practical roadmap for development of novel miRNA-based therapies to combat infectious disease by reducing the host inflammatory response and the downstream effects of pathogen infection.

2. miRNA biogenesis and mechanism of action

Most miRNAs are transcribed by RNA polymerase II as either polycistronic or monocistronic transcription units called primary miRNAs (pri-miRNAs), in which one or more hairpin structures with ~33bp stem regions and terminal loops are embedded. (Fig. 1) (Lee, et al., 2004) These pri-miRNAs are capped and polyadenylated and can be as long as several kilobases. (Cai, et al., 2004) Hairpin structures embedded within pri-miRNA transcripts are recognized and excised by the microprocessor complex in the nucleus, which consists of the RNase III-like enzyme Drosha and double-stranded RNA (dsRNA) binding protein DGCR8 (DiGeorge syndrome critical region gene 8). (Landthaler, et al., 2004, Lee, et

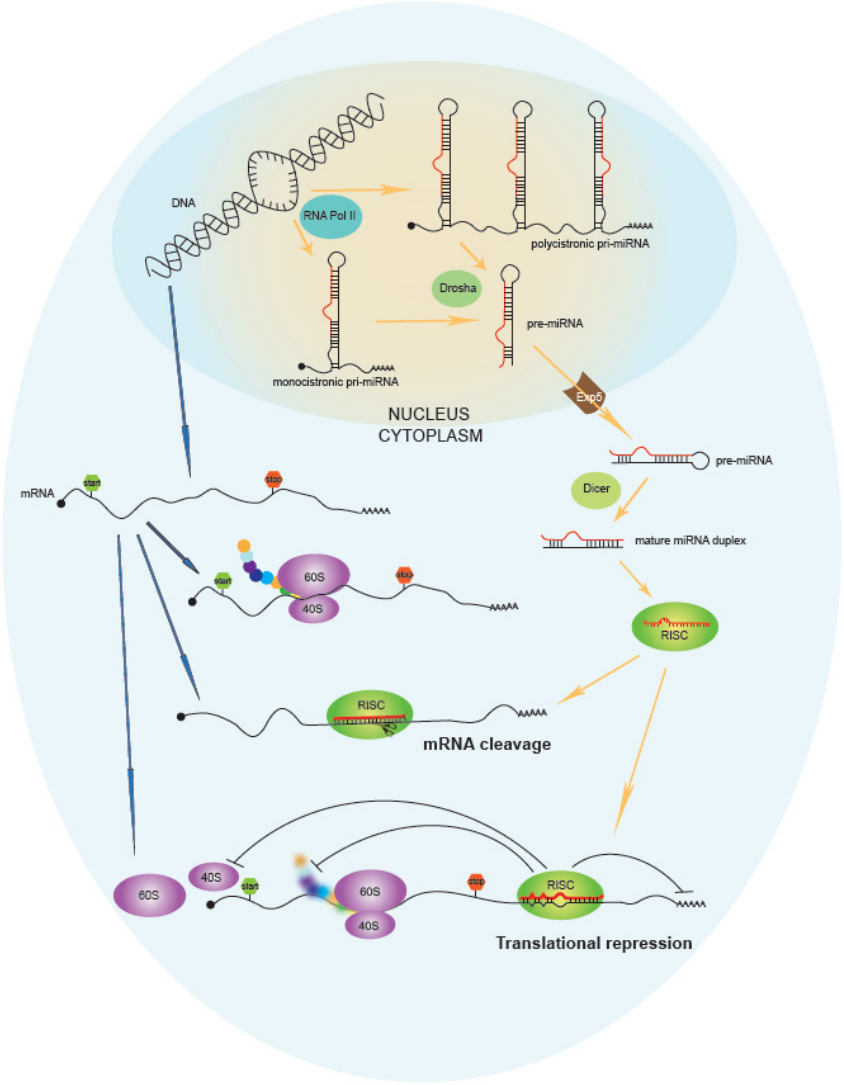

Fig. 1. miRNA biogenesis and mechanism of action. miRNAs are initially transcribed as either polycistronic or monocistronic long primary miRNA transcripts (pri-miRNAs), and then undergo a series of maturation steps including: (1) cleavage by the enzyme Drosha into the intermediate precursor miRNAs (pre-miRNAs), (2) transport from the nucleus to the cytoplasm via Exportin 5, and (3) final processing by the enzyme Dicer into the mature miRNA duplexes for loading into the RNA-induced silencing complex (RISC). Plant miRNAs pair extensively with their target mRNAs, resulting in direct cleavage of the mRNAs. Mammalian miRNAs exhibit partial complementarity with the 3′ untranslated region (UTR) of target mRNAs, causing translational repression, which may lead to mRNA degradation.

al., 2003) The released hairpin structure is typically ~65-70 nt and is referred to as a precursor miRNA (pre-miRNA). (Lee, et al., 2002) Pre-miRNAs are then exported into the cytoplasm by Exportin-5 (EXP5) in a Ran-GTP dependent manner. (Yi, et al., 2003) In the cytoplasm, the end opposite to Drosha cleavage in the mature miRNA is cleaved by another RNase III enzyme, Dicer, yielding a 22-25nt duplex. (Hutvagner, et al., 2001, Ketting, et al., 2001) Once the mature miRNA duplex is created, one strand of the duplex is loaded into a multi-protein complex, RNA-induced silencing complex (RISC), to direct subsequent miRNA:mRNA target interaction and gene silencing. (Bartel, 2004, Filipowicz, et al., 2008) This strand is called the guide strand (miRNA), while the other strand is termed the passenger strand (miRNA*), which usually undergoes degradation. There are cases where both strands can mediate subsequent gene silencing. The determination of guide/passenger strand is believed to depend on the thermodynamic stability of the base pairing at the ends of the duplex. The strand whose 5′ end displays less stability will become the guide strand. (Khvorova, et al., 2003) The catalytic component of RISC is the Argonaute (AGO) protein, which mediates binding and silencing of the target mRNAs. (Pillai, et al., 2004)

In general, miRNAs down-regulate translation by binding to the miRNA response elements (MREs) in the 3′UTR (3′ untranslated region) of their mRNA targets to cause inhibition of mRNA translation, and in some cases, mRNA destabilization. (Filipowicz, et al., 2008) Complementarity between miRNAs and MREs have been shown to be near perfect in plants, but only partial in animals. Multiple mechanisms of action have been proposed. AGO proteins in RISC may inhibit translation initiation by competing with eIF4E for mRNA m^7G cap-recognition. (Kiriakidou, et al., 2007, Mathonnet, et al., 2007) Alternatively, RISC may inhibit translation initiation by preventing the assembly of 80S ribosomes via recruitment of eIF6. (Chendrimada, et al., 2007) There is also evidence that RISC can repress translation post-initiation by causing ribosome drop-off or nascent polypeptide degradation during the elongation step. (Petersen, et al., 2006) Finally, miRNAs can accelerate mRNA destabilization. miRNA-associated targets were found to be enriched in P-bodies, compartmentalized cytoplasmic foci where mRNA decay occurs (Sheth and Parker, 2003), and to be associated with deadenylase, decapping enzymes and exonucleases. (Behm-Ansmant, et al., 2006, Liu, et al., 2005)

miRNA target identification in animals is relatively difficult because of imperfect miRNA:MRE complementarity. One important finding is the so-called “seed rule”, in which extensive Watson-Crick base paring between the “seed” region (2-7 nt from the 5′ end) of the miRNA and its target, remarkably reduces the number of false positive predictions. (Lewis, et al., 2003, Lim, et al., 2005) The seed rule has been widely applied as the fundamental criteria by most current prediction algorithms to screen for potential miRNA target genes. Nevertheless, considerable evidence exists to argue that the seed pairing is either not required or not sufficient for predicting miRNA:mRNA interactions. (Didiano and Hobert, 2006) Other features within 3′UTRs, in addition to seed pairing, have been demonstrated to be important determinants, including overall thermodynamic stability of miRNA:mRNA duplex, total number of MREs within the 3′UTR, accessibility of the MRE, position of the MRE related to the stop codon, and local AU rich elements. (Hon and Zhang, 2007, Kertesz, et al., 2007, Li, et al., 2008) Thus, current computational target prediction is far from established, and predicted target candidates need to be experimental verified.

Both biogenesis and function of miRNAs are subject to tight regulation. Almost every aspect of miRNA biogenesis, from transcription and processing to subcellular localization and stability, can be regulated in a sequence-specific and cell-specific manner. Such regulation is

believed to be important for many developmental and physiological processes. For example, the transcription factors Myogenin and MyoD1 induce expression of miR-1 and miR-133 specifically during myogenesis. (Rao, et al., 2006) During stem cell differentiation, the levels of pri-let-7 remain constant, while the levels of mature let-7 duplex increase. (Piskounova, et al., 2008) Interestingly, post-transcriptional suppression of let-7 in undifferentiated cells is mediated by its target Lin-28. Lin-28 not only blocks microprocessor cleavage in the nucleus by directly binding to the loop region of pri-let-7, (Piskounova, et al., 2008) but also prevents Dicer cleavage in the cytoplasm by promoting polyuridylation and degradation of pre-let-7. (Heo, et al., 2009) The nuclear export of miRNA is usually the rate-limiting step of miRNA biosynthesis. (Grimm, et al., 2006) Some pre-miRNAs such as human pre-miR-31, pre-miR-128, and pre-miR-105 are retained in the nucleus instead of being processed into mature miRNAs in certain cell types. (Lee, et al., 2008) Finally, although the degradation of miRNAs is not well-understood, the enrichment of guide strands but not passenger strands in the cells clearly indicates the existence of an as-yet unknown mechanism that quickly and selectively turns over these small RNAs. A family of exoribonucleases that degrades miRNAs have been identified in *A. thaliana*. (Ramachandran and Chen, 2008) Furthermore, under certain conditions, miRNA-mediated silencing can be reversed or blocked. (Kedde, et al., 2007, Schratt, et al., 2006) For example, mir-122-mediated repression of CAT1 (cationic amino acid transporter 1) can be alleviated in human cells lines as a response to starvation or other types of cell stress. (Bhattacharyya, et al., 2006)

3. miRNAs and the host immune system

Mammalian systems have developed a complex system of checks and balances to regulate gene expression in order to respond to pathogen infection. In the last several years, miRNAs are increasingly becoming implicated in the regulation of both immune cell development and function. Proper functioning of the immune system requires elaborate control of both innate and adaptive immune response in order to defend against various pathogens while maintaining self tolerance. miRNAs are required for normal immune system function by helping to maintain this balance. miR-146a is upregulated in human monocytes upon exposure to lipopolysaccharide (LPS), a cell wall component of Gram negative bacteria and established activator of innate immunity. Its upregulation is dependent on NF-κB, a key transcription factor that regulates practically all aspects of the innate immune response, such as synthesis of pro-inflammatory cytokines, including TNFα and IL-1β, and regulation of immune cell migration. Interestingly, TRAF6 (TNF receptor-associated factor 6) and IRAK1 (IL-1 receptor-associated kinase 1), two components of the Toll-like receptor 4 (TLR4) signaling pathway that act upstream of NF-κB, were found to be targets of miR-146a. These findings suggest that miRNAs function in the negative feedback regulation of TLR signaling in order to ensure appropriate strength and duration of the innate immune response. (Taganov, et al., 2006) Another inflammatory mediator, miR-155, is induced by LPS (Ceppi, et al., 2009, O'Connell, et al., 2007, Tili, et al., 2007) and nucleic acids, including poly(I:C) (polyriboinosinic:polyribocytidylic acid) and hypomethylated DNA (O'Connell, et al., 2007), implicating function in both bacterial and viral infection. miR-155 is proposed to fine tune inflammatory cytokine production through negative feedback loops by targeting TAB2 (Ceppi, et al., 2009), FADD (fas-associated death domain protein), IKKε (IκB kinase ε), and Ripk1 (receptor-interacting serine-threonine kinase 1). (Tili, et al., 2007)

In addition to innate immunity, the adaptive immune response is also subject to regulation by miRNAs, in particular miR-155. miR-155-deficient dendritic cells exhibited impaired ability in antigen presentation and T cell activation, suggesting its involvement in bridging innate and adaptive immunity. (Rodriguez, et al., 2007) miR-155 restricts Th2 but not Th1 lineage commitment after CD4+ T cell activation (Rodriguez, et al., 2007, Thai, et al., 2007), and is also required for the differentiation and proliferation of regulatory T helper cells, which function to self-limit the immune response. (Kohlhaas, et al., 2009, Lu, et al., 2009) Furthermore, miR-155 was induced in B lymphocytes upon activation and regulates the germinal center response and generation of immunoglobulin class-switched plasma cells. (Teng, et al., 2008, Thai, et al., 2007, Vigorito, et al., 2007) Naïve B lymphocytes express only immunoglobulin M (IgM) isotype antibodies on the cell surface as a result of V(D)J DNA recombination. Upon activation, B lymphocytes undergo somatic hypermutation (SHM), gene conversion (GCV), affinity maturation, and class-switched recombination (CSR) to produce a vast antibody repertoire with increased diversity, higher antigen-binding affinity, and different isotypes. miR-155 has been shown to regulate expression of activation-induced cytidine deaminase (AID), which catalyzes the SHM, GCV and CSR processes by deaminating cytosine to introduce U:G mismatches in Ig genes. (Teng, et al., 2008) Disruption of miR-155-AID interaction in vivo results in quantitative and temporal alteration of AID expression and defective antibody maturation. Another target of miR-155, the transcription factor PU.1, has been reported to be involved in the reduction of IgG1-switched plasma cells in a miR-155 deficient mouse model. (Vigorito, et al., 2007)
Several miRNAs have also been implicated in different immune development processes. miR-223 is activated by the myeloid transcription factors PU.1 and C/EBP (CCAAT/enhancer-binding protein) and has been shown to control granulocyte development. (Fazi, et al., 2005, Johnnidis, et al., 2008) miR-150 is an important regulator of B cell differentiation through targeting of the transcription factor c-Myb. (Xiao, et al., 2007, Zhou, et al., 2007) Finally, miR-181a modulates T cell receptor sensitivity and signaling strength during positive and negative selection, most likely through downregulation of phosphatases. (Li, et al., 2007)

4. miRNAs and viruses

Interestingly, miRNAs have been identified in various members of the herpesvirus family, such as Epstein-Barr virus (EBV), herpes simplex virus 1 (HSV-1), Kaposi's sarcoma-associated herpesvirus (KSHV), and human cytomegalovirus (HCMV), during both the latent and the productive stage of the viral life cycle. These viral miRNAs share the same biogenesis and execution pathways as their cellular counterparts, and downregulate either viral or host mRNAs in order to evade the host immune system or to control the transition from latent to the productive replication stage. (Cullen, 2009) For example, the degradation of EBV DNA polymerase mRNA by miR-BART2 (Barth, et al., 2008), the suppression of HSV-1 immediate early proteins ICP0 and ICP4 by miR-H2-3p and miR-H6 (Umbach, et al., 2008), and the downregulaton of HCMV viral immediate-early protein IE1 by miR-UL112-1 (Murphy, et al., 2008), help to establish and maintain viral latency. KSHV miRNA miR-K12-6-3p contributes to the formation of Kaposi's sarcoma tumors in vivo by downregulation of host gene THBS1 (thrombospondin 1). (Samols, et al., 2007) Downregulation of host MICB mRNA (major histocompatibility complex class I polypeptide-related sequence B), a ligand for natural killer cells, by HCMV miRNA miR-UL112-1 protects infected cells from natural

killer cells. (Stern-Ginossar, et al., 2007) Another host antiviral gene CXCL11 (CXC-chemokine ligand 11), a target of EBV miRNA miR-BHRF1-3, is downregulated to protect infected B cells from being targeted by cytotoxic T cells. (Xia, et al., 2008)

5. Targeting miRNA expression to modulate gene expression

Medical countermeasures that exploit miRNA function have focused on therapies for cancer. Different approaches have been developed to manipulate expression levels of miRNAs and determine downstream effects on disease. (Fig. 2) A number of studies have demonstrated that specific miRNAs exhibit altered expression levels in tumors, and that "normalization" of miRNA expression in cancer cells may have a therapeutic effect. To up-regulate miRNA expression, either miRNA mimics or miRNA expression vectors can be overexpressed in target cells and tissues. Mirna Therapeutics has reported the systemic delivery of mimics for miR-34 and let-7 via a neutral lipid emulsion, as a strategy for miRNA replacement therapy to inhibit tumor growth and metastasis in a mouse model. (Trang, et al., 2011, Wiggins, et al., 2010) Both miR-34 and let-7 are natural tumor suppressors that exhibit reduced expression in different cancers. Introduction of the mimics in mice led to ~60% reduction in tumor area compared to control mice. These results offer a novel therapeutic strategy for cancer by restoring miR-34 and let-7 expression to wild-type levels to reduce tumor growth. Given that miR-34 and let-7 levels are reduced in a number of different cancers, it may be the case that synthetic miRNA mimics can have broad applicability as anti-cancer agents.

Inhibitory agents that down-regulate miRNA expression, termed antagomirs, have been developed to pair with mature miRNAs through sequence complementarity and block miRNA-mediated gene regulation. (Hutvagner, et al., 2004, Krutzfeldt, et al., 2005) Development of antagomirs that contain locked nucleic acids (LNAs), a backbone modification in which the ribose moiety has been locked by an oxymethylene bridge connecting the C(2′) and C(4′) atoms of the ribose, have yielded unusually stable oligonucleotides with high duplex melting temperatures for more robust therapeutic studies. Their relatively small size, high affinity, and potential for systemic delivery without complicated delivery vehicles has established LNA oligos as a favorite platform for design of RNA-based drug candidates. Antagomirs against miR-16, miR-122, miR-192, and miR-194, conjugated to cholesterol, have been intravenously administered in mice, and corresponding miRNA levels exhibited marked reduction in multiple organs and tissues. A systemically administered unconjugated LNA-anti-miR-122 oligonucleotide led to specific, dose-dependent silencing of miR-122 in non-human primates. (Elmen, et al., 2008) This particular anti-miR was composed of 15 nucleotides, which covered the seed sequence in the 3′ UTR of miR-122 and adjacent nucleotides. In a recent study, 7-mer and 8-mer anti-miRs that only targeted the seed sequence exhibited potent inhibition of miR-21, which exhibits elevated levels in a variety of cancers. (Obad, et al., 2011) These short anti-miRs form strong hybrids with their target miRNAs and sterically block miRNA function. Injection of anti-miR-21 into mice yielded sequestration of the hybrid complex in the main organs and resultant upregulation of a miR-21 gene target. Their enhanced stability and relatively small size positions these seed-directed anti-miRs as potentially strong drug candidates to target specific miRNAs or miRNA families that function in disease onset.

Viral miRNAs are potential anti-viral candidates for therapeutic development and can serve as diagnostic markers for viral infection or specific stages of disease. The host miR-122,

which has two recognition sites in the 5′ UTR of the hepatitis C virus (HCV) genome, is required for virus replication. (Jopling, et al., 2005) Use of specific antagonists that target miR-122 resulted in a reduction in HCV in the liver of a primate model, demonstrating the therapeutic utility of this strategy.(Lanford, et al., 2010) The anti-miR-122 treatment provided continued efficacy in the animals up to several months after the treatment period with no adverse effects or evidence of viral rebound. Based on these studies, Santaris Pharma A/S has initiated the first miRNA-targeted Phase 2a clinical trial, based on the miR-122-inhibitory drug, Miravirsen, to assess safety and tolerability in up to 55 treatment-naïve patients infected with HCV. Designed using LNA technology, Miravirsen sequesters miR-122 from HCV, thus inhibiting replication of the virus. Secondary validation studies, including drug pharmacokinetics and effect on viral load, will be assessed. miRNA technology has also been applied to vaccine development for influenza virus. A miRNA-responsive element (MRE) was introduced into the viral nucleoprotein gene to control the level of viral attenuation via miR-124, which targets the MRE sequence. The resulting viruses produced a species-specific vaccine that generated high levels of neutralizing antibodies in the host. (Perez, et al., 2009)

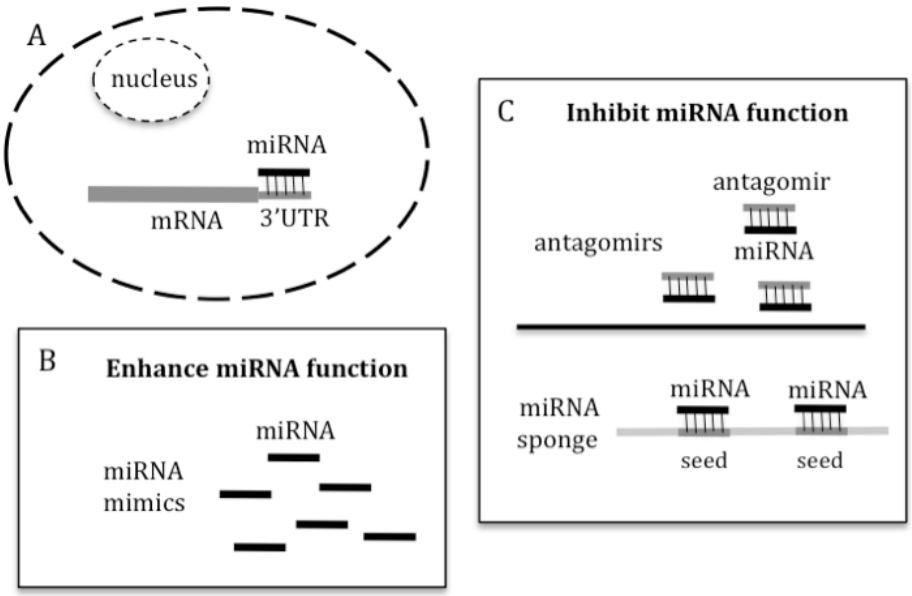

Fig. 2. (A) In general, miRNAs bind to the 3′ UTR of target genes to downregulate gene expression. (B) miRNA mimics provide increased numbers of miRNAs by overexpression or synthetic copies to enhance miRNA function. (C) Inhibition of miRNA function can be achieved by usage of (1) antagomirs, stable complementary oligonucletides, that hybridize to target miRNAs, or with (2) miRNA sponges, which provide alternative binding platforms to levels sequester miRNAs.

Artificial miRNA decoys, termed miRNA "sponges", provide alternative binding platforms for the miRNAs and inhibit their ability to bind and suppress their endogenous targets. The miRNA sponges often contain a strong promoter to drive expression of binding sites for the

miRNA either in a non-coding transcript or in the 3'UTR of a reporter gene, like GFP. (Brown and Naldini, 2009, Ebert, et al., 2007) Since the interaction between miRNAs and target genes is largely dependent on the seed region of the miRNA, design of the miRNA sponges that incorporate the seed sequence can interact with all members of a miRNA seed family. Both individual miRNAs and large seed families, such as miR-155 and the let-7 family, respectively, have been successfully targeted for continuous loss of miRNA function in multiple mammalian cell lines. (Bolisetty, et al., 2009, Kumar, et al., 2008) Compared to miRNA antisense oligonucleotides, a major advantage of miRNA sponges is the potential for stable integration for continual expression in the genome. Stable transfection of miRNA sponges in cultured cells led to partial derepression of miRNA target gene expression in a variety of cell systems, including mesenchymal stem cells (Huang, et al., 2010) and cancer cell models. (Bonci, et al., 2008, Valastyan, et al., 2009) A high expression level is often required for sufficient inhibition of endogenous miRNAs, given the much lower transgene copy number in stable lines compared to transient plasmid transfection. Forty integrated copies of a miRNA sponge targeting miR-223 driven by a weak promoter were needed to sufficiently suppress miR-223 expression. (Gentner, et al., 2009) Stable expression of sponges also enables miRNA loss-of-function studies that can span days to weeks. After 6 days, neurons that express anti-miR-92 sponges exhibited derepression of a potassium chloride cotransporter and electrophysiological changes in response to GABA treatment. (Barbato, et al., 2010) Both miR-144 and miR-451 were found to be required for erythropoiesis in bone marrow reconstitution experiments 3-4 weeks after transplantation of cells expressing miRNA sponges. (Papapetrou, et al., 2010)

6. RNAi therapeutics: Development of siRNAs for disease therapy

The potential development of new therapies for infectious disease using RNAi-based strategies has attracted the attention of biotechnology entrepreneurs. Multiple small companies have entered the field to transition RNAi from molecular biology tool to next-generation nucleotide-based drugs for treatment of disease. In the last ten years, there have been a growing number of clinical trials based on siRNAs, ~20 bp short interfering RNAs that silence target genes by directing their cognate mRNAs for degradation by the RISC complex. (Davidson and McCray, 2011, Vaishnaw, et al., 2010) Some programs involve local delivery of siRNAs to target tissues, including the eye, kidney, and liver, while others aim to achieve systemic delivery in the body. As with any novel strategy for drug development, there still remain technical challenges that need to be overcome before RNAi-based technology can successfully transition into robust therapies, including minimization of off-target effects (OTE) and systemic delivery of siRNAs in the body. A summary of these challenges and the methods that have been utilized to move the field of RNAi-based therapeutics forward is summarized in Table 1. Given that miRNA-based therapies will most likely face similar challenges that are currently being addressed by the more mature siRNA-anchored drug companies, we provide an overview of the strategies utilized in siRNA-based drug discovery.

6.1 Off-target effects

All RNAi-based therapeutics remain vulnerable to the potential of OTE, in which expression of non-targeted genes is unintentionally modulated. OTE can be categorized into sequence-dependent and sequence-independent effects. In the case of sequence-dependence, the

siRNAs can bind to bystander mRNAs that exhibit partial sequence complementarity but is unrelated to the target gene. This type of OTE can be mitigated through use of bioinformatics screening algorithms to design siRNA sequences that exhibit rare or no direct complementarity to other genome sequences aside from the target gene. In sequence-independent OTE, the siRNAs may have inherent immunostimulatory properties or the endogenous miRNA pathway may be saturated, especially in the case of overexpression of heterologous siRNAs. For example, RNAs have been shown to stimulate the Toll-like receptor (TLR) (Kleinman, et al., 2008) and retinoic acid inducible gene (RIG)-I pathways. (Yoneyama, et al., 2004) Since there does not exist any a priori knowledge about which signaling pathways are activated by specific siRNAs, it may be necessary to evaluate a panel of pro-inflammatory markers to downselect siRNAs with comparatively reduced immunostimulatory properties in preclinical assays. RNAi-based therapies also need to be cautious about the level of exogenous siRNAs expressed in the host. In mouse studies, PolII promoter-driven expression of plasmid-based short hairpin RNA (shRNA) constructs in the liver induced host mortality, which has been attributed to saturation of the transport factor, Exportin 5, that ferries miRNAs from the nucleus to the cytoplasm. (Grimm, et al., 2006) Thus, it is necessary to determine the lowest possible concentration of siRNAs that can still be therapeutically effective for introduction into the host. OTEs and non-specific immunostimulatory responses can also be mitigated using different siRNA backbone modifications, including 2′-O-Me modifications (Jackson, et al., 2006) or DNA substitutions. (Ui-Tei, et al., 2008) Once candidate siRNAs have passed a threshold efficacy level in *in vitro* cell culture studies, leads are then assessed using good laboratory practice (GLP)-compliant preclinical toxicological studies with animal models such as rodents and non-human primates.

6.2 siRNAs enter clinical trials

Alnylam Pharmaceuticals currently has several advanced RNAi-based therapeutic programs that target multiple diseases. Alnylam has completed Phase II clinical trials on ALN-RSV01, a siRNA-based inhaled treatment for respiratory syncytial virus (RSV) that targets the nucleocapsid encoding gene in the virus. (Alvarez, et al., 2009) Lung transplant patients exhibited improved symptom scores and overall lung function. Alnylam has also developed ALN-VSP, a cocktail for two siRNAs that target vascular endothelial growth factor and kinesin spindle protein, as a systemically delivered liver cancer therapy. In Phase I trials, ALN-VSP was well tolerated and reduced tumor blood flow in patients. Finally, Alnylam has initiated a Phase I study of ALN-TTR01, a siRNA therapeutic that targets transthryretin (TTR), a carrier for thyroid hormones and retinol binding proteins, which is mutated in hereditary TTR-mediated amyloidosis. Pre-clinical trials have demonstrated that ALN-TTR01 can cause regression of amyloid deposits and silence the TTR gene.

Other companies have multiple RNAi-based drugs in the R&D pre-clinical and Phase I pipelines, targeting a wide range of disease conditions, including age-related macular degeneration (AMD) and various cancers. (Vaishnaw, et al., 2010) In addition to the Alnylam siRNA drug against RSV, there have been some inroads into development of RNAi-based therapies to treat infectious disease. For example, Tacere Therapeutics has developed a RNAi-based cocktail that targets three separate conserved regions of the Hepatitis C virus (HCV) and can be delivered to liver cells via intravenous administration using encapsidation in an adeno-associated protein coat. In animal studies, this therapeutic agent targeted and cleaved HCV at three different sites simultaneously without toxicity. In a

pilot Phase I/II clinical trial, Benitec Ltd., in collaboration with City of Hope National Medical Center, demonstrated long-term expression of three RNA-based anti-HIV moieties (tat/rev short hairpin RNA, TAR decoy, and CCR5 ribozyme) in hematopoietic progenitor cells that support the development of an RNA-based cell therapy platform for HIV. (DiGiusto, et al., 2010) The gene-modified stem cells had been infused into HIV-positive patients via autologous bone marrow transplantation to treat AIDS-related lymphomas. In another study, intranasal delivery of siRNAs in a SARS coronavirus (SCV) rhesus macaque model was also effective in reducing SARS-like physiological symptoms, RNA expression of SCV genes, and lung histopathology associated with viral disease. (Li, et al., 2005)

Challenge	**Methods to address challenge**
RNA stability	Locked nucleic acids (LNA) Short (7-8-mer) seed-directed anti-miRs
Off-target effects	
Sequence-dependent	Bioinformatic screening algorithms
Sequence-independent	Evaluation of immunostimulatory properties Reduction of RNA expression levels Use of different RNA backbone modifications DNA sequence substitution
Systemic delivery	
Chemical modifications	Cholesterol Chitosan Lipophilic molecules (e.g. bile acids)
Packaging carriers	Lipid nanoparticles (LNPs, SNALPs) Multiple lipid bi-layer nanoparticles (Atuplex) Transferrin-decorated cyclodextrin particles Nanoparticles decorated with leukocyte receptor antibody Dynamic polyconjugates

Table 1. Transition of RNAi-based approaches to the therapeutic arena

It is also informative to describe siRNA-based clinical trials that were terminated prematurely due to conclusions that the study was unlikely to yield an effective drug. Bevasiranib (Opko Health Inc.) and AGN211745 (Allergan Inc.) were developed to target the vascular endothelial growth factor (VEGF) pathways to treat patients with AMD via the intravitreal route. The overgrowth of blood vessels behind the retina causes irreversible loss of vision. Despite initial positive reports of efficacy demonstrating reduced neovascularization upon direct ocular injection of VEGF siRNA (Shen, et al., 2006), both studies were terminated during Phase II/III of clinical trials, amid suggestions that the two siRNAs activated TLR3 to mediate its effects in preclinical models, rather than direct inhibition of target gene expression. (Kleinman, et al., 2008, Vaishnaw, et al., 2010)

6.3 Delivery of stable siRNAs into the body

A major technical challenge for RNAi-based therapy is drug stability in the body and efficient systemic delivery in sufficient quantity to have a therapeutic effect. Given their size and negative charge, siRNAs cannot easily cross the host cell membrane. Unmodified siRNAs injected into the body may be subject to RNase-mediated degradation and rapid renal excretion. Thus, therapeutic siRNAs are often chemically modified and/or packaged into carriers for delivery into the host. Various means of delivery have been tested in murine and non-human primate models, including in nanoparticles, complexed with polyconjugates, attached to cholesterol groups, or conjugated with cell surface receptors. (Table 1)

Tekmira Pharmaceuticals Corp. has developed stable nucleic acid lipid nanoparticles (LNPs, formerly referred to as SNALPs), composed of several non-covalently associated components, including an ionizable lipid, polyethylene glycol (PEG)-lipid, cholesterol, and a neutral lipid. LNPs containing siRNAs that target several Zaire Ebola (ZEBOV) viral proteins were delivered into a lethal non-human primate macaque model of ZEBOV-mediated hemorrhagic fever. (Geisbert, et al., 2010) All macaques given seven post-exposure treatments were protected against ZEBOV, demonstrating the efficacy of LNP-mediated siRNA therapy for emerging viral infections. Tekmira has also applied LNP-based delivery to a Phase I trial involving 23 patients with mild hypercholesterolemia. Alnylam Pharmaceuticals has also reported systemic delivery of siRNAs that target apolipoprotein B (ApoB), encapsulated in LNPs, by intravenous injection in cynomolgus monkeys. (Zimmermann, et al., 2006) A single injection was shown to last for more than 11 days, induced significant reductions in serum cholesterol and low-density lipoprotein levels, and resulted in greater than 90% target knockdown with no detectable toxicity. Silence Therapeutics has developed an independent nanoparticle approach, the AtuPlex technology, that embeds therapeutic siRNAs into multiple lipid bi-layer structures to provide systemic delivery to specific tissues. Pfizer and Quark Pharmaceuticals are currently testing delivery of therapeutic siRNAs using the Silence technology to treat AMD and diabetic macular edema in two separate Phase II trials. Pre-clinical data has indicated that therapy decreases onset of known endpoints in AMD.

Calando Pharmaceuticals has initiated a Phase I clinical trial that utilizes receptor-mediated delivery of siRNAs encapsulated in cyclodextrin particles decorated with transferrin. Cancer cells, which often overexpress the transferrin receptor, are thus more likely to take up the particles for targeted therapeutic delivery. The trial targeted a subunit of ribonucleotide reductase, an enzyme required for DNA synthesis, to inhibit cancer and tumor growth. Tumor biopsies from melanoma patients show the presence of intracellularly localized nanoparticles. Furthermore, mRNA and protein levels for ribonucleotide reductase in tumors were decreased compared to pre-dosing tissue. (Davis, et al., 2010) Another targeted receptor-based strategy is siRNA delivery to a specific class of leukocytes involved in gut inflammation. A cyclin D1 (Cyd-1)-targeted siRNA was loaded into stabilized nanoparticles, the surfaces of which incorporated an antibody specific for a receptor expressed by the leukocytes. The targeted siRNA-containing nanoparticles down-regulated the cyclin D1 target, suppressed leukocyte proliferation, and reversed experimentally-induced colitis in mice. (Peer, et al., 2008)

Cholesterol carriers enable improved siRNA uptake in the liver, with the cholesterol easily bound by low-density lipoprotein (LDL) in serum and robust LDL uptake in the liver.

(Soutschek, et al., 2004) An siRNA targeting apolipoprotein B (apoB) has been conjugated to cholesterol in order to load siRNAs into circulating LDLs for enhanced stability and to increase receptor-mediated uptake into target hepatocytes. (Soutschek, et al., 2004, Wolfrum, et al., 2007) ApoB siRNAs have also been complexed with dynamic polyconjugates, PEG, and the liver-targeting ligand N-acetyl galactosamine to achieve site-directed delivery and potent ApoB knock-down. (Rozema, et al., 2007) Lipophilic siRNAs can also bind high-density lipoprotein (HDL) and target to tissues with HDL receptors, such as gut and brain. (Chen, et al., 2010, Wolfrum, et al., 2007) Oral delivery of glucan-encapsulated siRNA particles has been reported to target Map4k4 in gut macrophages to protect mice from LPS-induced toxicity. (Aouadi, et al., 2009) Finally, the polymer chitosan has mucoadhesive properties and has been used for intranasal delivery of siRNAs specific to a BCR/ABL-1 junction sequence, into bronchiolar epithelial cells in mice, resulting in ~40% reduction of target gene expression. (Howard, et al., 2006)

7. Conclusions: Strategies and future of miRNA therapeutic applications

Recent advances in the understanding of miRNA structure and function has enabled development of novel miRNA-based strategies for combating human infectious disease. The technologies that have already been developed for stabilization and drug delivery of siRNA-based therapeutics will no doubt accelerate transition of miRNAs into the therapeutic arena. Given that miRNAs are thought to regulate tens to hundreds of genes in the cell, caution must be taken since there may be unintended downstream consequences on cell function by seemingly small alterations in miRNA expression. A recent development that may greatly advance anti-miR therapeutics is the silencing of miRNA families with short LNA antagomirs that specifically target the miRNA seed sequences. (Obad, et al., 2011) The relatively short 7-8 nucleotide lengths of these LNA sequences may bypass the need for carrier formulation for systemic administration in the host and reduce the manufacturing costs of RNAi therapeutics. Further research, including clinical trials, will determine the efficacy of these short antagomirs for treatment of human disease.

Several companies have been established to specifically develop high-impact medicines based on miRNAs, including Santaris Pharma A/S, Mirna Therapeutics, and Regulus Therapeutics. As aforementioned, Santaris has developed a LNA-based anti-miR-122 drug, Miravirsen, to inhibit HCV infection in Phase II clinical trials. Mirna Therapeutics has demonstrated intravenous administration of a neutral lipid emulsion to facilitate systemic delivery of tumor suppressor miRNA mimics modeled after the natural tumor suppressors let-7 and miR-34 to inhibit tumor growth. (Trang, et al., 2011) Regulus Therapeutics is focusing on both miR-21 as a potential target to reverse fibrosis and cancer onset and miR-122 to reduce cholesterol levels and inhibit HCV infection. Other studies in the laboratory have implicated miRNAs in key organ function. For example, the cardiac-specific miR-208 is required for cardiomyocyte hypertrophy, fibrosis and expression of bMHC in response to stress and hypothyroidism. (van Rooij, et al., 2007) This momentum in development of RNAi-based drug strategies represents an exciting time for translational research that links fundamental bioscience discovery in cancer and infectious disease to therapeutic treatment. The overall promise of miRNAs as a powerful new approach to induce sequence-specific inhibition of gene expression has generated enormous enthusiasm and hope in the biomedical community that miRNA-based therapeutic treatment of disease can become a reality in the near future.

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9. References

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8

Potentiation of Available Antibiotics by Targeting Resistance – An Emerging Trend in Tuberculosis Drug Development

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1. Introduction

Mycobacterial infections are one of the leading causes of death through disease world-wide (World Health Organization, 2010), encompassing infections such as tuberculosis (TB), leprosy, Buruli ulcers, and opportunistic non-tuberculosis mycobacterial (NTM) infections in immune-compromised individuals, especially patients with acquired immune deficiency syndrome (AIDS). The World Health Organization has estimated that one third of the world's population is currently infected with *Mycobacterium tuberculosis*, the causative agent of TB, although only ten percent of those infected will develop active disease (World Health Organization, 2010). Highest TB incidences are located in sub-Saharan Africa and Southeast Asia, coinciding with human immunodeficiency virus (HIV) hot spots (World Health Organization, 2010).

The extremely high level of intrinsic resistance to most antimicrobial drug classes exhibited by *M. tuberculosis* has left us with a very limited arsenal of useful anti-TB drugs (Nguyen & Thompson, 2006). The five available first-line drugs, isoniazid (INH), rifampicin (RIF), ethambutol (EMB), pyrazinamide, and streptomycin, are all more than sixty years old (Nguyen & Pieters, 2009, Nguyen & Thompson, 2006). Furthermore, the current standard regimen (DOTS) for TB is comprised of six to nine months of daily antibiotic treatment with a combination of four out of these five drugs, often leading to poor patient adherence and incomplete courses of treatment. The rapid rate of mutations occurring in bacteria in general, together with the frequent exposure of *M. tuberculosis* to sub-optimal doses of drugs, have granted ample opportunity for this pathogen to acquire additional resistance by amassing sequential mutations in drug-target encoding genes (Nguyen & Pieters, 2009, Nguyen & Thompson, 2006). Accordingly, we now face the problem of multiple drug resistant (MDR) and extensively drug resistant (XDR) *M. tuberculosis* strains. MDR strains exhibit resistance to at least the two most potent first-line drugs (RIF and INH). Besides RIF and INH, XDR strains are resistant to any fluoroquinolones and to at least one of the three injectable second-line drugs (capreomycin, kanamycin, and amikacin) (World Health Organization, 2006). Infections with such strains require further prolonged and aggressive treatment courses employing

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combinations of numerous second-line drugs that often exhibit toxic side effects and are expensive to administer (Dye, 2000, Nguyen & Thompson, 2006). Moreover, the spread of these infections is diminishing our already limited arsenal of effective antibiotics even further with which some XDR *M. tuberculosis* strains have become virtually untreatable with current medicines (Gandhi *et al.*, 2006, Jassal & Bishai, 2009, LoBue, 2009).

The current prevalence of drug-resistant strains poses a dire need for alternative TB therapies. Development of completely new TB drugs is both time-intensive and costly. Although a few compounds have made their way into pre-clinical or clinical stages, this approach thus far has provided us with no newly approved anti-TB drugs. On average, it takes twelve to fifteen years and US $500 million to get a new drug from the laboratory to the market (Bolten & DeGregorio, 2002). Clearly, the possibility that new resistant strains may rapidly occur and diminish the utility of a new drug after approval represents a significant risk factor for the development of anti-infective drugs. An alternative approach to this pathway is presented by the concept of "targeting resistance". This drug potentiation approach, which uses knowledge of resistance mechanisms to (re)sensitize pathogenic bacteria to already available drugs, may become an important trend in the new era of drug development for infectious diseases. The coadministration of existing drugs and inhibitors that suppress resistance mechanisms allows ineffective drugs to (re)gain their antimicrobial activity (Wright, 2000, Wright & Sutherland, 2007) (Figure 1). In the case of *M. tuberculosis*, this approach could be used to rescue and extend the utility of current TB drugs, or make use of other available drugs that are currently inactive against the bacillus. The extended lifespan of valuable approved antibiotics of known pharmacology, toxicology, and treatment schedule, represents a unique advantage of the drug potentiation approach.

This chapter will explore recent findings that suggest several available drugs as promising candidates for resistance-targeted potentiation. Future directions regarding this approach in TB-drug development will also be discussed.

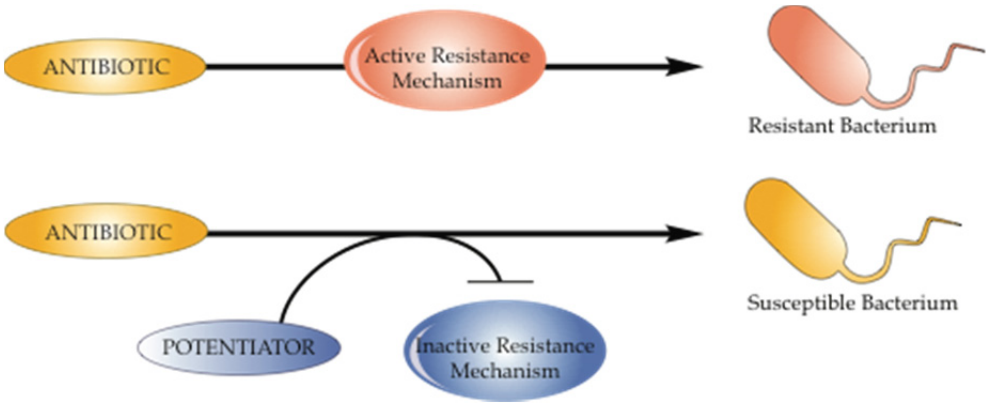

Fig. 1. Concept of drug potentiation by targeting resistance. An active resistance mechanism allows survival of bacterial pathogens in the face of an antibiotic(s). A potentiator that inhibits the resistance mechanism would (re)sensitize the bacteria to the antibiotic(s), thus enhancing antibacterial activity.

2. β–Lactams

The most widely used group of antibiotics today is the β–lactams, a broad class of drugs including penicillin and penicillin derivatives, cephalosporins, monobactams and carbapenems (Figure 2 A), that target bacterial cell wall synthesis at the peptidoglycan layer (Koch, 2003, Waxman *et al.*, 1980). Peptidoglycan is the major component of the cell wall in both Gram-positive and Gram-negative bacteria and is extensively cross-linked by penicillin-binding proteins (PBPs), lending it stability (Koch, 2003, Waxman *et al.*, 1980). β–lactam antibiotics are cyclic amides containing a hetero-atomic ring consisting of three carbon atoms, and one nitrogen atom (Figure 2 A), mimicking certain precursors of peptidoglycan (Koch, 2003, Waxman *et al.*, 1980). When PBPs mistakenly use β–lactams as their substrate rather than peptidoglycan precursors, the antibiotics are incorporated irreversibly into the PBP structure, inhibiting cross-linking activity (Koch, 2003, Waxman *et al.*, 1980), leading to consequent cell lysis in hypotonic environments (Beveridge, 1999, Lee *et al.*, 2001, Severin *et al.*, 1997).
Although serving as the only successful clinical example of potentiation through targeting resistance, inhibitors of β–lactamases have prolonged the life of β–lactams for more than thirty years (Drawz & Bonomo, 2010). Without these potentiators, many β–lactams would have long become useless against multiple bacterial pathogens. β–lactams such as penicillin are now commonly coadministered with β–lactamase inhibitors such as clavulanic acid, sulbactam, or tazobactam that prevent degradation of β–lactams, thus sustaining bacterial susceptibility to β–lactams.

2.1 β–Lactam resistance mechanisms in *Mycobacterium tuberculosis*

In the case of mycobacteria, resistance to β–lactams involves three main components: permeability of the mycobacterial cell wall (Chambers *et al.*, 1995, Jarlier *et al.*, 1991, Jarlier & Nikaido, 1990, Kasik & Peacham, 1968), affinity of the drugs to their target PBPs (Chambers *et al.*, 1995, Mukherjee *et al.*, 1996), and degradation by β–lactamase activity (Jarlier *et al.*, 1991, Quinting *et al.*, 1997). In addition, since *M. tuberculosis* is an intracellular pathogen, an effective TB drug must be able to penetrate the macrophage and phagosomal membranes to reach the bacilli residing within.
Although mycobacteria are classified as Gram-positive bacteria, their cell wall is extremely thick and multi-layered with varied hydrophobicity, posing an effective obstacle for the entry of most chemical compounds. The peptidoglycan network is covered by an arabinogalactan layer, both of which are hydrophilic and likely limit penetration of hydrophobic compounds (Brennan & Nikaido, 1995). On top of these aforementioned layers is another layer consisting of mycolic acids linked to acyl lipids, which forms a waxy, non-fluid barrier restricting transport of both hydrophobic and hydrophilic molecules (Liu *et al.*, 1995). Penetration by diffusion of β–lactams through the mycobacterial cell wall is hundreds of times slower than that of *Escherichia coli* (Chambers *et al.*, 1995, Kasik & Peacham, 1968). However, because of the extremely long generation time of *M. tuberculosis,* the slow rate of drug penetration is enough to allow for half-equilibration over the membrane well before the cell divides, making cell wall permeability and therefore drug penetration important but not a major determinant of β–lactam resistance (Chambers *et al.*, 1995, Quinting *et al.*, 1997).
As for drug target affinity, four major PBPs have been identified in *M. tuberculosis*, all of which bind β–lactams at therapeutically achievable concentrations (Chambers *et al.*, 1995). The 49-kDa PBP from *M. smegmatis* is also sensitive to several β–lactams at similar

concentrations (Mukherjee *et al.*, 1996). Therefore, target affinity does not significantly contribute to the mycobacterial β–lactam resistance. Cell division in *M. tuberculosis* is extremely slow, only occurring every 15-20 hours. This slow growth contributes both negatively and positively to drug resistance. Carbapenem antibiotics, which seem to be the most effective β–lactam with antimycobacterial activity, are relatively unstable and lose activity much faster than the mycobacterial growth rate (Watt *et al.*, 1992). It has however been shown that a daily antibiotic regimen can compensate for loss of activity and markedly increase growth inhibitions *in vitro* (Watt *et al.*, 1992).

With drug penetration and target affinity being negligible for β–lactam resistance in mycobacteria, degradation by β–lactamases constitutes the principal resistance mechanism. β–lactamase activity has been reported in all known mycobacterial species (Kasik, 1979), except the non-pathogenic *M. fallax*, which exhibits hypersusceptibility to β–lactams (Quinting *et al.*, 1997). Mycobacteria, including *M. tuberculosis*, export β–lactamases to the cell wall via the twin-arginine translocation (Tat) pathway (McDonough *et al.*, 2005, Voladri *et al.*, 1998), thus disruption of the Tat transporter leads to lower β–lactamase activity in *M. smegmatis* culture filtrates and increased β–lactam susceptibility (McDonough *et al.*, 2005). The major β–lactamase in *M. tuberculosis*, BlaC, is a member of the Ambler Class-A β–lactamases and exhibits broad substrate specificity, catalysing hydrolysis of both cephalosporins and penicillins (Voladri *et al.*, 1998, Wang *et al.*, 2006). This broad substrate specificity is attributed to the large and flexible substrate-binding site of this particular β–lactamase (Wang *et al.*, 2006). Two additional β–lactamase-like proteins, encoded by the *rv0406c* and *rv3677c* genes, have been identified to provide *M. tuberculosis* H37Rv with a lower β–lactamase activity (Nampoothiri *et al.*, 2008). Expression of these proteins in *E. coli* confers significant resistance to β–lactam antibiotics (Nampoothiri *et al.*, 2008).

In general, mycobacterial β–lactamases exhibit low-level activity compared to those of other pathogenic bacteria. However, because of the slow equilibration of β–lactams across the thick cell wall, this low β–lactamase activity is effective enough to provide protection to mycobacteria from β–lactam action (Jarlier *et al.*, 1991). When *M. fallax in trans* expresses the β–lactamase from *M. fortuitum*, MICs for β–lactams increase dramatically, indicating that β–lactamase-mediated degradation is the critical contributor to β–lactam resistance in mycobacteria (Quinting *et al.*, 1997). For most bacterial β–lactamases, β–lactams of the carbapenem subgroup are highly resistant to hydrolysis. Unfortunately, *M. tuberculosis* BlaC shows measurable activity with carbapenem compounds including imipenem, ertapenem, doripenem and meropenem, even though imipenem and meropenem seem somewhat more effective than other carbapenems and penicillins in antimycobacterial activity (Hugonnet & Blanchard, 2007, Tremblay *et al.*, 2010).

Interestingly, BlaC production in *M. tuberculosis* is β–lactam inducible, and controlled by a regulatory network that is also present in other Gram-positive bacteria (Sala *et al.*, 2009). The transcriptional repressor BlaI, a winged helix regulator, forms homodimers that bind DNA at specific recognition sites in the absence of β–lactam antibiotics (Sala *et al.*, 2009). BlaC is the only β–lactamase in *M. tuberculosis* whose gene is among the BlaI regulon (Sala *et al.*, 2009). Exposure to β–lactams dissociates BlaI from its DNA binding site, lifting its suppression on *blaC* transcription thus allowing the production of BlaC β–lactamase activity (Sala *et al.*, 2009).

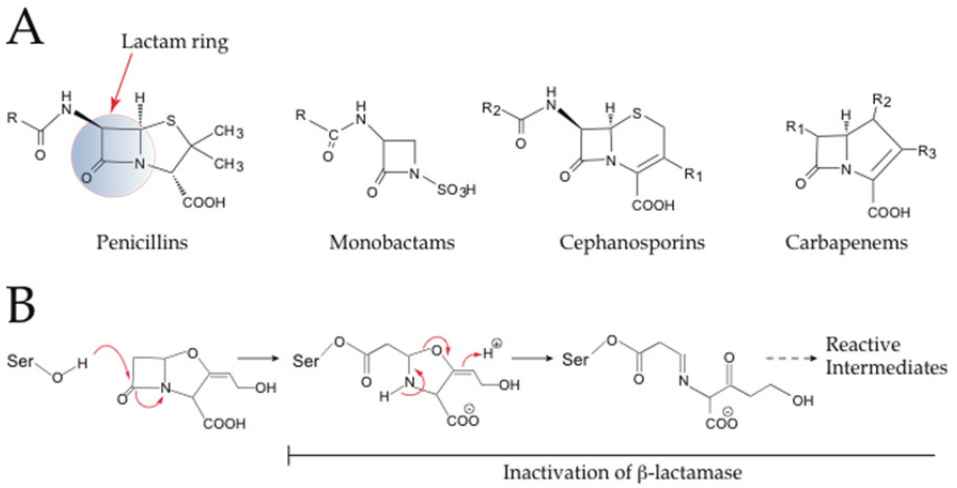

Fig. 2. (A) Structures of β–lactams. The β–lactam ring structure constitutes the base of all β–lactams while the secondary ring structure determines the class. (B) Mechanism of reaction between β–lactamase and clavulanate. The serine residue of the reactive site of β–lactamase reacts with the carbonyl group of clavulanate, followed by breakage of the amide bond that results in acylation. The acylation step is followed by the formation of an imine and secondary ring opening. Note that the ring opening step does not occur with all β–lactamase inhibitors.

2.2 Potentiation of β–lactams in mycobacteria

Attempts to promote the utility of β–lactams to treat TB and other mycobacterial infections have been continuously explored by many laboratories. Rather than ignoring these antibiotics, their antimycobacterial activity could be potentiated by coadministration with β–lactamase inhibitors, as routinely practiced for other bacterial infections. As mycobacterial susceptibility to β–lactams is quite high in the absence of β–lactamase activity (Flores *et al.*, 2005, Quinting *et al.*, 1997), effective chemical inactivation of β–lactamases should similarly increase β–lactam sensitivity in these bacteria. In fact, *in vitro* studies first showed that three FDA-approved inhibitors, sulbactam, tazobactam, and clavulanate, effectively inhibit nitrocefin degradation by purified BlaC protein (Hugonnet & Blanchard, 2007, Tremblay *et al.*, 2008). While sulbactam inhibits BlaC competitively and reversibly, tazobactam inhibits BlaC in a time-dependent manner with reappearing enzyme activity. Interestingly, clavulanate forms hydrolytically stable, inactive forms of the enzyme, completely and irreversibly inhibiting BlaC in a mechanism in which after acylation of clavulanate, a secondary ring-opening leads to reactive intermediates that occupy the active site of the enzyme (Figure 2 B) (Hugonnet and Blanchard 2007; Tremblay *et al.* 2008). Hence, clavulanate provides a potential lead for the development of effective β–lactam potentiators for TB.

Whereas data obtained from the aforementioned *in vitro* studies are promising, drugs used for TB must be able to penetrate the mycobacterial cell wall in order to exert their activity. A later *in vitro* study showed that meropenem/clavulanate combination is very effective in killing both aerobically and anaerobically grown *M. tuberculosis* (Hugonnet *et al.*, 2009).

More importantly, the drug combination is also effective against thirteen tested XDR *M. tuberculosis* strains (Hugonnet *et al.*, 2009). Furthermore, studies using mouse peritoneal macrophages infected with *M. tuberculosis* indicated that penetration through neither host macrophage nor phagosomal membranes appears to be a problem for β–lactams and/or β–lactamase inhibitors (Chambers *et al.*, 1995, Prabhakaran *et al.*, 1999). Significant reduction of mycobacterial counts in mouse macrophages upon treatment with various combinations of β–lactams and β–lactamase inhibitors within clinically achievable doses has been demonstrated (Chambers *et al.*, 1995, Prabhakaran *et al.*, 1999). Encouraging results were also reported in animal models. Although less effective than the first-line anti-TB drug INH, imipenem significantly reduced *M. tuberculosis* counts in the lungs and spleens of infected mice (Chambers *et al.*, 2005). As a result, imipenem doubled the survival rate of infected mice (35% mortality vs. 70%). While only a very few cases in which TB patients treated with β–lactams in conjunction with or without potentiators have been reported, the results were always promising. One study using imipenem alone in MDR-TB patients with poor predicted outcomes achieved a 70% cure rate (Chambers *et al.*, 2005). Another study showed that treatment of TB patients with an amoxicillin/clavulanate combination significantly reduced *M. tuberculosis* counts with early bactericidal activity comparable to patients treated with the frontline drug INH (Chambers *et al.*, 1998). Importantly, a case report recently described the successful recovery of an advanced XDR-TB patient treated with meropenem/clavulanate in conjunction with other drugs (Dauby *et al.*, 2011).

In summary, evidence obtained from numerous studies performed *in vitro*, in animals, and in humans, all support that β–lactams potentiated by β–lactamase inhibitors could provide an effective addition to the treatment of drug resistant TB.

2.3 Future perspectives

Much work remains to be done in potentiating β–lactams for the treatment of mycobacterial infections. More existing carbapenems should be tested in combination with various β–lactamase inhibitors. With crystal structures and kinetic data now available (Hugonnet & Blanchard, 2007, Hugonnet *et al.*, 2009, Tremblay *et al.*, 2010, Tremblay *et al.*, 2008), rational design or high throughput screening should be done to identify better inhibitors that specifically target BlaC or the other β–lactamases of *M. tuberculosis*. Similarly, sensitization of *M. tuberculosis* to β–lactams could be achieved by preventing dissociation of BlaI from its binding site on the *blaC* promoter, thereby repressing the expression of this major β–lactamase in the face of β–lactam exposure. In other bacteria, BlaI homologs are inactivated by proteolytic cleavage at a highly conserved Asparagine-Phenylalanine bond located in helix α5, which is also present in *M. tuberculosis* BlaI (Sala *et al.*, 2009). Although it has not been identified yet, the most likely candidate for the inactivating protease is predicted to be Rv1845c, a zinc metalloprotease encoded by a gene located adjacent to *blaI* (Sala *et al.*, 2009). If proteolysis of BlaI could be prevented by targeting Rv1845c with protease inhitors, the BlaI-mediated repression of *blaC* could be promoted to render the bacilli more susceptible to β–lactams. Similarly, inhibition of β–lactamase translocation by targeting the Tat transpoter system may also represent a novel strategy for β–lactam potentiation.

Since very promising results have been obtained with *in vitro* studies of MDR and XDR *M. tuberculosis* using β–lactamase inhibitors, more comprehensive and well-structured clinical trials with human MDR and XDR TB need to be done in order to affirm the efficacy of these

agents for TB treatment. Currently, a phase II clinical trial in 100 TB patients utilizing meropenem potentiated by clavulanate is being planned in South Korea (Drug Information Online, 2011, Science Centric, 2009). In addition, frequency of dosing will need to be determined to improve and maintain effective doses over longer periods of time. One of the major obstacles to the effective use of β–lactams in long course regimens is that currently used carbapenems have to be administered intravenously, leading to high costs of treatment due to necessary supervision by health care professionals as well as complicating patient compliance over the entire course of treatment.

3. Ethionamide

Ethionamide (ETH, 2-ethylthioisonicotinamide, or Trecator SC, Figure 3 A) is an important component of most current drug regimens used in the treatment of MDR-TB. While an effective drug against more than 80% of MDR-TB clinical strains, ETH has a low therapeutic index, or margin of safety, characterized by a narrow therapeutic effective concentration range (Sood & Panchagnula, 2003, Zimmerman *et al.*, 1984). In other words, it is more difficult to prescribe ETH treatment doses that ensure effective treatment outcomes and yet avoid toxic side effects (Burns, 1999). The lowest dose of ETH required to inhibit *M. tuberculosis* growth has been shown to elicit adverse side effects such as hepatitis and gastrointestinal ailments (Flipo *et al.*, 2011). Discovered in 1956, ETH is a structural thioamide analogue of INH and must be metabolically activated in order to form adducts with nicotinamide adenine dinucleotide (NAD). The ETH-NAD adducts subsequently inhibit InhA, the NADH-dependent enoyl-ACP reductase of the fatty acid biosynthesis type II system, allowing ETH to exert its activity against the synthesis of mycolic acids, the major component of the tubercle bacilli cell wall (Brossier *et al.*, 2011, Morlock *et al.*, 2003, Vannelli *et al.*, 2002, Vilcheze *et al.*, 2008, Zhang, 2005).

3.1 Ethionamide resistance mechanisms in mycobacteria

ETH activation requires an NADPH-specific FAD-containing monooxygenase, encoded by *ethA*, which oxidizes ETH to form the covalent ETH-NAD adducts. The active ETH-NAD adducts tightly bind to and inhibit InhA activity (Figure 3 A) (Brossier *et al.*, 2011, DeBarber *et al.*, 2000, Frenois *et al.*, 2004, Morlock *et al.*, 2003, Wang *et al.*, 2007). EthA was shown to catalyze the conversion of ketones to esters, suggesting its physiological function in mycolic acid metabolism of *M. tuberculosis* (Fraaije *et al.*, 2004). In the majority of ETH resistant *M. tuberculosis* isolates, mutations have been mapped to four principal catagories: (i) mutations that alter activity of EthA, (ii) mutations in *ethR*, the gene located adjacent to *ethA*, (iii) mutations in InhA that prevent binding of the activated drug, and (iv) mutations in the *inhA* promoter region that lead to InhA overexpression, (Banerjee *et al.*, 1994, Baulard *et al.*, 2000, Brossier *et al.*, 2011, DeBarber *et al.*, 2000, Morlock *et al.*, 2003). Besides these four main catagories, several additional genes (*ndh, mshA,* and *dfrA*) might also be involved in ETH resistance. For example, mutations in *ndh*, which encodes a NADH dehydrogenase, may result in an increased intracellular concentration of NADH that competitively inhibits the binding of ETH-NAD adducts to InhA (Vilcheze *et al.*, 2005). While the connection of *ndh* mutations and ETH resistance has been demonstrated in *M. bovis* BCG and *M. smegmatis*, it has not been observed in *M. tuberculosis* (Brossier *et al.*, 2011). *mshA* encodes a glycosyltransferase involved in the biosynthesis of mycothiol that may enhance the ETH activation by EthA (Brossier *et al.*, 2011, Vilcheze *et al.*, 2008, Xu *et al.*, 2011). Whereas *mshA*

mutations might be readily identified *in vitro* under ETH selection pressure, mutations in *mshA* only represent a minority among ETH resistant *M. tuberculosis* clinical isolates (Brossier *et al.*, 2011). Lastly, *dfrA* encodes the dihydrofolate reductase activity involved in folate biosynthesis. As it was suggested that dihydrofolate reductase is inhibited by INH adducts (Argyrou *et al.*, 2006), this enzyme may also be targeted by the adducts of ETH. Thus far, mutations in *dfrA* have not been identified among ETH resistant clinical isolates (Brossier *et al.*, 2011).

In summary, the reduced EthA-mediated activation of ETH represents the principal molecular mechanism contributing to ETH resistance. Indeed, *in trans* overexpression of the prodrug activator EthA in *M. smegmatis* leads to increased ETH sensitivity and inhibition of mycolic acid synthesis (Morlock *et al.*, 2003, Willand *et al.*, 2009) whereas attempts to overexpress EthA in *M. tuberculosis* have been unsuccessful (DeBarber *et al.*, 2000, Morlock *et al.*, 2003). In recent studies, it has been clarified that the production of EthA is negatively controlled by the transcriptional regulator EthR, encoded by an adjacent gene (Figure 3B) (Baulard *et al.*, 2000, Morlock *et al.*, 2003). *In trans* overexpression of *ethR* causes strong inhibition of *ethA* expression, whereas chromosomal inactivation of *ethR* stimulates ETH hypersensitivity (Dover *et al.*, 2004, Engohang-Ndong *et al.*, 2004). Furthermore, electrophoretic mobility shift assays and DNA footprinting analysis indicate direct interaction of EthR with the *ethA* promoter (Dover *et al.*, 2004, Engohang-Ndong *et al.*, 2004). EthR is a member of the TetR/CamR family of repressors that is suggested to sterically inhibit the interaction between RNA polymerase and the affected promoter (Engohang-Ndong *et al.*, 2004, Frenois *et al.*, 2004, Willand *et al.*, 2009). In fact, *M. tuberculosis* EthR was shown to cooperatively multimerize on a 55-bp operator, O_{ethR}, located within the *ethA* promotor, thereby repressing *ethA* expression (Frenois *et al.*, 2004, Vannelli *et al.*, 2002, Weber *et al.*, 2008).

Similar to other TetR/CamR repressors, recent X-ray crystallographic structures revealed that EthR exists as a homodimer organized by two functional domains, each composed of nine α-helices (Dover *et al.*, 2004, Frenois *et al.*, 2004, Willand *et al.*, 2009). The amino terminus of each DNA binding domain consists of a classical helix-turn-helix motif formed by α1, 2, and 3. The remaining six α-helices comprise the carboxy-terminus, which contains the ligand-binding site responsible for controlling the conformational changes that prevent binding of EthR to O_{ethR}. Interactions between α-helices of each monomer form a four-helix bundle resulting in dimerization of the repressor. The crystal structures also revealed a ligand cocrystallized with EthR (Frenois *et al.*, 2006, Frenois *et al.*, 2004). This ligand, hexadecyl octanoate (HexOc), occupies the hydrophobic tunnel of each monomer by means of hydrophobic interactions and hydrogen bonds (Willand *et al.*, 2009). In the presence of HexOc, the distance between the two DNA binding domains in the EthR structure is augmented by 18 Å. As a result, the conformational change impairs the ability of EthR to bind to its operator (Frenois *et al.*, 2006, Frenois *et al.*, 2004, Willand *et al.*, 2009). The ligand-binding domain, embedded in the core domain of each monomer, is characterized as a narrow hydrophobic tunnel rich in aromatic residues (Dover *et al.*, 2004, Willand *et al.*, 2009). More recently, two ETH resistant isolates expressing two unique mutations in EthR, Phenylalanine 110 changed to Leucine and Alanine 95 changed to Threonine, further illuminated the derepression mechanism of EthR. Both Phenylalanine 110 (located within the α5 helix) and Alanine 95 (located within the vicinity of helices α4 and α5) contribute to the ligand-binding domain (Brossier *et al.*, 2011). Based on this wealth of knowledge, recent

efforts are being made to develop compounds that could potentially interfere with EthR repressor function. Such inhibitors could therefore potentiate the antimycobacterial efficacy of ETH and possibly reduce its adverse side effects by allowing lower prescribed doses (Flipo *et al.*, 2011).

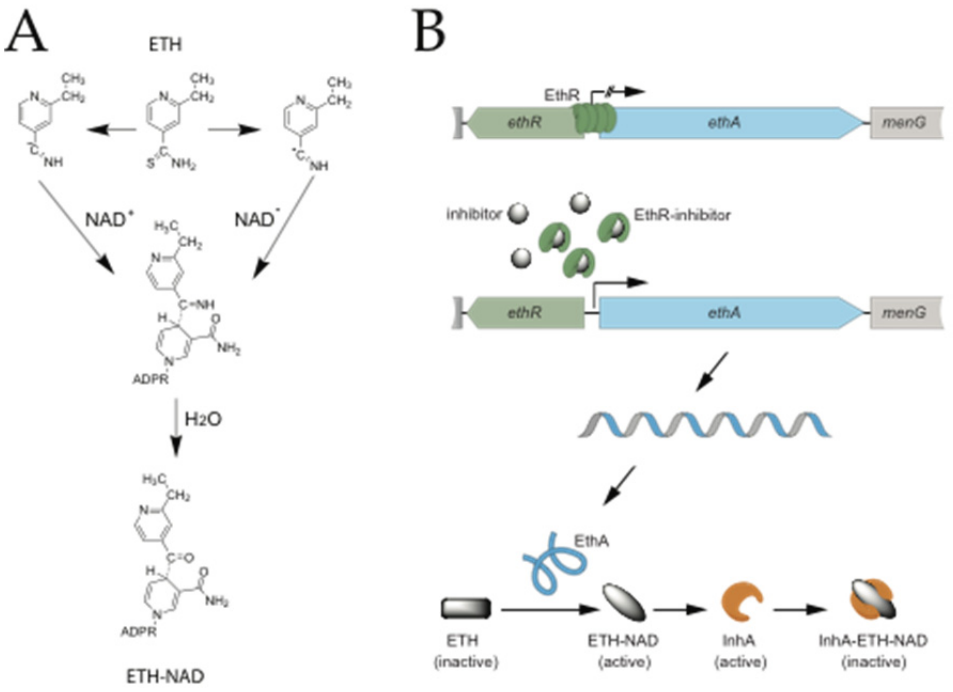

Fig. 3. Ethionamide activation and potentiation. (A) Model of ETH activation by EthA. ETH is first oxidized by EthA to a corresponding thioamide S-oxide that is further oxidized to form the final cytotoxic species. Although the latter oxidation steps remain unclear, it is postulated that thioamide S-oxide is converted to an imidoyl radical (right), which attacks NAD^+. Following hydrolysis and release of the amine group, the final ETH-NAD adducts are formed. Alternatively, the amidoyl anion (left) can serve as the intermediate before the NAD attack. Scheme redrawn from (Wang *et al.*, 2007). (B) Potentiation of ETH by targeting EthR. Binding of inhibitors releases EthR from its interaction with the *ethA* promoter. This allows for derepression of EthA expression, which is responsible for converting ETH to its active form ETH-NAD. The activated drug then binds to InhA and inhibits its activity in mycolate biosynthesis. EthR inhibitors could thereby function as ETH potentiators.

3.2 Potentiation of ethionamide in mycobacteria

Since ligand binding was shown to affect EthR function in repressing *ethA* expression, and increase susceptibility of *M. tuberculosis* to ETH (Flipo *et al.*, 2011, Frenois *et al.*, 2006, Frenois *et al.*, 2004, Willand *et al.*, 2009), much interest has been invested in determining whether synthetic compounds could be utilized to regulate DNA-binding activity of EthR (Weber *et al.*, 2008, Willand *et al.*, 2009). Since the DNA-binding domain of EthR is able to

accommodate a hydrophobic ester such as HexOc, initial attempts were made using several ketones to assay their ability to function as EthR ligands as well as to increase mycobacterial ETH sensitivity (Frenois *et al.*, 2004). *In vitro* experiments demonstrated synergy of benzylacetone and ETH on *M. smegmatis* growth (Fraaije *et al.*, 2004). Whereas benzylacetone itself did not display antimycobacterial activity, its addition to ETH used at subinhibitory concentrations (5 μg ml^{-1}) produced significant inhibition of mycobacterial growth (Frenois *et al.*, 2004).

As an intracellular pathogen, *M. tuberculosis* resides within phagosomal compartments of host macrophages (Nguyen & Pieters, 2005). Therefore, EthR inhibitors not only have to specifically target the repressor but must be able to reach the macrophages' cytosol (Weber *et al.*, 2008). To screen for drug-like ETH potentiators, an EthR-based reporter system was first developed by the Fussenegger group (Weber *et al.*, 2008). This elegant mammalian-based system allows for assessment of not only specificity and bioavailability of tested molecules, but also their cytotoxicity to the host cell. A library of hydrophilic esters, the primary products of EthA-catalyzed Baeyer-Villiger oxidation of ETH, was synthesized and tested for their ability to release EthR from its O_{ethR} operator within a mammalian cell, using the therein described reporter system. A licensed food additive, 2-phenylethyl-butyrate, was found to effectively regulate EthR activity as well as to increase *M. tuberculosis* susceptibility to ETH (Weber *et al.*, 2008). *In vitro* analysis of *ethA* transcripts by quantitative real time PCR verified that 2-phenylethyl-butyrate dissociates EthR from the *ethA* promoter in a dose-dependent manner. To assess bioavailability, the reporter system was transfected into human embryonic kidney (HEK) cells that were subsequently implanted into mice. In this animal model, orally administered 2-phenylethyl-butyrate effectively reached the target cells to activate the reporter gene. Most importantly, 2-phenylethyl-butyrate displayed synergistic effects with ETH on the growth inhibitory activity against pathogenic mycobacteria (Weber *et al.*, 2008).

From previous analyses of ligand-binding EthR crystal structures (Dover *et al.*, 2004, Frenois *et al.*, 2006, Frenois *et al.*, 2004), the hydrophobic interactions and hydrogen-bonding properties of the amphiphilic binding cavity was utilized to design a pharmacophore model as a means of isolating moderately lipophilic compounds that could potentially interfere with the repressor function of EthR (Willand *et al.*, 2009). The novel pharmacophore model was designed as a low-molecular weight structure consisting of two hydrophobic ends connected by a 4-6 Å linker. This would, in turn, allow for hydrogen bonding interactions with the tunnel's uncharged polar surface formed by Asparagine 179 and Asparagine 176 side-chains. From a library of drug-like compounds, 131 compounds fitting the pharmacophore model were selected and analyzed for properties relevant for drug development such as molecular weight, rotatable bonds, polar surface area, hydrogen bond donors and acceptors, etc. (Willand *et al.*, 2009). Surface plasmon resonance and co-crystallization assays emphasized several compounds with the ability to inhibit EthR-DNA interaction. Using this approach, BDM14500, a lead compound comprised of a 1,2,4-oxadiazole linker, was identified to inhibit EthR-DNA interaction by more than 50%. More importantly, inhibition activity of ETH on *M. tuberculosis* growth is significantly boosted by BDM14500 (Willand *et al.*, 2009). The primary data obtained from studies of BDM14500 allowed further development of improved EthR ligands. Two thiophen-2-yl-1,2,4-oxadiazole analogs of BDM14500, BDM31343 and BDM31381, were synthesized and subjected to surface plasmon resonance, co-crystallization, and ETH potentiation assays (Flipo *et al.*, 2011, Willand *et al.*, 2009). Kinetic analysis showed that BDM31343 and BDM31381 inhibit

the interaction of EthR and O_{ethR} with IC_{50} values in the nanomolar to micromolar range, indicating their potentially high efficacy. Indeed, in *M. bovis* BCG culture, BDM31381 treatment results in a 35-fold increase in the level of *ethA* mRNA. It is suggested that the efficacy of BDM31381 resides in its ability to form an energetically favorable orientation by generating a new hydrogen bond between the carbonyl of the ligand and the carboxamide of the Asparagine 179 side chain. In fact, MIC assays later confirmed that BDM31343 and BDM31381 are both more effective potentiators of ETH activity (Flipo *et al.*, 2011, Willand *et al.*, 2009). The addition of BDM31343 or BDM31381 (25 µM) respectively allows a 10 (0.1 vs 1 µg ml^{-1}) or 20 (0.025 vs 0.5 µg ml^{-1}) fold reduction in ETH concentration yet retains identical *M. tuberculosis* growth inhibition activity. In other words, BDM31343 and BDM31381 are able to potentiate ETH antimycobacterial activity by factors of 10 and 20, respectively (Willand *et al.*, 2009). *In vivo*, mice infected with *M. tuberculosis* were treated for 3 weeks with ETH alone or in conjunction with BDM31343 or BDM31381. Following treatment, the mycobacterial load in mouse lungs was quantified. Whereas the BDM31381/ETH combination had only a minor effect on bacterial load compared to control mice treated with ETH alone, the combination of BDM31343 with ETH resulted in a significant decrease of *M. tuberculosis* load with three times more efficiency than with ETH treatment alone. TB treatment with reduced ETH dosages by combining it with BDM31343 may thus allow for efficient elimination of the bacillus without severe side effects (Willand *et al.*, 2009).

3.3 Summary and future perspectives

Through the implementation of strategies including X-ray crystallography, pharmacophore modeling (Willand *et al.*, 2009), and synthetic mammalian gene circuits (Weber *et al.*, 2008), effective potentiators of ETH have been identified. While further *in vitro* and *in vivo* analyses of these compounds will need to be performed, it is expected that such potential molecules will boost activity and allow ETH to be reconsidered as a first-line anti-TB antibiotic (Weber *et al.*, 2008, Willand *et al.*, 2009). In addition, because ETH and INH inhibit the same target, InhA (Banerjee *et al.*, 1994), ETH potentiation might create an exponential boost for the anti-TB activity of INH and hence their combination. As the attrition rate of the developmental process is enormous (Bolten & DeGregorio, 2002), much work remains to be done in preclinical and clinical development and product approval stages in order to bring this concept to the clinics. Regardless of this risky process, the results obtained from these studies have showcased the potential of this approach in improving the efficacy of existing TB-drugs, thus extending their lifespan in TB treatment. Similar studies with other TB-drugs need to be encouraged, which will not only help to better understand their mechnisms of action and resistance, but also reveal further targets for the drug potentiation approach.

4. Antifolates

Folate is a generic name referring to a large group of chemically similar B vitamins that are essential for the existence of cells in all kingdoms of life. Whereas the synthetic form, widely used as a nutritional supplement, is called folic acid or vitamin B9 (pteroylmonoglutamic acid, PteGlu), most naturally occurring folate forms are derived from the reduced molecule tetrahydrofolate (H_4PteGlu, Figure 4). All of these compounds are comprised of three molecular components: a two-ring pteridine nucleus, a para-aminobenzoic acid (pABA) group, and one or more glutamate residues attached via amide linkages. These molecules vary by the C1 groups attached to the N-5 or/and N-10 positions of H_4PteGlu (Figure 4B).

Folates are important metabolites indispensable for the development and propagation of all organisms. H_4PteGlu derivatives are required in reactions that involve the transfer of one-carbon units (C_1 reactions, Figure 4A). These reactions are essential for the biosynthesis of purines, thymidine, glycine, panthotenate, methionine, and formyl-methionyl-tRNA, the initiator of protein synthesis in bacteria (Blakley, 1969, Green *et al.*, 1996, Selhub, 2002). Because these molecules are required for the synthesis of the building blocks of macromolecules such as nucleic acids and proteins, folate deficiency hinders cell division and consequently results in cell death. In addition, lack of folate derivatives also leads to defects in the recycling of homocysteine (Hcy, Figure 4) and S-adenosine methionine (SAM), which result in elevated homocysteine concentration (homocysteinemia) and reduced cellular methylation activities, respectively. Folates are particularly important during periods of rapid cell division and growth (Blakley, 1969, Green *et al.*, 1996).

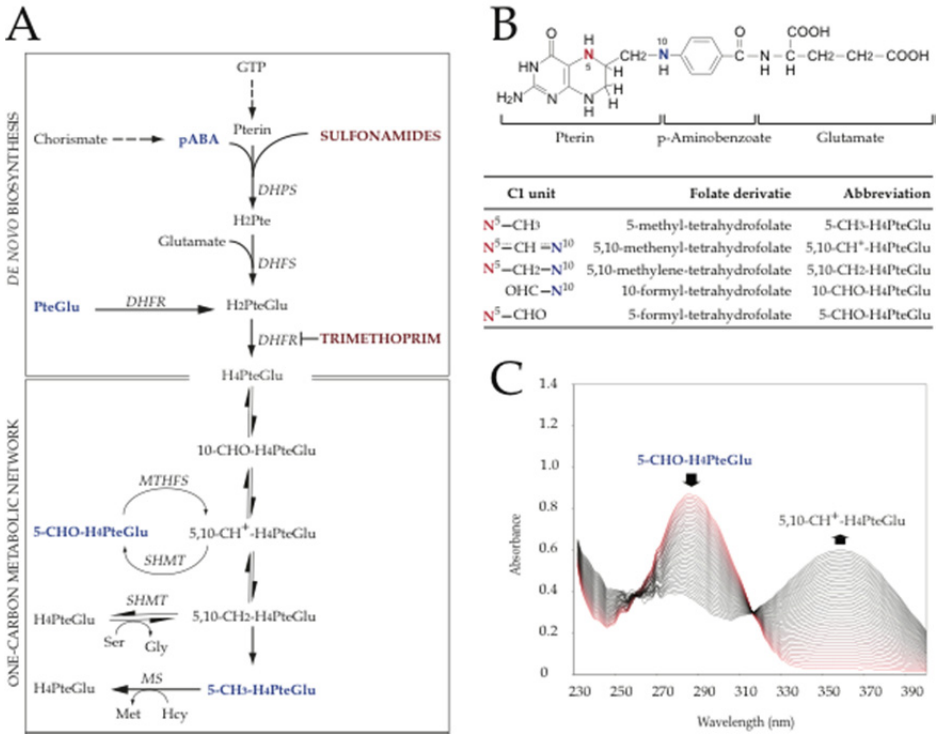

Fig. 4. Folate metabolism and antagonism in bacteria. (A) Simplified interconversions of folate derivatives in *de novo* folate synthesis and one-carbon metabolic network. DHFS, dihydrofolate synthase; Gly, glycine; Met, methionine; MS, methionine synthase; Pte, pteroate; Ser, serine. (B) Chemical structure of monoglutamylated tetrahydrofolate and its derivatives carrying C1 groups at various levels of oxidation attached to N-5 or/and N-10. Redrawn from (Waller *et al.*, 2010). (C) Scanning spectrophotometric analysis of MTHFS reaction, which converts 5-CHO-H_4PteGlu (Abs, 285nm) to 5,10-CH^+-H_4PteGlu (Abs, 360 nm), catalyzed by the *M. tuberculosis* MTHFS homolog, Rv0992c, a novel determinant of antifolate resistance.

Folate metabolism is generally divided into two stages: biosynthesis (upstream) and utilization (downstream) (Figure 4A). The upstream *de novo* folate biosynthesis involves: (i) pterin branch synthesizing the pteridine group from guanosine triphosphate (GTP), (ii) synthesis of pABA from chorismate, (iii) condensation of pteridine and pABA to form dihydropteroate (H_2Pte) and (iv) glutamylation which adds one or more glutamate groups to form dihydrofolate (H_2PteGlu) that is reduced to form H_4PteGlu. The downstream folate utilization is usually called one-carbon metabolism in which different active forms of H_4PteGlu participate in distinct reactions donating or accepting one-carbon units for the formation of purines, thymidine, glycine, panthotenate, methionine, and formyl-methionyl tRNA (Figure 4).

Because of the vital role of folates in multiple metabolic processes of the cell, folate antagonism has been used successfully in chemotherapeutic treatments of multiple diseases including cancers, malaria, psoriasis, rheumatoid arthritis, graft-versus-host disease, and bacterial infections (Bertino, 1971, Gorlick *et al.*, 1996, Vinetz, 2010). Folate antagonists (antifolates or *antifols*) have been used extensively for the treatment of infectious diseases from the late 1930s till 1960s, but their use has declined because of the emergence of resistant strains, their cytotoxicity, and most importantly the introduction of more effective drugs (Bertino, 1971, Libecco & Powell, 2004). Nevertheless, combination therapies using trimethoprim and sulfonamides to create synergistic effects are still used effectively today to treat some infectious diseases such as urinary tract infection, *Pneumocystis jiroveci* pneumonia, shigellosis, and for prophylaxis against recurrent and drug-resistant infections (Grim *et al.*, 2005, Libecco & Powell, 2004, Proctor, 2008). The absence of enzymes required for a complete *de novo* folate biosynthesis in humans and other mammals makes this pathway an attractive and potential target for the development of novel antimicrobial agents (Bermingham & Derrick, 2002). Whereas proteins participating in folate metabolism are well known, most current folate antagonists are thought to act on either the biosynthesis or the reduction of folate (Bermingham & Derrick, 2002, Gangjee *et al.*, 2007, 2008). Whereas trimethoprim and folate analogs such as methotrexate inhibit the reduction step through inhibition of dihydrofolate reductases (DHFR), sulfonamides and sulfone drugs are pABA analogs that outcompete pABA in the condensation with the pteridin group, catalyzed by dihydropteroate synthase (DHPS) (Bermingham & Derrick, 2002, Gangjee *et al.*, 2007, 2008).

4.1 Folate antagonism in chemotherapies of mycobacterial infections and antifolate resistance

The essentiality of folate-mediated one-carbon metabolism in fundamental metabolic and cellular processes has been recognized since the 1940s. Almost immediately after folates had been identified as essential metabolic cofactors, antifolate drugs that interfere with the folate pathway were developed and found to be effective antimicrobial and antineoplastic agents. As seen with other antibiotics, acquired resistance to antifolates in pathogenic bacteria also occurred rapidly following their introduction. These resistant forms are typically caused by mutations that alter either expression levels or protein structures of the targeted enzymes (Bertino, 1971, Libecco & Powell, 2004). DHFR can acquire resistance through point mutations of active-site residues, thus altering its affinity for trimethoprim (Adrian & Klugman, 1997, Volpato & Pelletier, 2009). While clinical resistant strains frequently show a diversity of mutations, residues that are most important for trimethoprim affinity are highly conserved among the isolates (Adrian & Klugman, 1997). For example, a point mutation in the DHFR gene that changes a conserved Isoleucine residue (Isoleucine 94 in *M. tuberculosis*

DHFR) to Leucine, confers 50-fold higher trimethoprim resistance in *Streptococcus pneumoniae* (Adrian & Klugman, 1997). This mutation is commonly found in DHFR from mammalian, parasitic and bacterial resistant isolates (Volpato & Pelletier, 2009). For sulfonamide and sulfone drugs, single point mutations at the Serine 53 or Proline 55 residues within DHPS are found in resistant isolates of *M. leprae* (Baca *et al.*, 2000, Kai *et al.*, 1999). The two affected residues are located in the drug binding region of *M. tuberculosis* DHPS and are highly conserved throughout bacteria and protozoa (Baca *et al.*, 2000). Combined mutations in DHFR and DHPS encoding genes have been known to confer resistance to all available antifolates (Bermingham & Derrick, 2002, Gangjee *et al.*, 2007, 2008). It is important to note that most current knowledge of trimethoprim and sulfonamide resistance comes from studies of bacteria distantly related to *M. tuberculosis*, and very limited information on mechanisms involved in antifolate resistance is available for mycobacterial species. With the increasing use of antifolates (Date *et al.*, 2010), a better understanding of antifolate resistance mechanisms in *M. tuberculosis* is urgently needed (Koser *et al.*, 2010).

Although much remains unknown about resistance mechanisms, antifolate drugs have been used to treat mycobacterial infections. For example, PAS (p-aminosalycilic acid) is currently used as a second-line drug for TB (Rengarajan *et al.*, 2004); the sulfone drug Dapsone has been used in monodrug regimens to treat leprosy for many decades (Doull, 1963). Interestingly, a recent study suggested that the frontline TB-drug INH may also target folate metabolism through the inhibitory action of its adducts on DHFR (Argyrou *et al.*, 2006). In addition, recent *in vitro* studies and a case report proposed that antifolate combinations such as those of co-trimoxazole (trimethoprim plus sulfamethoxazole) might be effective against TB, thus renewing much interest in the exploitation of antifolates to treat MDR and XDR-TB (Forgacs *et al.*, 2009, Ong *et al.*, 2010, Young, 2009). *M. tuberculosis* clinical strains isolated from TB patients were shown to be widely susceptible to clinically achievable concentrations of co-trimoxazole (Forgacs *et al.*, 2009), or sulfamethoxazole alone (Ong *et al.*, 2010). Importantly, the World Health Organization has recently called for widespread use of co-trimoxazole in the prophylactic treatment of HIV-AIDS patients to prevent opportunistic infections (Date *et al.*, 2010). While this practice shows promise, it is likely to expose infectious agents, including *M. tuberculosis,* to antifolates more frequently, which could lead to selection of resistant strains, thus shortening the lifespan of this powerful family of drugs (Vinetz, 2010). As in the case of β–lactams, strategies for potentiation of antifolates should be readily available to counterattack upcoming resistant strains, thereby extending their utility for TB treatment.

4.2 Potentiation of antifolates in mycobacteria

A method for boosting antifolate efficacy by utilizing combinations of drugs that target individual steps in folate biosynthesis is already in place. Trimethoprim is commonly coadministered with sulfonamides, for example sulfamethoxazole in the co-trimoxazole combination, to achieve synergy (Libecco & Powell, 2004) (Figure 4). However, in many cases including that of *M. tuberculosis*, the synergistic effect of trimethoprim on sulfonamides remains questioned and inconclusive (Forgacs *et al.*, 2009, Ong *et al.*, 2010, Suling *et al.*, 1998). In addition, bacterial strains resistant to both trimethoprim and sulfonamides have readily been isolated (Bermingham & Derrick, 2002, Gangjee *et al.*, 2007, 2008). Therefore, novel potentiation approaches targeting resistance mechanisms might be

more effective in both potentiating available antifolates and preventing the emergence of resistant strains.

A recent study aimed at targeting intrinsic antifolate resistance in mycobacteria might reveal valuable targets for such resistance-targeted potentiation approaches (Ogwang *et al.*, 2011). To identify novel antifolate resistance determinants, a genetic screen was first employed using a saturated transposon-insertion library of *M. smegmatis*. These mutants are systematically tested for increased antifolate susceptibility, followed by chemical complementation using folate derivatives of both the *de novo* synthesis and the one-carbon interconversion network. This chemogenomic profiling approach allows for identification of novel determinants previously unknown to function in mycobacterial intrinsic antifolate resistance (Ogwang *et al.*, 2011). Using this non-bias screen, the genome-wide collection of antifolate resistance determinants in mycobacteria (mycobacterial antifolate resistome) was found to be composed of fifty resistance determinants (unpublished data).

A novel determinant identified from this screen was further characterized in a recent report (Ogwang *et al.*, 2011). The *M. smegmatis* mutant presented in this report exhibits hypersusceptibility to several combinations of trimethoprim/sulfonamides tested (Ogwang *et al.*, 2011). For example, its MIC to trimethoprim/sulfachloropyridazine is 64 fold lower than that of the parental *M. smegmatis* strain. The transposon insertion was mapped to a gene encoding a hypothetical protein with low homologies to 5,10-methenyl-tetrahydrofolate synthases (MTHFS, also called 5-formyl-tetrahydrofolate cyclo-ligase, EC.6.3.3.2) from other organisms, including the prototype MTHFS first described in humans (Ogwang *et al.*, 2011). Cross-species *in trans* expression of the human MTHFS was shown to restore antifolate resistance to the *M. smegmatis* mutant. A series of genetic knockout and complementation studies indicated that the disrupted gene encodes a MTHFS activity required for mycobacterial intrinsic antifolate resistance (Ogwang *et al.*, 2011). Absence of MTHFS enzymatic activity results in the inability to metabolize folinic acid (5-formyl-tetrahydrofolate, 5-CHO-H_4PteGlu) along with the reduced metabolism of 5-methyl-tetrahydrofolate (5-CH_3-H_4PteGlu), two major folate derivatives in the cell (Ogwang *et al.*, 2011). 5-CHO-H_4PteGlu is formed by the hydrolysis of 5,10-CH^+-H_4PteGlu catalyzed by serine hydroxymethyltransferase (SHMT, Figure 4A) (Holmes & Appling, 2002, Stover & Schirch, 1990), whereas MTHFS is the only enzyme known to recycle 5-CHO-H_4PteGlu back to 5,10-CH^+-H_4PteGlu in an irreversible, ATP-dependent reaction (Figure 4C). As a consequence of MTHMS absence in mycobacterial cells, polyglutamylated forms of 5-CHO-H_4PteGlu are elevated up to 80 fold, whereas the corresponding polyglutamylated forms of 5-CH_3-H_4PteGlu are reduced (Ogwang *et al.*, 2011). Interestingly, 5-CHO-H_4PteGlu is the only H_4PteGlu derivative whose biological function remains largely unknown (Stover & Schirch, 1993). Although it is well known chemically and widely used as a medical agent, 5-CHO-H_4PteGlu does not appear to function as a cofactor in any of the one-carbon metabolic reactions thus far known (Stover & Schirch, 1993). Because 5-CHO-H_4PteGlu is known as the most stable form of reduced folate species in nature, and its presence is increased in plant seeds and fungal spores, it was suggested that it might function as a folate storage form required for these dormant states of life (Kruschwitz *et al.*, 1994, Shin *et al.*, 1975, Stover & Schirch, 1993). In mammals and yeasts, 5-CHO-H_4PteGlu comprises 3-10% of total folate, whereas its presence may account for up to 50% of total folate in plant mitochondria during photorespiration when the glycine to serine flux is accelerated (Goyer *et al.*, 2005, Roje *et al.*, 2002). *In vitro*, 5-CHO-H_4PteGlu is also a potential inhibitor of SHMT and other enzymes of the one-carbon metabolism, thus it may potentially serve to regulate these metabolic

reactions (Roje *et al.*, 2002, Stover & Schirch, 1991). Deletion of MTHFS in *Arabidopsis* leads to a 2-8-fold increased accumulation of total 5-CHO-H_4PteGlu, 46-fold accumulation of glycine, reduced growth and delayed flowering (Goyer *et al.*, 2005). In human cells, overexpression of MTHFS lowers folate levels and increases folate turnover, suggesting that MTHFS may also function as a folate-degrading enzyme (Anguera *et al.*, 2003).
The role of MTHFS in intrinsic antifolate resistance was found not only in mycobacteria but also in *E. coli*, a Gram-negative bacterium (Nichols *et al.*, 2011, Ogwang *et al.*, 2011), suggesting that this determinant functions ubiquitously among bacteria. Indeed, further work confirmed that *rv0992c*, the gene that encodes the MTHFS homolog in *M. tuberculosis*, is also required for antifolate resistance via its MTHFS enzymatic activity (Figure 4C). Pharmaceutical inactivation of MTHFS activity is therefore expected to sensitize *M. tuberculosis* to classical antifolates, including those current TB-drugs that happen to target folate pathways (PAS, INH, etc.). This intervention may also allow for reduction of effective therapeutic doses, thereby minimizing the cytotoxicity of classical antifolates which has been an issue for their widespread use in the clinics. Work is underway to identify specific inhibitors of *M. tuberculosis* MTHFS by rational design and high throughput screening, as well as to characterize their antifolate potentiation activity against *M. tuberculosis*.

4.3 Future perspectives

Fundamental studies of molecular mechanisms conferring both acquired and intrinsic antifolate resistance in *M. tuberculosis* and related mycobacteria should be further conducted. Knowledge obtained from these studies will be essential for strategic implementations of antifolate use for TB, and will reveal valid targets for the resistance-targeted potentiation of classical antifolates.
A potential problem for the development of MTHFS inhibitors might be their nonspecific inhibition towards human MTHFS. However, the low homologies of MTHFS proteins indicate the possibility to identify species-specific inhibitors. Trimethoprim, which specifically inhibits bacterial DHFR but not the human counterpart, represents an encouraging example for such possibilities. Interestingly, a recent work showed that *ygfA*, the gene that encodes the MTHFS homolog in *E. coli*, is required for the formation of drug persisters during antibiotic treatments (Hansen *et al.*, 2008). Although it remains to be characterized if the function of *ygfA* in antibiotic persister formation is related to its MTHFS activity, a similar role for *M. tuberculosis rv0992c* during TB latent infection is under investigation.
Although *in vitro* studies and a case report suggested that co-trimoxazole could be used for TB treatment (Forgacs *et al.*, 2009, Ong *et al.*, 2010, Young, 2009), more comprehensive well-designed trials with TB patients should be done to evaluate the efficacy of this antifolate combination. These trials should also address if these drugs may help to shorten the current TB regimens. In addition, new combinations using co-trimoxazole and PAS and/or INH should be tested against *M. tuberculosis* both *in vitro* and in patients.

5. Conclusions and future prospects

The primary goal of this chapter is to assess an emerging approach in TB drug development that uses knowledge of resistance mechanisms to sensitize *M. tuberculosis* to available, approved antibiotics (Figure 1) (Wright, 2000, Wright & Sutherland, 2007). Specific inhibitors that suppress resistance mechanisms would boost the efficacy of current anti-TB

drugs, or potentiate the antimycobacterial activity of currently non-TB antibiotics, thus making use of drugs that are already available but have never been used for TB treatment before. Proofs of concept have been made in recent years to demonstrate the feasibility of this approach in potentiating the antimycobacterial activity of important antibiotics such as β–lactams, ethionamide, and antifolates. It is anticipated that this trend will become increasingly important in the future of drug development, not only for TB but any disease treated by chemotherapies. As the rate of drug resistance expansion appears far beyond that of the current drug developmental process, it is logical that such sustainable approaches should be promoted to improve the utility and protection of those effective agents.

Besides targeting antibiotic resistance mechanisms, currently approved drugs should be tested systematically against *M. tuberculosis*, especially drug resistant strains. Recent work showed that many antibiotics that had been thought to be inactive against TB might be effective as chemotherapeutic agents for the disease (Forgacs *et al*., 2009, Hugonnet *et al*., 2009, Ong *et al*., 2010). In addition, drug-drug interactions among current combinatorial regimens for TB need to be further investigated. Most of the antibiotic combinations developed thus far are mainly aimed at minimizing the development of resistance, but disregard possible synergistic or antagonistic effects. Future drug combinations that minimize antagonistic effects but maximize synergy among the drugs used may not only reduce harmful clinical doses but also shorten treatment schedules, which would help to prevent the evolution and spread of antibiotic resistance.

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9

Antitubercular Drugs Development: Recent Advances in Selected Therapeutic Targets and Rational Drug Design

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1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is a remarkably successful pathogen that has latently infected a third of the world population (Zhang et al., 2006). Infection occurs via aerosol, and inhalation of a few droplets containing *M. tuberculosis* bacilli is enough for lung infection (Hassan et al., 2006). After infection, *M. tuberculosis* pathogenesis occurs in two stages. The first is an asymptomatic state that can persist for many years in the host, called latent TB. The second stage requires only a weakened immune response to become activated (Zhang, 2004), then the bacteria begins replicating and causing characteristic symptoms such as cough, chest pain, fatigue and unexplained weight loss. If left untreated, the disease eventually culminates in death. The emergence of Human Immunodeficiency Virus (HIV) and the resultant Acquired Immune Deficiency Syndrome (AIDS) pandemic underlined the importance of reactivation of the disease and its potentially catastrophic outcome since over 50% of deaths among HIV-infected patients results from co-infection with *M. tuberculosis* with the two pathogens inducing each other's replication, thus accelerating the collapse of the immune system (Cole & Alzari, 2007).

While it is impossible to determine the exact number of cases, the latest World Health Organization (WHO) survey estimates that close to 2 million deaths occur every year, that there are approximately 8 million new cases annually, and that every third individual on the planet has been exposed to or infected by *M. tuberculosis* (Dye, 2006; Cole & Alzari, 2007).

Although TB can be treated and even cured with chemotherapy, treatment is exceedingly lengthy and takes 6-9 months (Blumberg, et al., 2003). In addition to significant toxicity, lengthy therapy also causes poor patient compliance, which is a frequent cause for selection of drug resistant and often deadly multidrug resistant TB (MDR-TB) bacteria (Zang et al., 2006).

Currently, TB chemotherapy is made up of a cocktail of first-line drugs, isoniazid (INH), Rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB), which are given for six

months (Blumberg et al., 2006). If this treatment fails as a result of bacterial drug resistance or intolerance to one or more drugs, second-line drugs are used, such as *para*-aminosalicilate (PAS), kanamycin, fluoroquinolones, capreomycin, ethionamide and cycloserine. These are generally less effective or more toxic with serious side effects (Blumberg et al., 2006). This second-line treatment can also result ineffective since MDR-strains that exhibit resistance to these second-line drugs are currently on the rise (Zhang & Amzel, 2002)

Treatment is also made quite difficult by the presence of metabolically silent, persistent or dormant bacteria within host lesions. These are not susceptible to the anti-mycobacterial drugs that usually kill growing but not persistent bacteria (Zhang, 2004). While there are many reasons for drug resistance, including prescription of inadequate regimens, an uncertain drug supply, and ineffective drugs, duration of lengthy treatments is one of the major contributors because some TB patients prematurely stop their therapy after an initial, rapid heath improvement, thereby favoring the emergence of drug-resistant strains (Cole & Alzari, 2007)

2. Anti-TB drug targets

Despite the relative efficacy of current treatment, the various antibiotics that constitute first- and second-line drugs for TB therapy target only a small number of core metabolic processes such as Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) synthesis, cell wall synthesis, and energy metabolism pathways (Zhang, 2005). New classes of drugs with additional drug targets that are difficult to overcome by mutation are urgently needed (Hansan et al., 2006). Desirable new targets should be involved in vital aspects of bacterial growth, metabolism and viability whose inactivation would lead to bacterial death or an inability to persist, thus therapy could be shortened and drug resistant strains could be eliminated or drastically reduced (Mdluli & Spigelman, 2006; Duncan, 2004). Moreover, targets involved in the pathogenesis of the disease process should also be considered for drug development (Zhang et al., 2006; Palomino et al., 2009).

The discovery of the complete genome sequence of TB bacteria helped to identify several important drug targets (Cole et al., 1998). Various groups have used this genomic information to identify and validate targets as the basis for development of new Anti-TB agents. Besides, mycobacterial genetic tools, such as transposon mutagenesis, gene knockout, and gene transfer, greatly facilitate target identification.

2.1 Cell wall biosynthesis related targets

Cell wall biosynthesis is a particularly good source of molecular targets because the biosynthetic enzymes do not have homologues in the mammalian system (Mdluli & Spigelman, 2006). The cell wall of *M. tuberculosis* is very important for its survival within constrained conditions such as those inside of human macrophages. The biosynthesis of the cell wall components involves many important stages and different enzymes that are absent in mammals and could be attractive drug targets (Khasnobis et al., 2002; Brennan & Crick, 2007; Sarkar & Suresh, 2011). Recently, the 2C-methyl-D-erytrol 4-fosphate (MEP) pathway was found (Eoh et al., 2009) as a potential drug target since the end product of the pathway leads to the formation of isoprenoids, which are responsible for the synthesis of several cell wall components (Mahapatra et al., 2005; Anderson et al., 1972).

Peptidoglycan biosynthesis is another source of potential drug targets. For instance, alanine racemase and D-Ala-D-Ala-ligase catalyze the first and second committed steps in bacterial

peptidoglycan biosynthesis, and since these steps are essential for important polymers, they are good drug targets. Both alanine racemase and D-Ala-D-Ala ligase are inhibited by D-cycloserine, a second line anti-TB drug (Strych et al., 2001; Feng & Barletta, 2003).
Another good drug target is the pyridoxal 5'-phosphate containing enzyme Alr that catalyzes the racemization of L-Alanine into D-Alanine, a major component in the biosynthesis of peptidoglycan (LeMagueres et al., 2005).
Arabinogalactan biosynthesis, a novel arabionofuranosyl transferase that catalyzes the addition of the first key arabinofuranosyl redisude of the galactan core, is not sensitive to EMB, but is essential for viability (Sassetti et al., 2003). The ribosyltransferase that catalyzes the first committed step in the synthesis of decaprenyl-phosphoryl-D-arabinose, the lipid donor of mycobacterial d-arabinofuranosyl residues, has also recently been characterized and shown essential for growth (Huang et al., 2005)

2.2 Mycolic acid biosynthesis related targets

Within the mycobacteria lipid metabolism, mycolic acids are essential structural components of the mycobacterial cell wall (Brennan, 2003). The early stage of fatty acid biosynthesis, which generates the precursors of mycolic acids, is a rich source of antibacterial targets (Heath et al., 2001). It is also the site of action of INH and ethionamide (Quemard et al., 1995; Larsen et al., 2002). *M. tuberculosis* has both types of fatty acids synthase (FAS) systems found in nature, FAS-I and FAS-II. FAS I is the system responsible for de novo synthesis of C16-C26 fatty acids and the FAS II system extends these fatty acids up to C56 chains to make precursors of mycolic acids, which are essential for growth.
Since enoil-ACP reductase (InhA) is the target of INH, it is reasonable to assume that all steps in the FAS-II pathway will be essential for the viability of *M. tuberculosis*. Many of the individual enzymes of the FAS-II system have been expressed, purified and characterized (Kremer et al., 2001; Choi et al., 2000; Scardale et al 2001; Benerjee et al 1998; Marrakchi et al.,2002; Marrakchi et al., 2000; Slayden & Barry, 2002).

2.3 Energy production related targets

Isocitrate lyase (ICL) is an important enzyme in this category and also an important drug target. ICL is involved in energy production via the metabolism of acetyl-CoA and propionial CoA of the glyoxilate pathway. Inactivation of the *icl* gene leads to attenuation of both persistent and virulent strains of *M. tuberculosis*. However, *M. tuberculosis* has a salvage pathway, so a suitable anti-TB drug for this target must address both the main and salvage pathways (McKinney et al., 2000; Savi et al., 2008)

2.4 Amino acid biosynthesis related drug targets

Amino acid biosynthesis is another important target for developing anti-TB drugs. The shikimate pathway is very important and is involved in the synthesis of aromatic amino acids in algae, fungi, bacteria, and higher plants; however, it is absent in the mammalian system (Sarkar & Suresh, 2011). The final product of the shikimate pathway, chorismate, is a key biosynthetic intermediate involved in generating aromatic amino acids and other metabolites. The entire pathway is essential in *M. tuberculosis* (Parish & Stoker 2002). This feature makes the pathway an attractive target for developing anti-TB drugs with minimum cross reactivity (Ducati et al., 2007). Other enzymes of this pathway are also likely to be essential, and shikimate dehydrogenase (Magalhaes et al., 2002), and 5-enolpyruvylshikimate 3-phosphate synthase (Oliverira et al., 2001) have been characterized

in detail. The biosynthesis of non-aromatic amino acids is also emerging as a potential drug target. The impact of amino acids such as lysine (Pavelka & Jacobs, 1999), proline, tryptophan and leucine (Smith et al., 2001) is evident from the fact that knocked out *M. tuberculosis* strains of the genes required for amino acid biosynthesis showed less virulence (Pavelka et al., 2003; Smith et al., 2001). Another attractive target of the lysine biosynthesis pathway is the enzyme dihydrodipicolinate reductase, for which potent inhibitors have been identified (Paiva et al., 2001).

2.5 Cofactor-related drug targets

Several cofactor biosynthetic pathways and pathways requiring some cofactors are good candidates for identification of new drug targets. Folate derivatives are cofactors utilized in the biosynthesis of essential molecules including purines, pyrimidines, and amino acids. While bacteria synthesize folate de novo, mammals must assimilate preformed folate derivatives through an active transport system (Mdluli & Spigelman, 2006). Dihydrofolate reductase, which catalyses the reduction of dihydrofolate to tetrahydrofolate, a key enzyme in folate utilization whose inhibition may affect the growth of *M. tuberculosis* (Gerum et al., 2002), and dehydropteroate synthase are validated targets of the widely used antibacterial sulfonamide, trimethoprim (Huovinen et al., 1995).

Two enzymes involved in the de novo biosynthesis of NAD that affects the $NADH/NAD^+$ ratio upon which *M. tuberculosis* is dependent, have been studied as possible drug targets (Bellinzoni et al., 2002). Genomic analysis studies have suggested that the riboflavin biosynthesis pathway is essential in *M. tuberculosis* (Morgunova et al., 2005) and the lumazine synthase pathway has been validated as a target for anti-TB drug discovery.

2.6 DNA metabolism

Differences in mammalian and mycobacterial thymidin monophosphate kinase have been studied and exploited in an attempt to find selective inhibitors for this drug target (Haouz et al., 2003; Vanheusden et al., 2002). Other targets are ribonucleotide reductases that catalyze the first committed step in DNA synthesis and have differences with corresponding mammalian enzymes (Yang et al., 1994; Yang et al., 1997); DNA ligases, that play an important role in the replication and repair of DNA, are classified as NAD^+ or ATP dependent. NAD^+ dependent ligases are only found in some viruses and eubacteria (Mdluli & Spigelman, 2006). LigA is essential for growth of *M. tuberculosis* (Gong et al., 2004) and inhibitors that distinguish between the two types of ligases and have anti-TB activity have been identified (Srivastava et al., 2005). DNA gyrase has also been validated as a target for *M. tuberculosis*, since this is the only type II topoisomerase that it possesses (Cole et al., 1998). Its inhibition by fluoroquinolones results in highly mycobactericidal activity.

2.7 Menaquinone biosynthesis

It appears that menaquinone is the only quinone in *M. tuberculosis*, so its biosynthesis is essential for growth. The menaquinone pathway is not present in humans, and bacterial homologues of MenA-E and MenH have been described in *M. tuberculosis*, so this pathway is another promising drug target (Meganathan, 2001).

2.8 Other potential drug targets in *M. tuberculosis*

The tubercle bacillus produces no less than 20 cythochrome p450 enzymes, some of which appear to play essential roles (Cole & Alzari, 2007). Antifungal azole drugs target these

enzymes and the cytochrome p450 homologues in the bacteria. Drugs like miconazole and clotrimazole are active against *M. tuberculosis* (McLean et al., 2007; Ahmad et al., 2006; Sun et al., 1999 TD). Subsequent crystallization studies of the *M. tuberculosis* cytochrome p450 enzyme system evoked studies to evaluate new drugs (Leys et al., 2003).
Peptide deformylase inhibitors may be effective against *M. tuberculosis* since peptide deformylase catalyzes the hydrolytic removal of the B-terminal formyl group from nascent proteins. It is a metalloprotease essential for maturation of nascent polypeptides in bacteria but not essential for humans, making it an attractive target for antibacterial drug development (Teo et al., 2006); however, it has little effect on slow growing TB bacteria (Khasnobis et al., 2002).
Another important set of emerging drug targets are the components of the siderophore biosynthesis of *M. tuberculosis* (Monfeli et al., 2007). Upon infection, as a part of the defense mechanism, the host has several mechanisms to withdraw or control the free extracellular, as well as intracellular, iron concentration (Weinberga & Miklossy, 2008; Ferreras et al., 2005). Mycobacteria have an unusual reliance on serine/threonine protein kinases as the main component of signal transduction pathways (Av-Gay & Everett, 2000), and there is considerable activity around this transduction system since some of these enzymes are essential for growth (Fernandez et al., 2006). *M. tuberculosis* synthesizes mycothiol in a multistep process involving four enzymatic reactions for protection against the damaging effects of reactive oxygen species. This pathway is absent in humans, and it has been shown to be essential to *M. tuberculosis* (Sareen et al., 2003).

3. Rational drug design

One of the design strategies for new anti-TB compounds is based on the development of analogs of first-line and/or second line drugs. In this section we review the strategies employed and analyze structure-activity relationships (SAR), which have led to the development of new anti-TB agents. In addition, we review new pharmacophore groups. One problem that must be considered in the design of anti-TB compounds is that there is a subpopulation of bacteria in a persistent non-replicating state. This is considered a major contributing factor to long drug treatments for TB. For this reason, it is important to determine if compounds have potential activity against these bacteria at the onset of design. We should also consider the physicochemical properties that directly affect the pharmacokinetics and pharmacodynamics of drugs. An example of this is the influence of stereoisomers on biological activity, because individual enantiomers have significant differences in activity, although sometimes the activity of some enantiomers cannot be explained.

3.1 Isoniazid derivatives

One of the strategies frequently used in medicinal chemistry to develop new drugs is "hybridization", a method that has been proposed particularly for new anti-TB drugs. An example is the design of molecules based on INH or PZA, incorporating NR1R2 groups derived from a second anti-TB molecule or possibly other nucleophilic groups to provide anti-TB activity. With special interest compounds 1 and 2 (figure 1) were obtained. These could be considered prodrugs because they contain two conventional drugs that are bound by a CH fragment. Although the results of activity are very similar to those presented by INH and PZA, the hydrolysis of new compounds ensures prolonged release of the active drugs (Imramovsky et al., 2007).

A variety of compounds derived from INH that include mostly a hydrazine fragment have been determined. Following this strategy and considering the inclusion of an oxadiazole moiety, Navarrete et al, developed new agents with high anti-TB activity (3, figure 1). Due to the substitution in 5-position on the oxadiazole ring, the compounds obtained showed high lipophilicity, hypothesizing that this lipophilicity could facilitate passage of these compounds through the *M. tuberculosis* bacterial membrane (Navarrete-Vazquez et al., 2007). Also, structural modification of the hydrazide moiety on INH (4, figure 1) provided lipophilic adaptations of the drug that blocked the N-acetylation process, obtained high levels of *in vitro* activity against *M. tuberculosis* and macrophages infected, as well as low toxicity (Hearn et al., 2009).

Another strategy in drug design is the formation of molecules that mimic the natural substrate of an enzyme. Delaine et al designed a new series of bi-substrate-type inhibitors based on a covalent association between molecules mimicking the INH substrate and the NAD cofactor that could provide compounds with a high affinity and selectivity for the INH catalytic site (5 and 6, figure 1). In these compounds, the authors determined that incorporating a lipophilic component into the nicotinamide hemiamidal framework provides more active derivatives (Delaine et al., 2010).

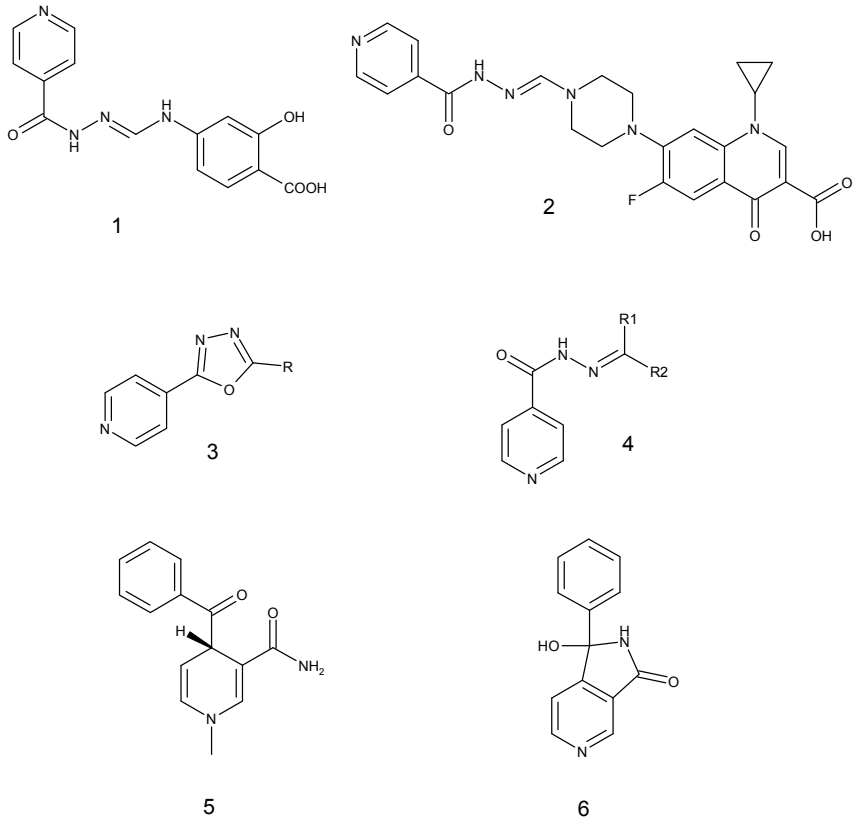


Fig. 1. Structure of compounds derivatives of anti-TB first line drugs.

3.2 Ethambutol derivatives

Amino alcohols that include EMB, which is used for pharmacological TB treatment, are an important class of compounds with various applications. This compound has been widely studied determining that the 1,2-ethylenediamine moiety is the EMB pharmacophore, possibility due to chelate bond formation with divalent metal ions such as copper. Based on EMB, a second-generation agent has been developed, a compound called SQ109 (7, figure 2), which is being tested in clinical trials. It is a drug that exhibits potent activity against *M. tuberculosis* strains, including multidrug resistant strains *in vitro* and *in vivo*. Unfortunately, SQ109 has poor bioavailability of only 12% and 3.8% in rats and dogs, respectively. Studies indicate that this compound undergoes oxidation, epoxidation and *N*-dealkylation, which cause its low bioavailability; therefore strategies have been designed to improve its bioavailability minimizing this first-pass effect. Prodrugs based on carbamate groups are a good option for reducing this effect. Considering this Meng and colleagues developed a new series of analogues based on carbamate prodrugs of SQ109 (8, figure 2) that provide good chemical stability as substrates of plasma esterase. The results of bioavailability of these compounds show a five-fold increase of the SQ109 reference compound (Meng et al., 2009). Alternatively, Zhang has carried out the synthesis of new analogues of S2824 (9, figure 2), a second-generation compound derived from EMB. The results show that new analogues with a homopiperazine ring (10, figure 2) have high *in vitro* activity against both sensitive and drug-resistant *M. tuberculosis* strains (Zhang et al., 2009).

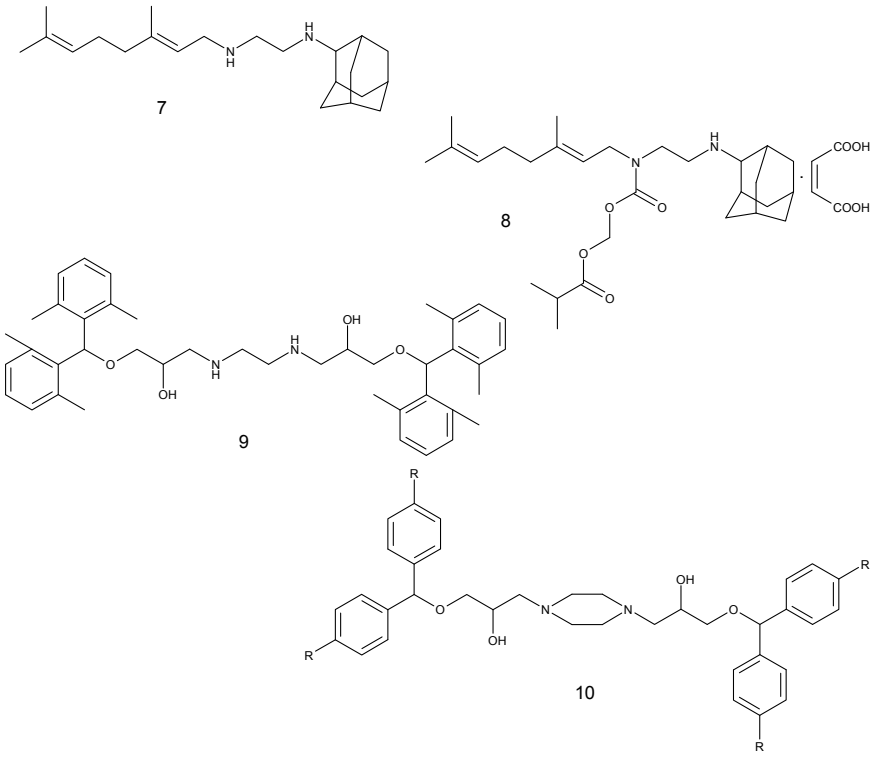


Fig. 2. Structure of SQ109 and analogs.

In the design of new 1,2-diamine derivatives (11, figure 3) compounds with 35 times more activity than EMB have been synthesized. Interestingly, studies show that they do not have the same target as EMB. An SAR analysis has determined that the presence of an β-hydroxy group on the amine increases anti-TB activity; however, the distance between oxygen and nitrogen atoms in EMB are the same as between both atoms in the hydroxyethylamine suggesting a good relationship between both structures (12, figure 3). In a new series of EMB analogs obtained by Cunico et al, it was determined that the sulfonamide moiety reduces activity against *M. tuberculosis*, and that the amino alcohol moiety on hidroxyethylsulfonamide is crucial for anti-TB activity, where the presence of a carbamate moiety leads to a loss of activity. Consistent with this, it has been reported that if compounds lose the basicity of the amino group (12, figure 3), this results in a loss of activity (Cunico et al., 2011). Finally, EMB has served as a proposal for tripartite hybridization (chloroquine, isoxyl and ethambutol) for the development of new anti-TB agents (13, figure 3), which exhibit high activity against *M. tuberculosis* (Nava-Zuazo et al., 2010).

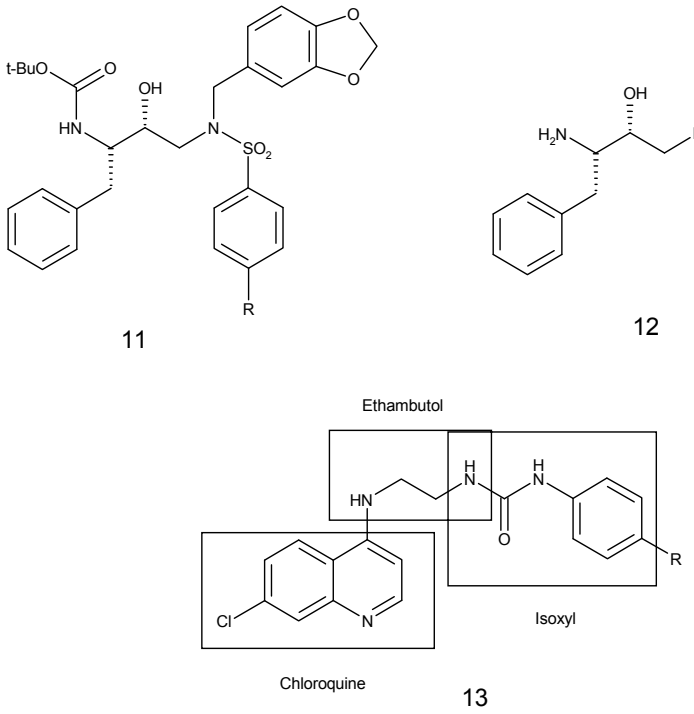


Fig. 3. Ethambutol analogs as anti-TB agents.

3.3 Salicylanilides derivatives

salicylanilides (SAL) derivatives have been of great interest in medicinal chemistry, although their mechanism of action still unknown. It is postulated that they serve as epidermal growth factor receptor protein kinase (EGFR PTK) inhibitors. Such compounds have generally been designed to compete with adenosine triphosphate (ATP) in binding

with the catalytic domain of tyrosine kinase. Recent studies specify that selective inhibitors of interleukin-12p40 production also have a specific role in the initiation, expansion, and control of the cellular response to TB. Following the development of SAL derivatives, Imramovskỳ´s group obtained a series of compounds (14, figure 4) with activity similar to INH. Through a SAR study, they established that positions R1 and R2 showed Cl and Br atoms that are necessary for high activity against TB and that the benzyl and isopropyl substituent at R3 increases activity (Imramovskỳ et al., 2009).

In addition, in various SAL derivatives that have been developed it has been shown that electron withdrawing groups on the salicyloyl ring and hydrophobic groups on the anilide ring, as well as the 2-hydroxy group, are essential for optimal antimicrobial effect. Halogen-substituted SAL in both parties maintains the requirements and forms of more active derivatives that show anti-TB activity. However, its unsuitable physical properties led to the generation of prodrugs of SAL derivatives with better bioavailability, and due to a high degree of lipophilicity, more efficient transport through *M. tuberculosis* cell membranes. Considering this, Imramovskỳ and colleagues obtained compounds (15, figure 4) with interesting activity against *M. tuberculosis*. They showed a level of inhibition of 89%-99%and an MIC of 3.13 μg/mL. Although, they demonstrated that lipophilicity is a secondary parameter in anti-TB activity, they also demonstrated that in these compounds the stereoisomer effect is important for anti-TB activity; however, in this case the difference is not determined for individual R/S isomers (Imramovskỳ et al., 2009).

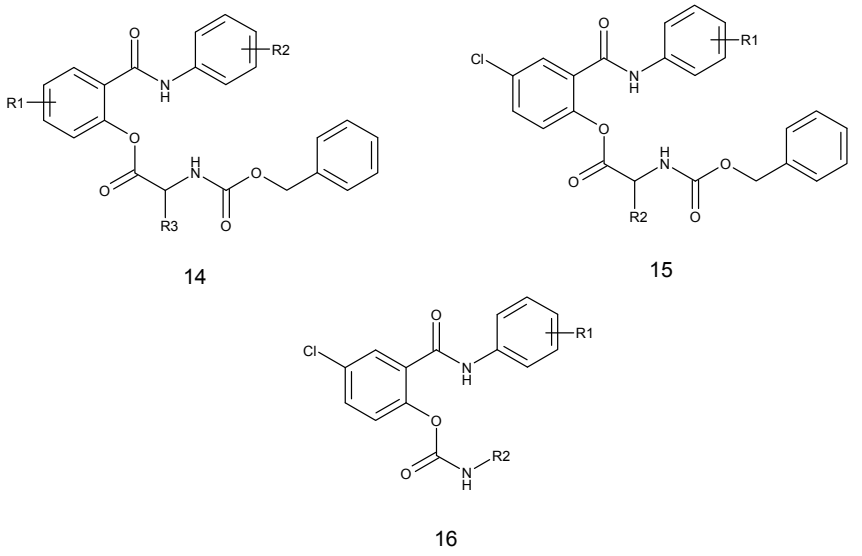
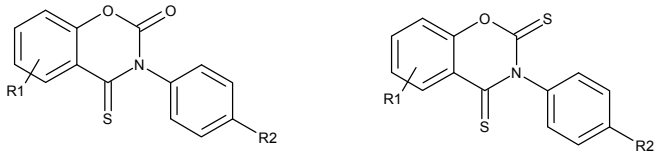


Fig. 4. General structure of salicylanilides derivatives with anti-TB activity.

Using the hybridization strategy, Ferriz et al obtained a new series of derivatives with SAL and carbamate groups, which have been used as antibacterial and antiviral agents. Thus the hybridization of two moieties could produce a new series with changes in their pharmacokinetic and pharmacodynamic properties. Ferriz et al postulated that carbamate could be protecting these molecules against first-pass metabolism, increasing their activity

profile. The series obtained show that Cl atoms at 3 and 4-position on the aniline ring increase *M. tuberculosis* biological activity. Interestingly, the presence of an alkyl chain also increases the biological activity of these compounds, which suggests the importance of carbamate group (16, figure 4). Although these kinds of compounds are consistent with the Lipinski rules, it is speculated that due to their high lipophilicity, these molecules have high permeability, making their release more effective (Ferriz et al., 2009).
Another strategy using SAL derivatives has been the formation of cyclic derivatives, which could serve as antibacterial agents with a dual inhibition system. Thus, following this design strategy a new series of benzoxazinediones derivatives was obtained, where a thioxo group replaced one or two oxo groups. The substitution of an oxo group by the thioxo group (17, figure 5) strongly increased anti-TB activity, although a second substitution with the thioxo group had only a small effect on activity (18, figure 5) (Petrlikova et al., 2010).



17 18

Fig. 5. General structure of 1,3-benzoxazine derivatives.

3.4 Quinoline derivatives

A quinoline ring is one of the moieties frequently used in new drug design. It has been considered a pharmacophore for the design of anti-TB agents. Diarilquinoline, denominated TMC207 (19, figure 6), is an adenosine ATP synthase inhibitor that is one of the most important quinoline derivatives with anti-TB activity. TMC207 is currently in Phase II clinical trials. Also, butanamide has been established as an important pharmacophore with good antibacterial activity and the carbohydrazone moiety is also known as a pharmacophore group. Based on the above, the design of new quinoline derivatives with active carbohydrazine and butanamide moieties in 3 and 4-position, respectively, has been carried out. The SAR study of these compounds shows that the presence of a trifluoromethyl group at 8-position increases activity; however, the introduction of a fluoro group in 6-position partially decreases activity (20, figure 6) considering these type of compounds non-toxic (Eswaran et al., 2009). Following the development of mefloquine analogs (21, figure 6) in a series of compounds (22, figure 6), good anti-TB activity has been attributed to the presence of pharmacologically active heterocyclic groups such as pyrazole, imidazole, and indole rings on the quinoline ring. Surprisingly, compounds with a hetoaromatic pyrazole ring have activity against resistant strains, which can be attributed to the presence of substituents (electron donating groups) that stabilize the pyrazole ring, making the quinoline ring a more active entity (Eswaran et al., 2010).
The conformational restriction-like strategy in flexible drugs is extensively used in medicinal chemistry. This helped determine steric requirements of receptor-drug interaction and identification of new structures with high efficiency and selectivity. Based on this, Goncalves et al studied the conformational restriction of the piperidinyl ring of mefloquine through the construction of an oxazolidine ring and different substituents on the phenyl ring (23, figure 6).

Conformational restriction showed that the introduction of an oxazolidine core in the mefloquine structure enhances anti-TB activity. Although, the activity of these compounds is affected by substituents on the aromatic ring bound to C-17 of the oxazolidenyl nucleus. Compounds that show hydroxyl or methoxyl groups, which are both electron donators and capable of forming strong hydrogen bonds, in general are active. In contrast, with one exception, compounds with nitro or halogenated groups (electron withdrawing groups and capable of forming only weak hydrogen bonds), are inactive (Goncalves et al., 2010). Thus, mefloquine has been used to design anti-TB agents. Modifications in previous reports included introduction of a hydrazone linker into mefloquine at 4-position, substitution of a piperidine with a piperazine ring and extension of the basic terminus of the piperazine ring at 4-position. Additionally, isoxazole is emerging as one of the most powerful hits in high-throughput screening (HTS) against *M. tuberculosis*. Both types of compounds show an aromatic ring, a two-atom linker and a five or six member ring. Hybridization strategies have been the basis for the design of new chemical entities by Mao et al (24, figure 6). One problem that has been detected in this type of compounds is poor penetration of acid derivatives through the *M. tuberculosis* cell wall. It is suggested that these compounds may act as prodrugs when ester derivatives generate acid derivatives (24, figure 6). SAR studies of these compounds show that when a methyl group replaces a trifluoromethyl group, it is 10 times less active, suggesting that electronic effects may play an important role in anti-TB activity. Additionally, steric effects can affect anti-TB activity. Subsequently, making use of drug design strategies, the authors included ester bioisosteres, such as amides and oxadiazole, although none of these bioisosteres showed better activity than ester derivatives. It was determined that 2 and 8-trifluoromethyl groups on quinoline ring (24, figure 6) are essential for anti-TB activity against replicative bacteria (Mao et al., 2009).

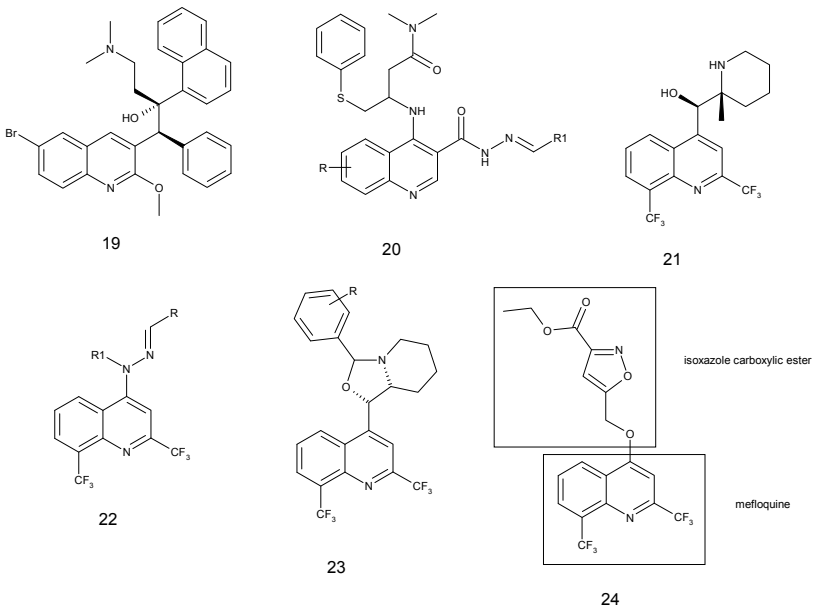
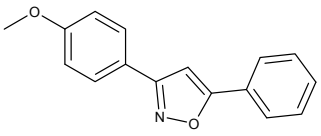


Fig. 6. Quinoline as scaffold for designing new anti-TB agents.

Isoxazole derivatives have also been reported as anti-TB agents, in particular compound 25 (figure 7) with an activity of 2.9 μM, which is comparable to INH and (RIF) (Kini et al., 2009). Thus, quinoline and oxazole ring hybridization has been used to develop a series of new anti-TB agents (26, figure 7) which have good activity due to the presence of aryl substituents at 2-position on quinoline ring. SAR studies show that the introduction of a 1,3-oxazole ring significantly increases activity, obtaining compounds that are more potent than INH (Eswaran et al., 2009). In search of a new moiety that confers anti-TB activity with low cytotoxicity, Yang and colleagues reported methoxybenzofuro[2,3-b]quinoline derivatives (27, figure 7), compounds that have a potent *M. tuberculosis* growth inhibition of 99% at low concentrations (0.20 μg/mL) and very low cytotoxicity against VERO cells with an Inhibitor concentration 50 (IC50) value of > 30.00 μg/mL (Yang et al., 2009).

Several studies have analyzed modifications in the quinolone ring, mainly at 3, 6 and 7-position. Wube et al proposed a new strategy for anti-TB agent development. They made a modification in the 2-position, including an aliphatic side chain with various degrees of unsaturation, lengths chains, and double bond positions (28, figure 7). Their results showed that increasing the chain length enhances anti-TB activity, showing optimal activity with 14 C atoms. If there is an increase of more carbon atoms in the chain, activity decreases dramatically. This behavior has also been described for ciprofloxacin derivatives where lipophilicity could play an important role in anti-TB activity. Other research has determined that the saturated aliphatic chain has less activity than unsaturated analogues. This means that unsaturation of an aliphatic chain is an essential structure for *in vitro* anti-TB activity (Wube et al., 2010).



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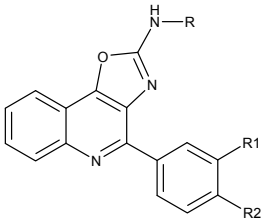

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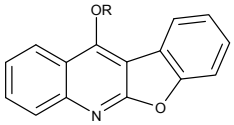

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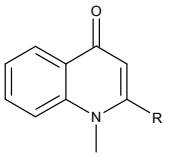

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Fig. 7. Quinoline and oxazole derivatives as anti-TB agents.

On the other hand, both phenazine and quinoxaline rings are considered bioisosteres of the quinoline ring. In this focus, phenazine derivatives are a class of useful compounds for new anti-TB agent development, particularly Tubermicyn B and Clofazimine (phenazine derivatives). Likewise, De Logu et al developed new agents that show activity (29, figure 8) in a concentration range of 0.19 to 3.12 mg/L against *M. tuberculosis*-resistant clinical isolates. Interestingly, they found that this series of compounds were ineffective in

inhibiting the growth of INH resistant strains. Compounds that had exocyclic groups, which confer different lipophilic and electronic properties, but with a size similar to INH, such as the phenylamide methyl lipophilic group in 4-position, were the most active. In contrast, the same group in 3-position reduced activity 100-fold. Also, phenazine derivatives with electron withdrawing groups in 2 and 3-position have values with similar biological activity. These results show the importance of the arylic moiety as a pharmacophore for phenazinecarboxamide anti-TB agents. While phenazine´s mechanism of action is still unknown, it is hypothesized that it could act as a cellular superoxide bismutase inhibitor. We know that the compound Lomofungin (1-carbomethoxy-5-formyl-4,6,8-trihydroxyphenazine) is capable of inhibiting DNA-dependent RNA polymerase, with both options being possible mechanisms of action of phenazine derivatives (De Logu et al., 2009).
Quinoxalines are compounds with a broad spectrum of biological activities. Quinoxaline-*N*-oxide derivatives are known as *M. tuberculosis* bioreductor agents. In this type of compounds missing *N*-oxide groups have led to the loss of anti-TB activity. In this sense, Monge´s group developed over 500 derivatives of quinoxaline (30, figure 8), demonstrating the importance of this group for generating a new class of anti-TB drugs. Interestingly, this research group determined that the quinoxaline compounds obtained have activity on non-replicating bacteria, which could lead to shorter anti-TB therapies (Vicente et al., 2008).
Finally, a compound denominated ER-2 is a new analogue of quinoline derivatives (31, figure 8) that is a gyrase supercoiling inhibitor that has potency similar to Ciprofloxacin with a minimum inhibitory concentration 90 (MIC90) of 0.5 μg/mL (Sainath et al, 2009).

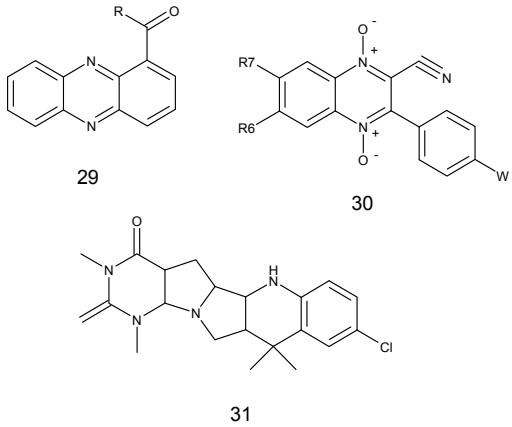

Fig. 8. General structure of phenazine-1-carboxamides, quinoline and quinoxaline derivatives.

3.5 Azoles derivatives

One of the most important strategies for effective anti-TB agent design has been the development of cell wall biosynthesis inhibitors. Azole derivatives have shown interesting anti-TB antimicrobial activity, inhibiting the bacteria by blocking lipid biosynthesis and/or additional mechanisms. Thus, by hybridization of 1,2,4-triazoles and a thiazole moiety, new anti-TB agents were discovered (32, figure 9). These molecules with a highly electronegative part at the sulfhydryl groups have emerged as new anti-TB compounds. Particularly, Schiff

bases derivatives probably due to its ability to increase penetration into the bacterial cell (Shiradkar et al, 2006).

Benzimidazole is an important pharmacophore in drug discovery. Gill et al propose 1,2,3-triazole and benzimidazole ring hybridization as design strategies of new anti-TB agents. They have also considered the use of electron withdrawing groups in the benzimidazole ring, which are present in molecules with anti-TB activity. They obtained compound 33 (figure 9) that could be considered a lead series. Their optimization led to determine that substitutions with electron withdrawing groups produce a loss of anti-TB activity (Gill et al., 2008). A new strategy of hybridization between benzimidazole and a 1,2,4-triazole ring has obtained a series of compounds (34, figure 9). Using a SAR study, it was determined that these compounds enhance biological activity by increasing electronegativity of the molecule, but surprisingly when a trifluoromethyl group (high electronegativity) was introduced, it produced a substantial loss of activity, which could be due to a delay in intracellular transport (Jadhav et al., 2009). Following with the use of a benzimidazole ring as a drug design, Klimešova and cols replaced a nitrogen atom with a corresponding oxygen atom (isosteric) (35, figure 9) to obtain a series of benzylsulfanyl benzoxazole derivatives. They consider alkylsulfanyl derivatives of pyridine, benzimidazole and tetrazole as new anti-TB agents, which present anti-TB activity due to the presence of the alkylsulfanyl group bound to an electron deficient carbon atom in the heterocycle ring. Thus, a SAR study of these compounds indicates that anti-TB activity is attributed to the presence of a benzyl moiety at 2-position on the benzoxazole ring, denoting that anti-TB activity is not affected by electron withdrawing or electron donating susbstituents on the benzyl moiety. It is important to note that the presence of two nitro groups on benzyl led to the most active compound (MIC 2 μmol/L), which may be related to compounds such as PA-824 and OPC-67683, that also show nitro groups. Research postulated as a mechanism of action the generation of active species that act on biochemical targets. Additionally, regression coefficient values for log P show that anti-TB activity increases when lipophilicity decreases (Klimesova et al., 2008).

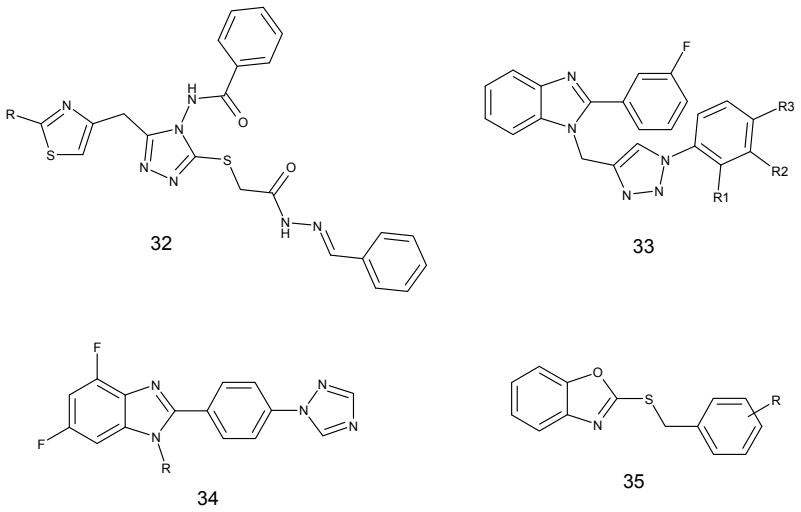

Fig. 9. Triazole and benzimidazole scaffold for designing new anti-TB agents.

Another strategy using 1,2,4-triazole and 1,3,4-thiadiazole rings, led to the development of new anti-TB agents (36, figure 10). Guzeldemerci et al obtained compounds that inhibit 90% *M. tuberculosis* with a concentration greater than 6.25 µg/mL. In addition, the benzothiazole moiety has been recognized for anti-TB design. Both benzothiazole and 1,2,4-triazole moiety were considered to obtain new structures based on hybridization (37, figure 10). Benzothiazole derivatives with 4-methoxy groups showed the best anti-TB activity; however, compounds obtained by hybridization with the 1,2,4-triazole-benzothiazole moiety with the best activity were those with an electron withdrawing substituent (Cl) on the benzothiazole ring (Patel et al., 2010).
Another moiety considered in anti-TB agent design has been isopropylthiazole. Based on this a series of isopropylthiazole derived triazolothiadiazoles, triazolothidiazines derivatives, and mannich bases were developed. The SAR study of the thiadiazoles series (38, figure 10) shows that these compounds have excellent activity against *M. tuberculosis* when they have fluorinated (highly electronegative) substituents that increase molecule lipophilicity, producing hydrophobic molecule interactions with specific binding sites on either receptors or enzymes (Suresh Kumar et al., 2010).

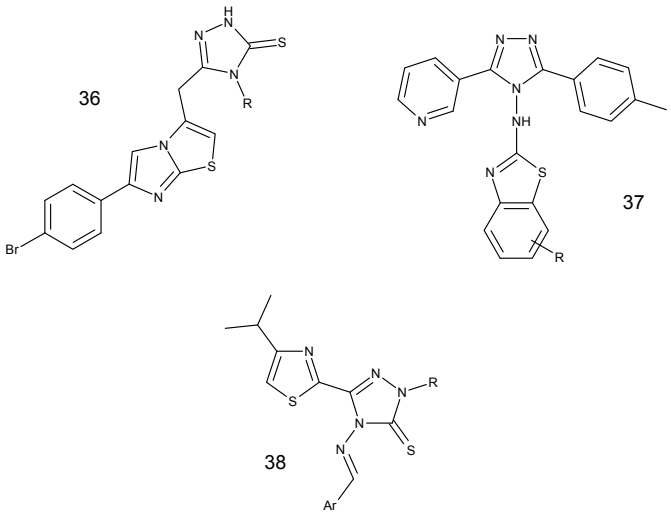

Fig. 10. Triazole derivatives as anti-TB agents.

One of the strategies employed in the development of new drugs is *in silico* screening based on drug structure, structural data of protein and a virtual library of compounds. With this strategy Izumizono et al identified 5 classes of compounds that have an affinity for the active site of enoyl-acyl carrier protein reductase. They determined that these compounds have a structural skeleton of dibenzofuran, acetoamide, triazole, furyl and methoxy phenyl groups (figure 11) that completely inhibit *M vanbaalenii* growth with no toxic effect on mammalian cells. Binding mode prediction determined that compounds 39, 40 and 41 form common hydrogen bonds with amino acid Lys 165 of the active site of the reductase protein. Lys 165 is an amino acid residue that is known to form hydrogen bonds with INH. This shows that hydrogen bond formation with Lys 165 tends to be effective in the design of new drugs. In drug-interaction, the triazole group of compound 39 forms hydrogen binds with

the active side, and the methoxy and sulfonyl groups in compound 40 and the sulfonyl group in compound 41, respectively, form hydrogen bonds with Lys 165 (Izumizono et al., 2011).

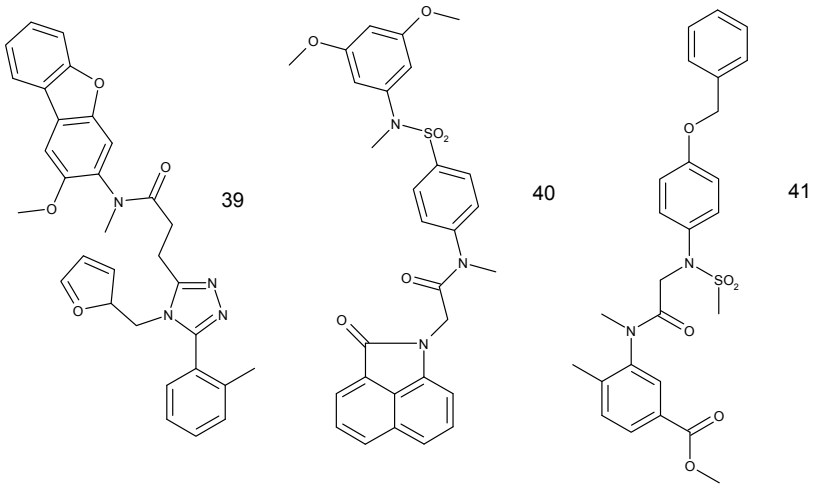

Fig. 11. Dibenzofurane, triazole, methylphenyl and acetamide moiety in compounds with anti-TB activity.

Other derivatives of azoles are pyrazoles. Their activity has been tested against *M. tuberculosis*. SAR studies show that the presence of a *para*-chlorobenzoyl moiety in C4-position on the pyrazole ring is essential for anti-TB activity. Results of a series of pyrazole derivatives, generally show that cyclohexylthio substituted pyrazole derivatives are more active than arylthio substituted systems. An excellent activity is presented when a *para*-nitrophenilthio ring is incorporated on a pyrazole ring (42, figure 12) (Manikannan et al, 2010).

Thiazoles are compounds that contains sulfur and nitrogen atom in its structure, and have been the basis of clinically used compounds. Therefore Samadhiya and colleagues consider it a basis of anti-TB agent design. In one study, which synthesized a series of new thiazoles (43, figure 12), it was demonstrated through SAR analysis that compounds with nitro groups show greater biological activity on *M. tuberculosis* than compounds with Cl and Br atoms, although these derivatives (Cl and Br) have better activity than other compounds. Finally, they found that the activity of the compound depends on the nature of the substituent groups (electron withdrawing) with the following sequence NO_2 > Cl > Br > OCH_3 < OH > CH_3 (Samdhiya et al., 2010).

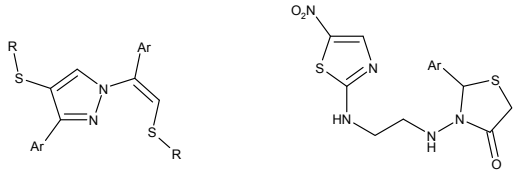

Fig. 12. General structure of pyrazoles and thiazoles derivatives as anti-TB agents.

On the other hand, hybridization of Spiro compound and pyrrolo[2,1-b]thiazole, an unusual ring with different biological properties, particularly permitted the obtention of pyrrolothiazoles derivatives (44, figure 13) that present a MIC of 0.007 μM against *M. tuberculosis*, being more potent than INH and Ciprofloxacin (Karthikeyan et al., 2010) .

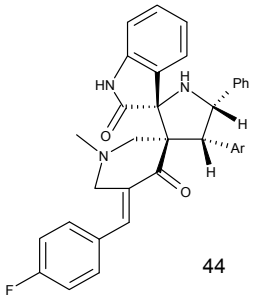

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Fig. 13. Spiro-pyrrolothiazoles derivative with anti-TB activity.

3.6 Hydrazides/hydrazones derivatives

Hydrazide/hydrazone is a class of compounds that have been considered for new anti-TB drug design. An example is diflunisal, a hydrazide/hydrazone derivative, which has dual effect acting with antimicrobial/anti-inflammatory properties. Furthermore, in thiazolylhydrazone derivatives, SAR studies have found that substitutions on the phenyl ring affect anti-TB activity (45, figure 14) (Turan-Zitouni et al., 2008). Another example of a thiazolylhydrazine is compound 46 (figure 14), which has high anti-TB activity with a IC50 of 6.22 μg/mL and low toxicity (CC50> 40 μg/mL). Here, a pyridyl moiety plays a direct role related to anti-TB activity (Turan-Zitouni et al, 2010). Pyridine is a moiety known in the design of anti-TB agents. Considering this, and using hybridization technique, Sankar et al developed a series of compounds with potential anti-TB activity (47, figure 14), although in many cases as this, the use of this technique did not produce any agent with excellent activity against *M. tuberculosis* (Sankar et al, 2010).

New designs have been made by molecular hybridization of E-cinamic acid and guanylhydrazones. Based on an empirical analysis of SAR, Bairwa and colleagues determined that electronic and steric parameters have an important role in the activity of these compounds on *M. tuberculosis* (48, figure 14).They remain the basis of new anti-TB agents (Bairwa et al, 2010).

3.7 Nitrogen heterocyclic derivatives

Purines are an important group in the design of anti-TB agents. In these compounds (49, figure 15), activity depends on the substituents present in C2, C6 and N9 of the purine ring (Correia et al., 2009). In 6,9-disubtituted purine derivatives, activity increases substantially when a Cl atom is introduced in the 2-position. Interestingly, purine derivatives with thienyl substituents exhibit better activity in non-replicating bacteria, although in these compounds a Cl atom in 2-position is not beneficial for activity. Additionally, it has been determined that purine N-9 is important for activity, in the case of purine C-8, an atom can be exchanged without losing activity and a change in purine N-7 results in a loss of activity, although there are 7-deazapurines derivatives (50, figure 15) that could be compared with RIF (Khoje et al., 2010).

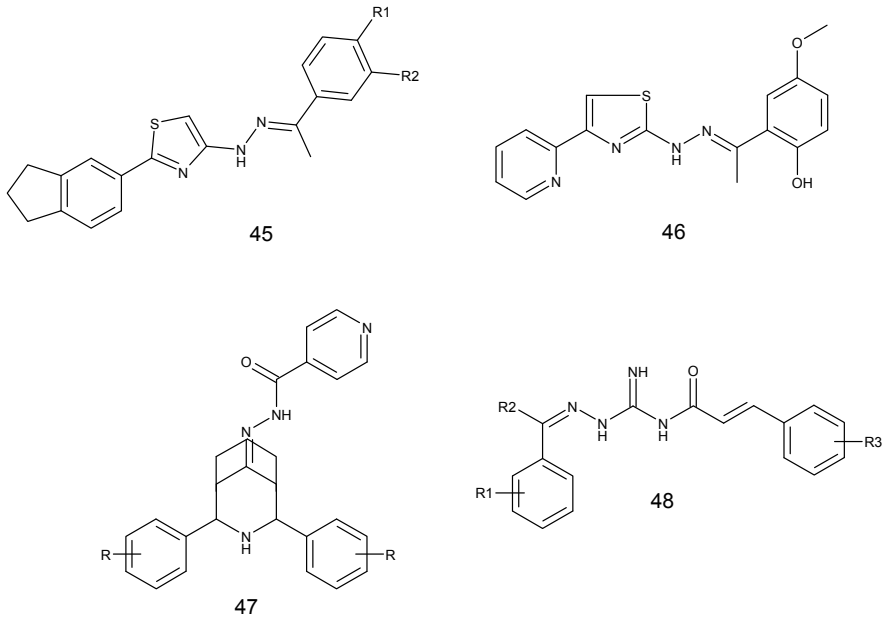


Fig. 14. Hydrazone derivatives as anti-TB agents.

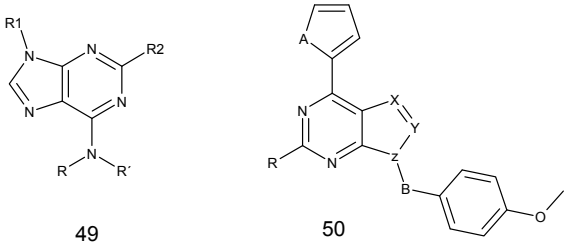
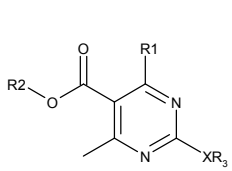


Fig. 15. Purine derivatives as anti-TB agents.

Heterocycles with one nitrogen atom, especially pyrimidines have potential therapeutic applications as anti-TB agents, but there are few reports. For this reason, the design of new pyrimidine derivatives is a viable option (51, figure 16). However, neither compound has an activity comparable to reference drugs, although it has been described that the substituent nature in 2-position can modulate cytotoxic activity (Singh et al, 2011).

On the other hand, thymidine monophosphate kinase of *M. tuberculosis* (TMPKmt) is a prominent target for the development of anti-TB drugs. TMPK is the last specific enzyme for dTTP synthesis and is a key enzyme in *M. tuberculosis* metabolism. This enzyme is different from human enzyme analogs (22% homology). TMPK inhibitors have been developed with single or multiple chemical modifications of the pyrimidine moiety and thymidylate sugar. In particular benzyl-thymine derivatives have been remarkable TMPK inhibitors, which has led to the proposal of new modifications such as: chain length in *para*-position on the benzyl ring, saturation of the alkyl chain, functionalization of the chain group and substitution at 5-

position of the core base. This has led to more selective compounds on TMKP that correspond to benzyl-pyrimidines substituted by a chain length of 4 carbons and a terminal carboxylic acid function. Docking of molecule 52 (figure 16) on TMPKmt showed that the hydrogen of the thymine and acid group can interact with Arg95 (Gasse et al., 2008).



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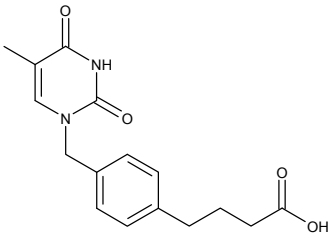
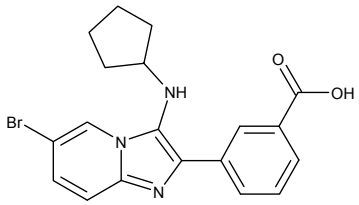


52

Fig. 16. General structure of pyrimidine derivatives as anti-TB agents.

Pyridine derivatives have also been described as anti-TB agents, an example is compound 53 (figure 17), which presents inhibitory activity with an IC50 value of 0.38 μM, suggesting that its possible mechanism of action is through glutamine synthetase inhibition. This would be the first inhibitor compound not derived from amino acids (Odell et al, 2009). Another series of pyridine derivatives were developed by Fassihi et al who synthesized compound 54 (figure 17), a potent anti-TB agent with activity similar to RIF. The results of these compounds showed that an imidazole group as a substituent is equivalent to a nitro phenyl group, which has been reported in anti-TB agents derived from 1,4-dihydropyridinecarboxamides (Fassihi et al., 2009).



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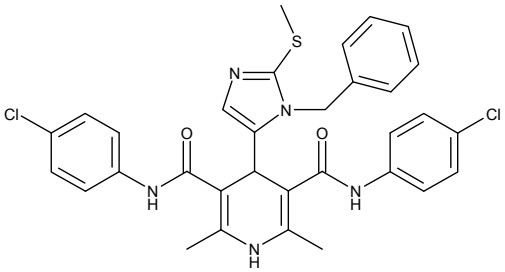

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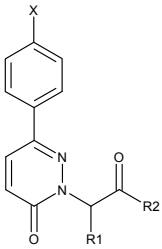
Fig. 17. Pyridine derivatives with potential activityanti-TB.

Another important heterocyclic for the design of anti-TB agents is the pyridazine moiety. In these compounds a relationship between Br, Cl and CH_3 substituents, respectively, with Br and vinyl has been found with a favorable anti-TB activity. In these compounds there is an influence of the substituents X in *para*-position on the aromatic ring, where the activity is increased in the following order: CH_3 < Cl < Br with the activity being affected by the R1 substituents, where the most active compounds have a CH_3 group (55, figure 18) (Mantu et al., 2010).

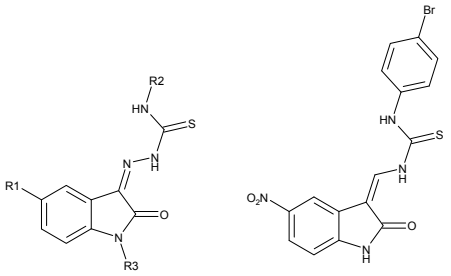


55

Fig. 18. *N*-substituted-pyridazinones derivatives.

3.8 Other derivatives

Several studies indicate that thiosemicarbazone derivatives can be used in TB therapy and prophylaxis. Previous studies of 1H-2-thiosemicarbazoneindolinone derivatives indicate that halogenation of R1, elongation of the alkyl chain in R2, substitutions of the alkyl chain in R2 with cyclohexyl or phenyl, and the presence of a substituent in R3, are more efficient for increasing anti-TB activity, while R1 substitutions with a nitro group produce the most active compounds. The presence of a morpholine ring in Schiff bases substituted in R1 with a nitro group also has a significant impact on anti-TB activity. The results of biological activity of this new series indicate that the elongation of the alkyl chain increases activity. This enhanced activity is related to lipophilicity properties and confirmed by values of Log P compounds. Also, replacement of the alkyl chain in R2 and phenyl unsubstituted ciclohexyl has led to more active compounds (56, figure 19). The absence of substitutions at N1 on the indole ring and increased lipophilicity appear to be responsible for high activity against *M. tuberculosis* (Guzel et al., 2008). An example of thiosemicarbazone-derived compounds that have exhibited important anti-TB activity with an IC50 value of 2.59 uM/mL, is compound 57 (figure 19) (Karali et al., 2007).

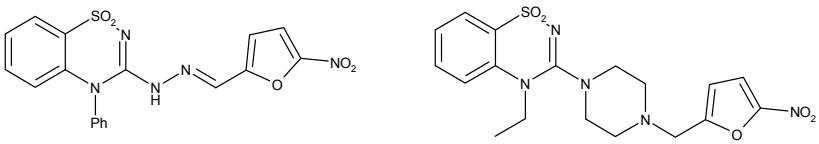


56 57

Fig. 19. General structure of 1H-indole-2,3-dione 3-thiosemicarbazone with anti-TB activity.

Other moieties used in the design of anti-TB agents are phenazine and benzothidiazine. In particular, benzothidiazine 1,1-dioxide constituents are an important class of anti-TB agents (58, figure 20). A SAR study of this series of compounds indicates that the furan/thiophene group linked to benzothidiazine through a methylen bridge exhibits good activity against TB. It is important to point out that a conjugated thiophene derivative shows moderate

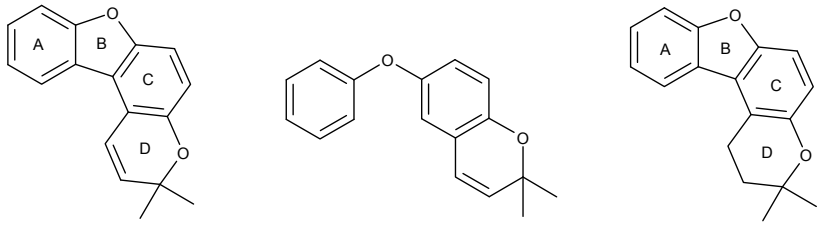
activity and is enhanced when it presents a nitrofuran group. However, elimination of the methylene group with a carbonyl group leads to a dramatic loss of activity. Finally, Kamal et al postulated piperazine-benzothidiazine with methylene linkage (59, figure 20) as an attractive moiety for the design of anti-TB agents (Kamal et al., 2010).



58 59

Fig. 20. Benzothiadiazine derivatives as anti-TB agents.

The creation of a hybrid compound has been a frequent strategy for the design of anti-TB agents. One example is compound 60 (figure 21), formed from dibenzofuran and 2,2-dimethylpyran subunits. SAR studies and modifications of benzofurobenzopyran have demonstrated less active compounds such as compound 61, where the furan B ring is replaced by an ether linker, a single carbon-carbon bond, a carbonyl group, a hydroxymethylene or a methylene group. Even modifications such as acylation and bromination in 5-position on the C ring have produced inactive compounds, thus, it has been suggested as a basis for the pharmacophore structure of compound 60. In this sense, Termenzi et al has carried out the synthesis of more derivatives of compound 60, finding that substitutions with a hydroxy, methoxy, or halogen group on benzofurobenzopyran increases anti-TB activity. Although, hydroxy compounds with good activity showed, unfortunately, cytotoxic activity on VERO cells. Halogenated compounds with a Cl or Br atom in 8, 9 and 11-position, exhibit increased potency compared with compound 60. SAR analysis shows that electronic effects of substituents on the A ring play a dramatic role in anti-TB activity. In addition, potency was significantly decreased when the A ring was substituted by an electron withdrawing group. In contrast, electron donating group substitutions such as hydroxy or methoxy show a significant increase in activity (62, figure 21). While all compounds showed a possible mechanism of action of interaction with lipid biosynthesis of the *M. tuberculosis* cell wall, a specific compound was an epoxy-mycolate synthesis inhibitor (Termentzi et al., 2010).

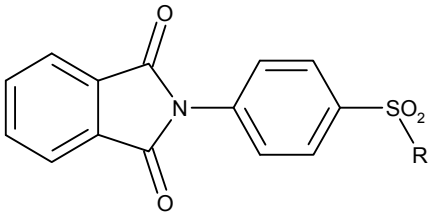


60 61 62

Fig. 21. Structure of benzofurobenzopyrane as anti-TB agents.

Other compounds containing a phthalimide moiety have been described as biophoro to design new prototypes of drug candidates with different biological activities. It has been

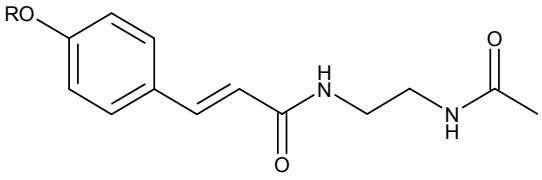
shown that hybridization of both phthalimide (Thalidomide) and sulfonamide (Dapsone) moiety leads to compounds with activity against *M. leprae*. In this sense, the design of new products such as anti-TB agents is interesting. SAR study of a series of derivatives showed that if the pyrimidine ring is substituted in any position or changed by an isosteric, this decreases activity on *M. tuberculosis*. Amino group substitutions by another phthalimide ring also lead to a decrease in anti-TB activity (63, figure 22). Modifications in the pyridine ring decrease anti-TB activity. Introduction of a phthalimide group by molecular hybridization did not produce compounds with an activity similar to INH; however, it allows for compounds with MIC values similar to PZA (Santos et al., 2009).



63

Fig. 22. Phthalimide derivatives as anti-TB agent.

Among families of compounds that act as inhibitors of the FAS-II system we can mention diphenyl ether systems that interact with enzyme-cofactor binary complex, but, recently new compounds such as indols, benzofuran and cinnamic acid derivatives have been reported. Development of new cinnamic acid derivatives would focus on more specific FAS-II inhibitors. From a series of compounds developed (figure 23) it was determined that addition of an alkyl chain increases anti-TB activity. The best results are associated with an acceptable lipophilicity parameter that appears when a geranyl chain is incorporated. This led to compound 64, the most active substance with an MIC of 0.1 µg/mL (Yoya et al., 2009).



64

Fig. 23. Cinnamic derivatives.

It has also been shown that amide derivatives of fatty acids have anti-TB activity. Due to their nature these compounds are designed to penetrate bacterial cells, which can be useful for studying the mechanism of INH resistance as this can also be due to factors such as mutations in unknown genes, decreased permeability, or increased efflux (D´Oca et al., 2010).

4. Drugs in clinical trials

In drug design, bicyclic nitroimidazofurane derivatives that have anti-TB activity, such as CGI-17341 (65, figure 24) have been developed; however, this compound is mutagenic. This has led to the development of PA-824 (66, figure 24), which is currently in phase II clinical studies and has a long half-life. Its mechanism of action is to inhibit *M. tuberculosis* cell wall lipids and protein synthesis; however, it also inhibits non-replicating bacteria. Additionally, it was reported that PA-824 is a prodrug that is metabolized by *M. tuberculosis* before exercising its effect and may involve bioreduction of aromatic nitro groups to generate a radical intermediate nitro.

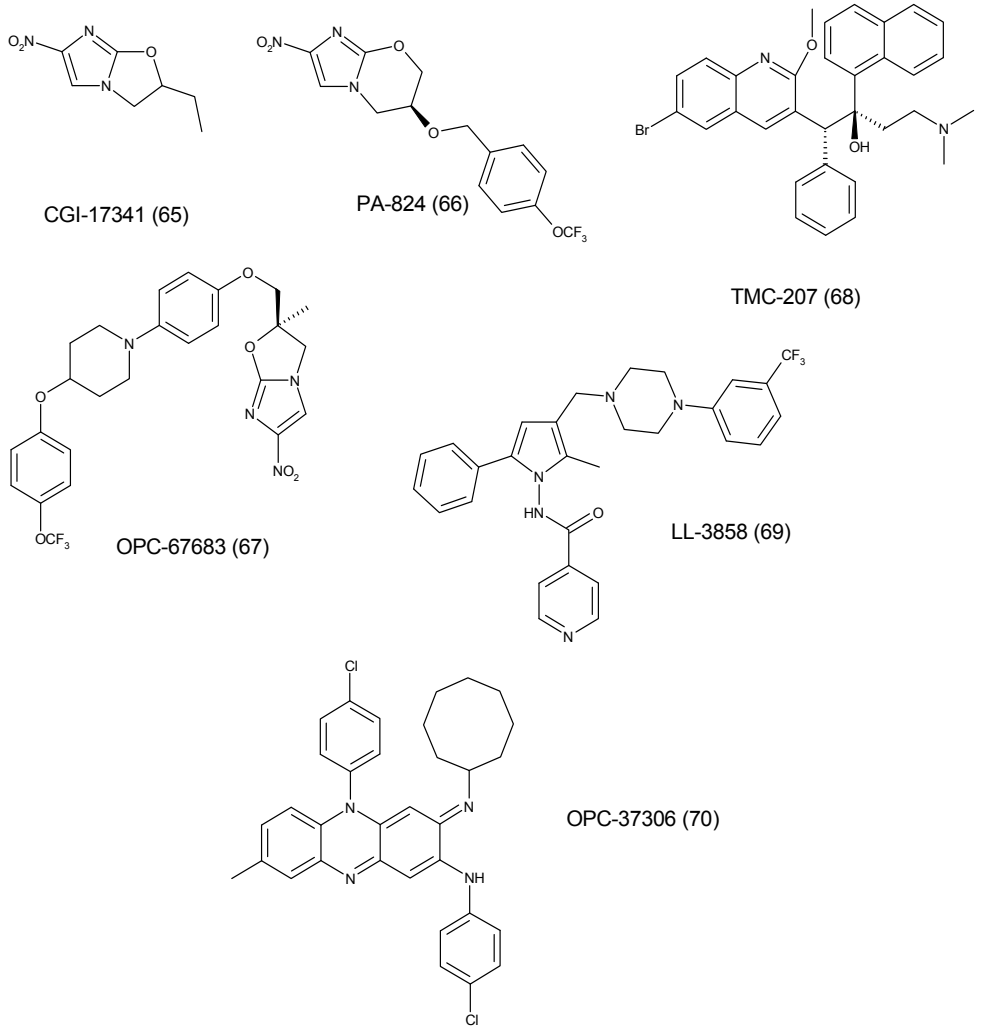


Fig. 24. Anti-TB compounds in clinical trials.

The interest in derived oxazoles as anti-TB compounds led to the development of OPC-67683 (67, figure 24), which has excellent activity *in vitro* in sensitive and resistant *M. tuberculosis* strains. It has a long half-life and its mechanism of action involves inhibition of the synthesis of keto-mycolic, and methoxy-mycolic acid, although is possible another possible mechanism of action or interaction with another drug target in *M. tuberculosis*. OPC-67683 also acts as a prodrug, since *M. tuberculosis* metabolizes it and produces as a product desnitro-imidazooxazole metabolite.

TMC207 (68, figure 25) is a quinoline derivative with potent anti-TB activity in susceptible, DR and XDR strains. It is well absorbed in humans with a long half-life and is currently in phase II clinical studies. Its mechanism of action involves inhibition of ATP synthase that binds the *M. tuberculosis* membrane and there is a synergistic effect between TMC207 and PZA. Other compounds with very promising anti-TB activity are LL-3858 and OPC-37306 (69 and 70, figure 25) (Rivers et al., 2008). Some other examples of anti-TB compounds in clinical trials are showed in table 1 (Janin, 2007; Palomino et al., 2009; Shi & Sugawara, 2010)

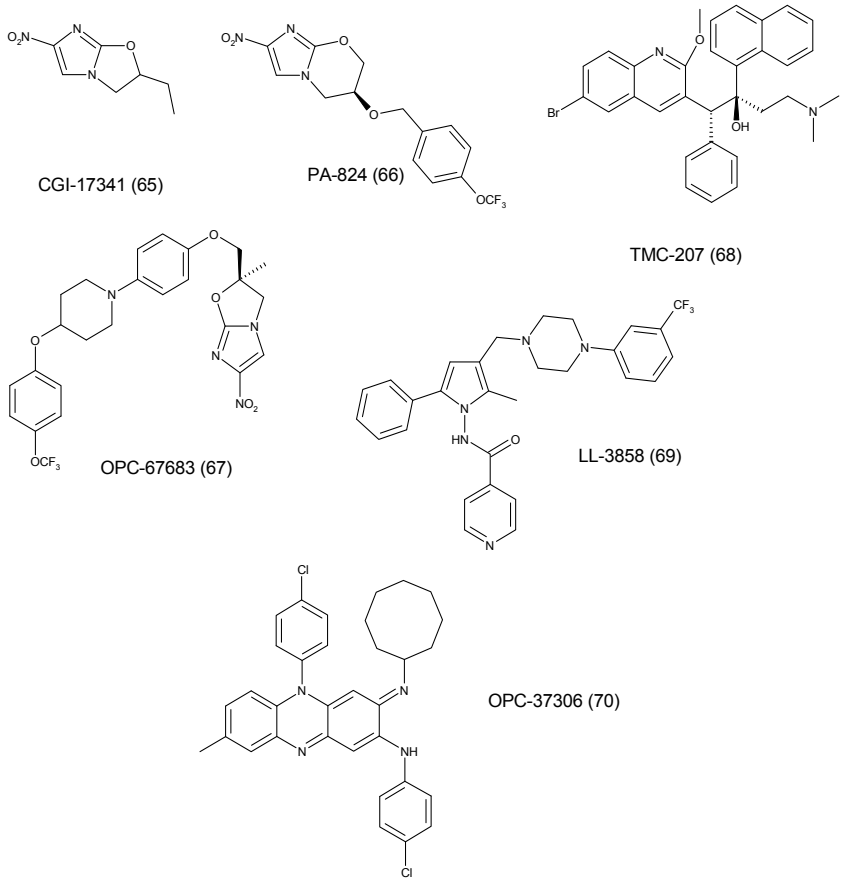


Fig. 25. Anti TB compounds in clinical trials.

Compound	Funding	Target	Mechanism of action	Resistance mechanisms	Clinical trial phase
a) Nitroimidazoxacines					
PA-824	GATB	F420 dependent nitroreductase	Inhibition of proteins and cell wall biosynthesis	Rv0407, Rv3547, Rv3261 and Rv3262 mutations	II
OPC-67683	Otsuka	Nitroreductase	Inhibition of mycolic acid and cell wall biosynthesis	Rv3547 mutations	II
b) Fluoroquinolones					
Moxifloxacin	Bayer, CDC, NIH, FDA	DNA girase	Inhibition of DNA biosynthesis	*gyrA* mutations	III
Gatifloxacine	NIH	DNA girase	Inhibition of DNA biosynthesis	*gyrA* mutations	III
c) Diarilquinolines					
TMC207	Tibotec	F1F0 ATP sintetase	Inhibition of ATP synthesis and disruption of membrane potential	*atp*E mutations	II
d) Oxazolidinones					
Linezolid	NIH, Pfizer	Ribosome	Inhibition of protein biosynthesis	rRNA 23S mutations	Pre-trial
e) Dietilamins					
SQ109	Sequella	Un known	Inhibition of cell wall biosynthesis	Unknown	I/II
f) Pirrols					
LL3858	Lupin	Unknown	Unknown	Unknown	I

Table 1. Some compounds under clinical trials

5. Conclusion

Tuberculosis remains the leading infectious disease worldwide, despite the availability of TB chemotherapy and the BCG vaccine. This is further demonstrated by the fact that half a year of treatment with multiple drugs is needed. Recent genetic and genomic tools as well as high-throughput screening, and structure-based drug design strategies have allowed the discovery of new anti-TB drugs. These are increasingly receiving more attention, and a large number of new compounds or derivatives from existing drugs are under investigation. With this and a better understanding of the unique biology of TB, more targets will be validated, and hopefully a pattern will emerge that will help us reach the goals of more potent compounds that allow multiple stages and drug targets to be addressed.

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10

Insight into the Key Structural Features of Potent Enoyl Acyl Carrier Protein Reductase Inhibitors Based on Computer Aided Molecular Design

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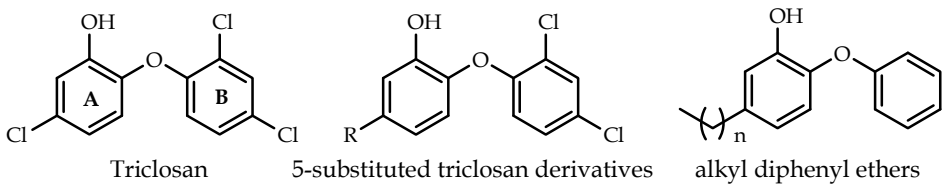
1. Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is one of the leading reason of mortality and is still spread worldwide, indicated by more than 9 million incident cases of TB in 2009 (World Health Organization, 2010). Current standard treatment regimens of TB are severely hampered by multidrug resistant tuberculosis (MDR-TB), extensively drug-resistant tuberculosis (XDR-TB) and HIV co-infection with TB (WHO, 2010). This fact prompts the research to develop novel and more potent drug candidates to treat *M. tuberculosis* strains resistant to existing drugs. The enoyl acyl carrier protein reductase (InhA) of *M. tuberculosis* catalyzing the NADH-specific reduction of 2-trans-enoyl-ACP (Quemard et al., 1995) is an attractive target for designing novel antibacterial agents (Campbell et al., 2001; Heath et al., 2004; White et al., 2005; Zhang et al., 2004; Wen et al., 2009; Wright et al., 2007). InhA has been identified as the primary target of isoniazid (INH), one of the most effective first-line anti-TB drugs (Rozwarski et al., 1998; Vilcheze et al., 2006; Dessen et al., 1995; Lei et al., 2000; Johnsson et al., 1995; Quemard et al., 1996). InhA is inhibited by the active adduct of INH (INH-NAD) (Timmins et al., 2006; Johnsson et al., 1997) which is covalently formed between NAD+ and the reactive acyl radical of INH generated by the activation of catalase-peroxidase (KatG) (Saint-Joanis et al., 1999; Zhao et al., 2006; Metcalfe et al., 2008; Sinha et al., 1983; Nguyen et al., 2001; Heym et al., 1993; Johnsson et al., 1994). The mutations in KatG have been linked to the major mechanism of INH resistance (de la Iglesia et al., 2006; Banerjee et al., 1994). To overcome the INH resistance associated with mutations in KatG, compounds that directly inhibit the InhA enzyme without requiring activation of KatG have been developed as new promising agents against tuberculosis (Freundlich et al., 2009; am Ende et al., 2008; Boyne et al., 2007; Sullivan et al., 2006; He et al., 2006; He et al., 2007; Kuo et al., 2003). Triclosan, 5-chloro-2-(2,4-

dichlorophenoxy)phenol as shown in Fig.1, has been shown to inhibit InhA without the requirement for KatG-mediated activation (Parikh et al., 2000; Kuo et al., 2003). Because of the remarkable properties of triclosan, a series of triclosan derivatives with modifications at the 5-chloro of triclosan, 5-substituted triclosan derivatives shown in Fig.1, was synthesized in order to optimize the potency of triclosan against InhA (Freundlich et al., 2009). Furthermore, using structure-based drug design, three lipophilic chlorine atoms of triclosan were removed, and one chlorine atom of ring A was replaced by an alkyl chain of varying length resulting in the alkyl diphenyl ethers shown in Fig. 1 (Sullivan et al., 2006). The most efficacious triclosan derivatives in the two classes of 5-substituted triclosan and alkyl diphenyl ether derivatives are more potent than the parent compound triclosan. Importantly, a subset of these triclosan analogues displays high efficacy against both INH-sensitive and INH-resistant strains of *M. tuberculosis* more than those of isoniazid. Because of the remarkable property of 5-substituted triclosan derivatives and alkyl diphenyl ethers, their structural requirements for a better therapeutic activity against tuberculosis in both cases of drug-sensitive and drug-resistant strains of *M. tuberculosis* are fascinating and need to be thoroughly examined. Therefore, in the present study, a structure based drug design using molecular docking calculations was applied to investigate the important drug-enzyme interactions of 5-substituted triclosan derivatives and the related alkyl diphenyl ethers in the InhA binding pocket. Moreover, approaches based on 2D and 3D QSAR methods, HQSAR (Hologram QSAR), CoMFA (Comparative Molecular Field Analysis) and CoMSIA (Comparative Molecular Similarity Indices Analysis) (Cramer et al., 1998; Klebe et al., 1994; Tong et al., 1998) have been used to elucidate the relationship between the structures and the activities of these compounds. A powerful guideline for designing novel and highly effective antitubercular agents is the consequence of these investigations.



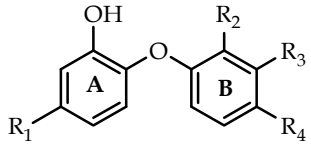
Triclosan 5-substituted triclosan derivatives alkyl diphenyl ethers

Fig. 1. The chemical structures of triclosan and its derivatives

2. Materials and methods of calculations

2.1 Data sets and InhA inhibitory activity

Chemical structures and experimental biological activities expressed as IC_{50} (the half maximal inhibitory concentration) of 17 compounds of 5-substituted triclosan derivatives (Freundlich et al., 2009) and 12 alkyl diphenyl ether derivatives (am Ende et al., 2008; Sullivan et al., 2006) were selected for the present study. All chemical structures of these compounds were constructed using standard tools available in GaussView 3.07 program (Gaussian, Inc., 2006) and were then fully optimized using an *ab initio* quantum chemical method (HF/3-21G) implemented in the Gaussian 03 program (Gaussian, Inc., 2004). The compounds were divided into a training set of 25 compounds and a test set of 4 compounds for the model development and model validation, respectively. The representatives of the test set were manually selected and are covering the utmost range of activity and structural diversity of direct InhA inhibitors in the data set.



Cpd.	R_1	R_2	R_3	R_4	IC_{50} [nM]	Log(1/ IC_{50})
1	Cl	Cl	H	Cl	1100	2.96
2*	CH_3	Cl	H	Cl	800	3.10
3	$CH_2(C_6H_{11})$	Cl	H	Cl	110	3.96
4	CH_2CH_3	Cl	H	Cl	120	3.92
5	$(CH_2)_2CH_3$	Cl	H	Cl	91	4.04
6*	$(CH_2)_3CH_3$	Cl	H	Cl	55	4.26
7	$CH_2CH(CH_3)_2$	Cl	H	Cl	96	4.02
8	$(CH_2)_2CH(CH_3)_2$	Cl	H	Cl	63	4.20
9	$CH_2CH(CH_3)CH_2CH_3$	Cl	H	Cl	130	3.89
10	CH_2(2-pyridyl)	Cl	H	Cl	29	4.54
11	CH_2(3-pyridyl)	Cl	H	Cl	42	4.38
12	CH_2(4-pyridyl)	Cl	H	CN	75	4.12
13	*o*-CH_3-C_6H_5	Cl	H	Cl	1300	2.89
14	*m*-CH_3-C_6H_5	Cl	H	Cl	870	3.06
15	$CH_2C_6H_5$	Cl	H	Cl	51	4.29
16	$(CH_2)_2C_6H_5$	Cl	H	Cl	21	4.68
17	$(CH_2)_3C_6H_5$	Cl	H	Cl	50	4.30
18	$(CH_2)_5CH_3$	H	H	H	11	4.96
19	CH_2CH_3	H	H	H	2000	2.70
20*	$(CH_2)_3CH_3$	H	H	H	80	4.10
21	$(CH_2)_4CH_3$	H	H	H	17	4.77
22	$(CH_2)_7CH_3$	H	H	H	5	5.30
23	$(CH_2)_{13}CH_3$	H	H	H	150	3.82
24*	$(CH_2)_5CH_3$	NO_2	H	H	180	3.74
25	$(CH_2)_5CH_3$	H	NO_2	H	48	4.32
26	$(CH_2)_5CH_3$	H	H	NO_2	90	4.05
27	$(CH_2)_5CH_3$	NH_2	H	H	62	4.21
28	$(CH_2)_5CH_3$	H	NH_2	H	1090	2.96
29	$(CH_2)_5CH_3$	H	H	NH_2	55	4.26

*The test set compounds

Table 1. The chemical structures and IC_{50} values of 5-substituted triclosan and alkyl diphenyl ether derivatives against InhA

2.2 Molecular docking calculations

The X-ray crystal structures of InhA complexed with 2-(2,4-dichlorophenoxy)-5-(2-phenylethyl)phenol (5-substituted triclosan derivative) and 5-octyl-2-phenoxyphenol (alkyl diphenyl ether derivative) with pdb codes of 3FNH and 2B37, respectively, were employed for molecular docking calculations of compounds 1-17 and compounds 18-29, respectively. Docking calculations of the data set were carried out by the Autodock 3.05 program using Lamarckian Genetic Algorithm (LGA) (Morris et al., 1998). Docking parameters were used as default values, except for the number of docking runs which was set to 50. The docking calculation was validated by reproducing the X-ray conformation of the ligand as well as the orientation in its pocket. The root mean-square deviation (RMSD) value between the original and docked coordinates lower than 1Å is acceptable. The ligand pose with the lowest final docked energy was selected as the best binding mode of 5-substituted triclosan and alkyl diphenyl ether derivatives. Then, the conformations of all compounds were used according to this binding mode for CoMFA and CoMSIA setups.

2.3 CoMFA and CoMSIA techniques

CoMFA and CoMSIA, 3D-QSAR methods, are successfully used to derive a correlation between the biological activities of a set of compounds with a special alignment and their three-dimensional descriptors. In both CoMFA and CoMSIA, a set of compounds is aligned and the structurally aligned molecules are represented in terms of fields around the molecule (three-dimensional descriptors). CoMFA and CoMSIA are based on the assumption that changes in biological activities of compounds are related to changes in molecular properties represented by fields around the molecule. Therefore, the structural alignment of compounds is an important prerequisite for the setup of appropriate CoMFA and CoMSIA models. In the present study, the reasonable binding modes of the compounds in the data set obtained from the validated docking calculations were employed for the molecular alignment. SYBYL 8.0 molecular modeling software (Tripos, Inc, 2007) was used to construct CoMFA and CoMSIA models. CoMFA descriptors, steric and electrostatic fields, were calculated using a sp^3 carbon probe atom with a formal charge of +1 which was placed at the intersections in a grid with the spacing of 2Å. The maximum steric and electrostatic energies were truncated at 30 kcal/mol. Five CoMSIA descriptors, steric, electrostatic, hydrophobic, hydrogen bond donor and hydrogen bond acceptor fields, were derived with the same grid as used for the CoMFA field calculation. There are no energy cutoffs necessary for CoMSIA calculations because a distance-dependent Gaussian type potential was used in contrary to the procedure of CoMFA calculations. CoMFA and CoMSIA descriptors were set as independent variables and log ($1/IC_{50}$) values were used as dependent variables in the partial least square (PLS) analysis to derive a linear relationship between molecular descriptors and activities. The cross-validation was performed using the leave-one-out method with a 2.0 kcal/mol column filter to minimize the influence of noisy columns. A final non cross-validated analysis with the optimal number of components was sequentially performed and was then employed to analyze the results. The non-cross-validated correlation coefficient (r^2) and the leave-one-out (LOO) cross-validated correlation coefficient (q^2) were used to evaluate the predictive ability of the CoMFA and CoMSIA models. Contour maps were created to visualize the molecular areas responsible for the biological effects.

2.4 HQSAR

Hologram QSAR (HQSAR) does not require information about the three-dimensional geometry of the inhibitors. Hence, in contrary to CoMFA and CoMSIA methods, HQSAR needs no molecular alignment. Each compound of the data set was converted into all possible molecular fragments including linear, branched, cyclic, and overlapping fragments in the size of 4-7 atoms. Molecular fragment generation utilizes the fragment distinction parameters including atoms (A), bonds (B), connections (C), hydrogen atoms (H), chirality (Ch) as well as hydrogen donor and acceptor properties (DA). The generated molecular fragments are counted in bins of a fixed length array to produce a molecular hologram. PLS statistical method was employed to establish a correlation of the molecular hologram descriptors with the biological data. The HQSAR module of SYBYL 8.0 was employed for the HQSAR study. The same training and test sets as for CoMFA and CoMSIA studies were used. The most convenient model was selected based on the best cross-validated r^2 to determine these structural subunits which are important for the biological activities.

3. Result and discussion

3.1 The X-ray crystal structures of 5-substituted triclosan and alkyl diphenyl ether derivatives

To probe the interaction of 5-substituted triclosan and alkyl diphenyl ether derivatives with InhA, the X-ray crystal structures of these compounds complexed with InhA have been solved (Freundlich et al., 2009; Sullivan et al., 2006). To compare the conformational change of InhA complexed with the different ligands, the ligand-unbound InhA (pdb code 1ENY) and InhA bound with 2-(2,4-dichlorophenoxy)-5-(2-phenylethyl)phenol (pdb code 3FNH) and 5-octyl-2-phenoxyphenol (pdb code 2B37), compounds 16 and 22, respectively, are superimposed as shown in Fig. 2.

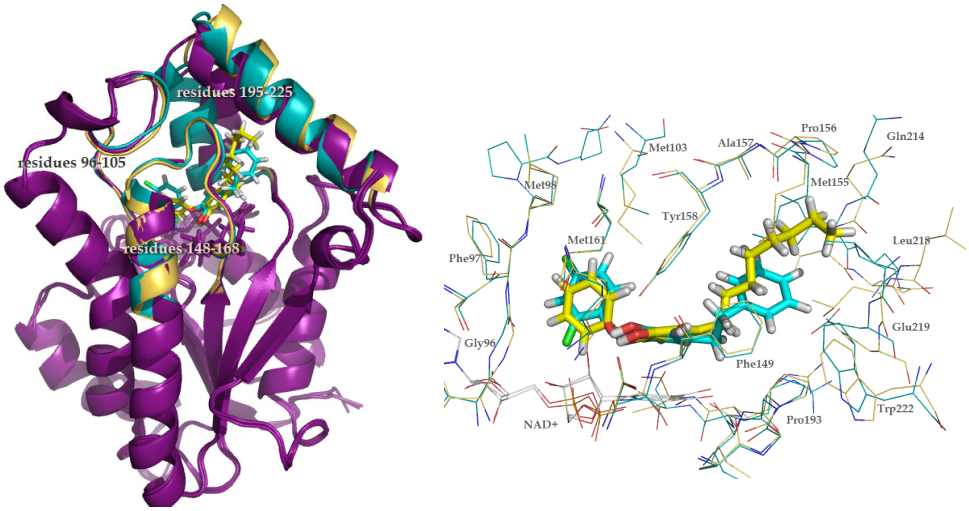


Fig. 2. Superimposition of ligand unbound InhA, InhA bound with compound 16 (cyan) and compound 22 (yellow) with pdb codes of 1ENY, 3FNH and 2B37, respectively. InhAs are colored by purple, whereas residues 96-105, 148-168 and 195-225 complexed with compounds 16 and 22 are colored by cyan and yellow, respectively.

The binding residues within 6Å apart from compounds 16 and 22 consist of residues 96-105, 148-168 and 195-225. As compared with the ligand-unbound InhA, only positions of residues 195-225 including two α-helixes and one loop of the InhA bound with compounds 16 and 22 have been changed to accommodate the binding of two different ligands. On the other hand, the binding residues 96-105 and 148-168 of the InhA bound with these compounds are located in the same position compared to those of the ligand-unbound InhA as shown in Fig. 2. These results imply that residues 195-225 could be sufficiently flexible for the binding of different ligands, whereas residues 96-105 and 148-168 are more rigid, which are consistent with the mobility of these residues investigated by means of molecular dynamics simulations performed in one of our previous investigations (Punkvang et al., 2010). With regard to the binding modes of compounds 16 and 22 in InhA binding site, the B rings of these compounds are bound in a similar orientation and buried with the rigid residues 96-105, 148-168 and the pyrophosphate group of NAD+. The aromatic B ring of compound 22 interacts with the methyl side chain of Met161 to form the methyl-π interaction, whereas that of compound 16 loses this interaction. The hydroxyl group at the A ring of compounds 16 and 22 form two hydrogen bonds with Tyr158 as well as the 2´-hydroxyl group of NAD+. The substituents at the A rings of compounds 16 and 22, the ethyl phenyl and the octyl chain, respectively, are occupied with the flexible residues 195-225. The first four carbons of the octyl chain of compound 22 superimpose well with the ethyl phenyl of compound 16 and interact with the flexible residues of Phe149, Tyr158, Pro193, Met199, Ile215, Leu218, Glu219 and Trp222, respectively. The last four carbons of the octyl chain of compound 22 interact with Met155, Pro156, Ala157, Gln214, whereas these interactions are lost for binding of compound 16. The presence of the methyl-π interaction and more interactions with the flexible residues 195-225 of the substituents at the A rings of compound 22 may be accounted for higher activity of compound 22 as compared with that of compound 16.

3.2 Docking calculations of 5-substituted triclosan and alkyl diphenyl ether derivatives

Molecular docking calculations using the Autodock 3.05 program have been successfully applied to investigate the binding modes of all 5-substituted triclosan and alkyl diphenyl ether derivatives in the InhA binding pocket. The RMSD between the docked and crystallographic conformations is lower than 1Å, indicating that molecular docking calculations are rendering high reliability for reproducing the binding mode of all 5-substituted triclosan and alkyl diphenyl ether derivatives. All predicted binding modes of these inhibitors that are similar to the binding modes as those of the above X-ray crystal structures are shown in Fig. 3.

The B rings are surrounded by the more rigid residues 96-105 and 148-168, whereas the A rings and their substituents are surrounded by the flexible residues 195-225. The hydroxyl groups at the A ring of all 5-substituted triclosan and alkyl diphenyl ether derivatives could create the same hydrogen bonding pattern with NAD+ and Tyr158. The major modification of 5-substituted triclosan and alkyl diphenyl ether derivatives is the variation of substituent R_1 at the A ring. The increasing length of an alkyl chain at the substituent R_1 results in the decreasing IC_{50} values for InhA inhibition of both 5-substituted triclosan and alkyl diphenyl ether derivatives, compounds 2, 4-6, 19-22. Compound 22 bearing the octyl chain at the A ring is the most active compound in the data set. To compare the binding modes of compounds containing alkyl chains of different lengths at the position R_1, the predicted

binding modes of compounds 18-20 containing C6, C2 and C4 alkyl chains, respectively, are superimposed on the X-ray binding mode of compound 22 as shown in Fig. 4.

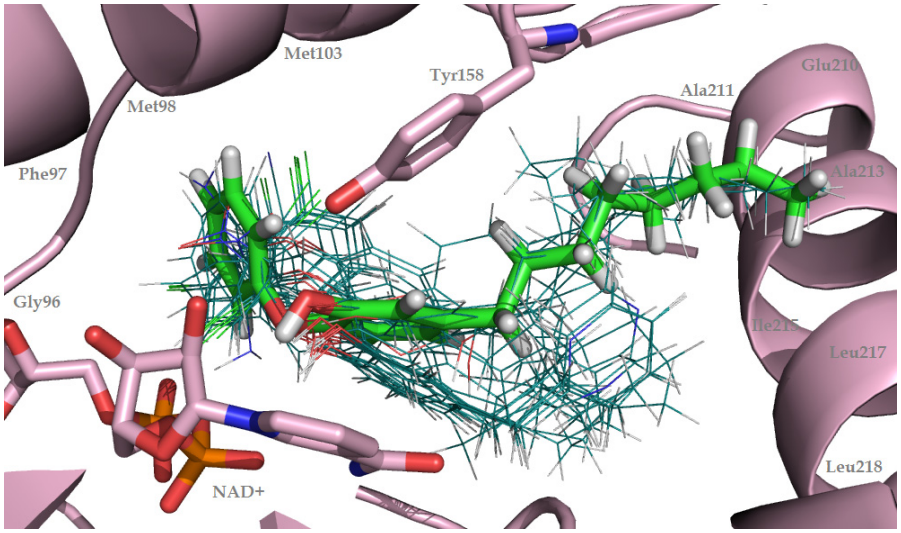


Fig. 3. Superposition of all predicted binding modes of 5-substituted triclosan and alkyl diphenyl ether derivatives (green line) in InhA (pink) derived from molecular docking calculations and the X-ray structure of compound 22 (green stick)

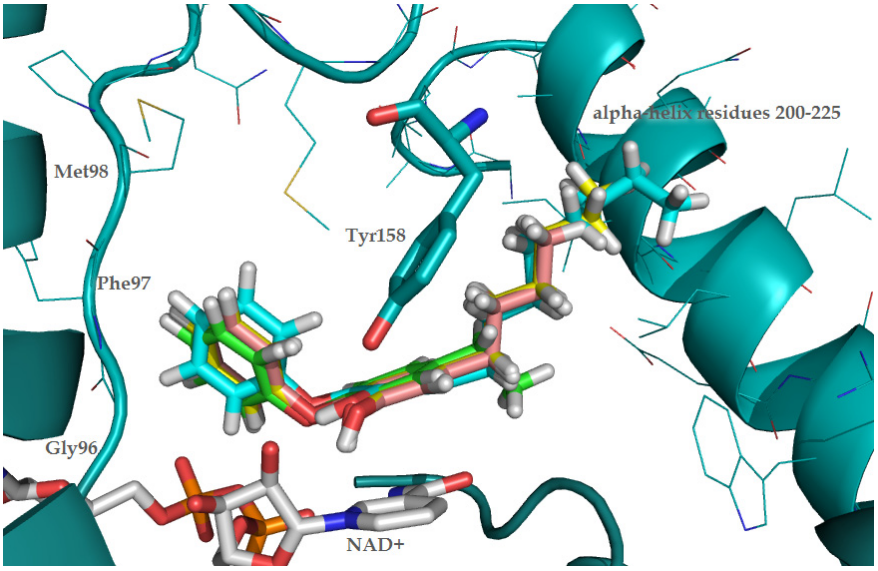


Fig. 4. The predicted binding modes of compounds 18 (yellow), 19 (green) and 20 (orange) containing C6, C2 and C4 chains at the substituent R_1, respectively, and the X-ray binding mode of compound 22 (cyan) in the InhA binding pocket (cyan)

The longer octyl chain of compound 22 could more closely interact with α-helix residues 200-225, whereas C6, C2 and C4 alkyl chains of compounds 18-20 are far from these residues, particularly the C2 alkyl chain of compound 19. This result may explain the higher activity of compound 22 bearing the longer alkyl chain at the position R_1 as compared with those of compounds 18-20. However, when the octyl chain of compound 22 was enlarged to a C14 chain resulting in compound 23, there is a corresponding increase in IC_{50} value for InhA inhibition from 5 nM to 150 nM. In contrast to the C8 chain of compound 22 which lies in a linear conformation in InhA binding pocket, the C14 chain of compound 23 forms a U-like shape within InhA binding pocket and slightly interacts with α-helix residues 200-225 as shown in Fig. 5. Moreover, the B ring of this compound loses the methyl-π interaction with Met161. These results may be accounted for the lower potency of compound 23. In the case of compounds 13 and 14, where phenyl rings are directly attached to the A ring at position R_1, the activity against InhA of these compounds are lower than those of compounds 15-17 which have a linker between the A ring and the phenyl ring at substituent R_1. Based on molecular docking calculations, the phenyl substituents of compounds 13 and 14 overlap with an ethyl linker of compound 16 and are surrounded by the rigid residues 148-168. To avoid the steric clash with these residues, a change of the binding conformation of compounds 13 and 14 has occurred, leading to a loss of the π-π stacking of the A ring with nicotinamide ring of NAD+ as compared with that of compound 16. Moreover, a linker phenyl substituent of compound 16 could create more hydrophobic interactions with the hydrophobic residues in the flexible residues 195-225. These results may explain why compounds 13 and 14 are of lower potency compared to compounds 15-17.

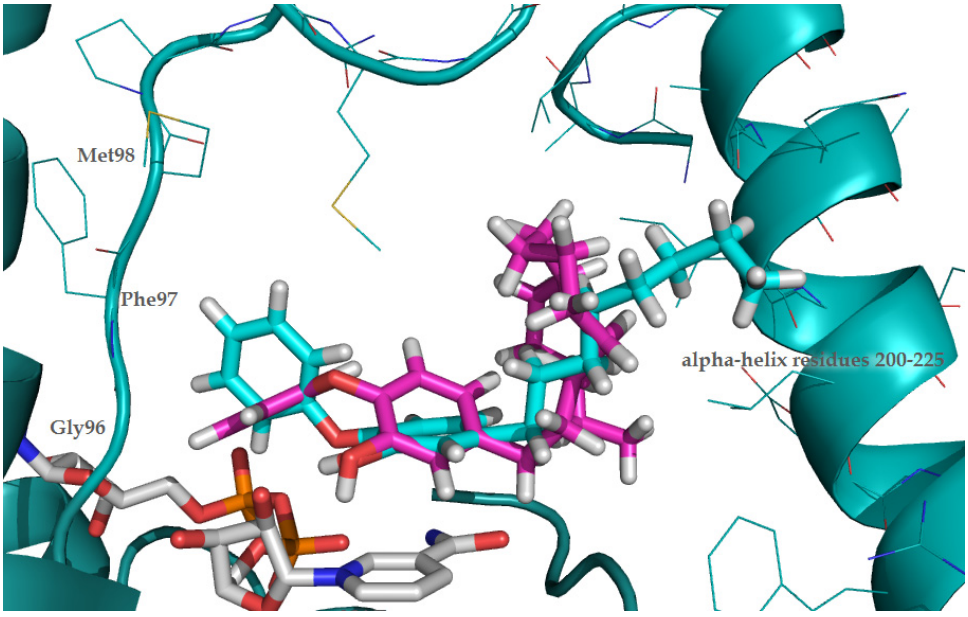


Fig. 5. The predicted binding modes of compound 23 (pink) and the X-ray binding mode of compound 22 (cyan) in InhA binding pocket (cyan)

The presence of NO_2 and NH_2 groups at the B ring of the alkyl diphenyl ether derivatives giving compounds 24-29 results in lower activities (increasing of IC_{50} values) for InhA inhibition of these compounds as compared with that of compound 18. Based on molecular docking calculations, the A rings and hexyl substituents of compounds 24-29 overlap well with that of compound 18 as shown in Fig. 6. However, the NO_2 and NH_2 substituents at the B ring of compounds 24-29 induce the position change of B rings of these compounds to avoid the steric conflict with the rigid residues Gly96, Phe97, Met98, Met161 and the pyrophosphate group of NAD+. Because of this reorientation, the B rings of compounds 24-29 lose the methyl-π interaction with Met161 as compared with compound 27. Therefore, the lower activities against InhA of alkyl diphenyl ether derivatives which contain the NO_2 and NH_2 groups at the B ring may be a consequence of the loss of the methyl-π interaction of the B rings of these compounds.

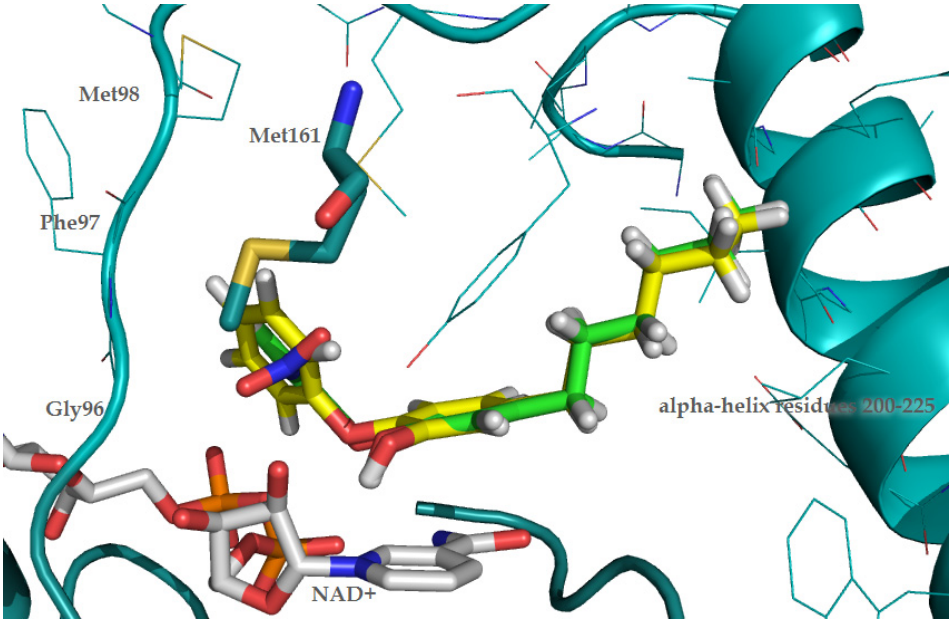


Fig. 6. Superposition of the predicted binding modes of compounds 18 (yellow) and 24 (green)

3.3 CoMFA, CoMSIA and HQSAR models

The results of the PLS analyses of CoMFA, CoMSIA and HQSAR models are summarized in Table 2. QSAR models 1, 3 and 5 derived from the PLS analyses of all compounds in the training set show a poor q^2. To improve the quality of these QSAR models, compound 19 was considered as an outlier resulting in better q^2 values of models 2, 4 and 6. However, HQSAR model 6 still shows a poor q^2 of 0.27. To modify this model, compounds 23 and 28 were omitted from the training set which yields the satisfying HQSAR, model 7. Based on a good q^2, models 2, 4 and 7 were selected as the final CoMFA, CoMSIA and HQSAR models, respectively. The final CoMFA model composing the steric and electrostatic fields gives q^2 of 0.66 and r^2 of 0.99. In the case of the final CoMSIA model including the steric, electrostatic

and hydrophobic fields, a higher q^2 value of 0.73 as compared with that of the final CoMFA model was obtained. This result indicates that the final CoMSIA model performs better in the prediction than the final CoMFA model, which is an indication for the fact that beyond steric and electrostatic effects, hydrogen bonding may be an additional contribution. Among the considered descriptors of the final CoMSIA and CoMFA models, the electrostatic fields of both models are the most important parameter influencing the IC_{50} values of the 5-substituted triclosan and alkyl diphenyl ether derivatives in the training set. With regard to the best HQSAR model generated based on the combination of the different fragment types, atom (A), bond (B) and connection (C), this model shows q^2 value of 0.74 with r^2 value of 0.97 which are in the same level with those of the final CoMSIA model.

	Models	Statistical parameters						Fraction
		q^2	r^2	N	s	SEE	F	
	CoMFA							
1	S/E	0.38	0.91	3	0.56	0.21	73.19	48/52
2	**S/E**	**0.66**	**0.99**	**6**		**0.05**	**698.63**	**39/61**
	CoMSIA							
3	S/E/H	0.45	0.89	4	0.54	0.24	39.27	24/46/30
4	**S/E/H**	**0.73**	**0.99**	**6**		**0.07**	**277.19**	**19/57/24**
	HQSAR							
5	A/B/C	0.14	0.32	1	0.63	0.56	-	-
6	A/B/C	0.27	0.43	1	0.54	0.47	-	-
7	**A/B/C**	**0.74**	**0.97**	**6**		**0.12**	-	-

Table 2. Summary of statistical results of CoMFA, CoMSIA and HQSAR models, N, optimum number of components; s, standard error of prediction, SEE, standard error of estimate; F, F-test value; S, steric field; E, electrostatic field; H, hydrophobic field; A, atom; B, bond; C, connection

3.4 Validation of the QSAR models

Satisfyingly good correlations between actual and predicted activities of the training set based on the final CoMFA, CoMSIA and HQSAR models are depicted in Fig. 7. The predicted activities of the training set derived from the final QSAR models are close to the experimental activities indicating the high degree of correlation between the actual and predicted activities. In order to assess the external predictive ability of selected QSAR models, InhA inhibition activities of the test set were predicted. The IC_{50} values of test set compounds predicted by the final CoMFA, CoMSIA and HQSAR models are within one logarithmic unit difference from the experimental values except those of compound 2 as presented in Fig. 7. This result reveals that all selected QSAR models are reliable to predict the activity of external data set. Therefore, the final CoMFA, CoMSIA, and HQSAR models can be utilized for designing new direct InhA inhibitors with improved activity.

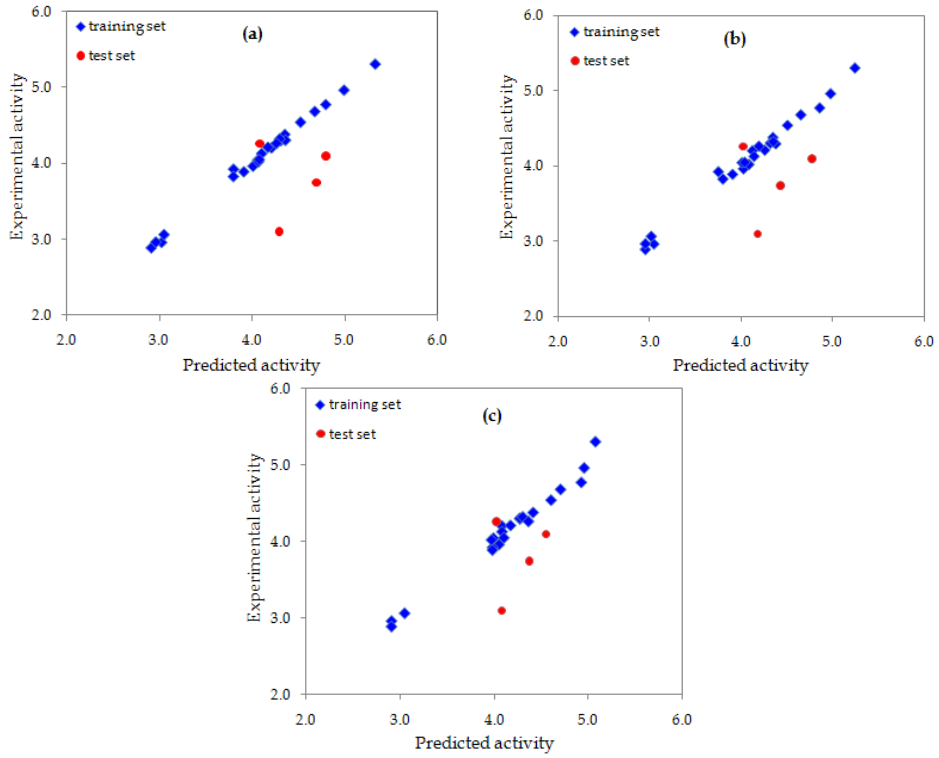


Fig. 7. Plots between the actual and predicted activities of the training and test sets derived from the final CoMFA (a), CoMSIA (b) and HQSAR (c) models, respectively

3.5 CoMFA and CoMSIA contour maps

To reveal the importance of molecular descriptor fields, steric, electrostatic and hydrophobic fields, on the InhA inhibition activities of 5-substituted triclosan and alkyl diphenyl ether derivatives, CoMFA and CoMSIA contour maps were established and depicted in Fig. 8 and 9, respectively. CoMFA and CoMSIA contour maps are merged with the InhA pocket complexed with compounds 16 and 22 in order to link the structural requirement for better activity of 5-substituted triclosan and alkyl diphenyl ether derivatives visualized by CoMFA, CoMSIA contour maps toward the interaction of these compounds in InhA binding pocket. Green and yellow contours indicate areas where favorable and unfavorable steric bulks are predicted to enhance the antitubercular activities of the direct InhA inhibitors. Blue and red contours indicate regions where electropositive and electronegative groups lead to increasing antitubercular activity, respectively. Purple and white contours represent areas where the hydrophobic group and the hydrophilic group, are predicted to favour the biological activities. Compounds 16 and 22 were selected as the template for graphic interpretation of CoMFA and CoMSIA models. CoMFA model shows three yellow contours surrounding the B rings of compounds 16 and 22 buried in the pocket consisting of the rigid residues Gly96, Phe97, Met98, Met161 and NAD+ as shown in Fig. 8(a). These contours indicate that the substituent of the B ring should be small in order to increase the enzyme

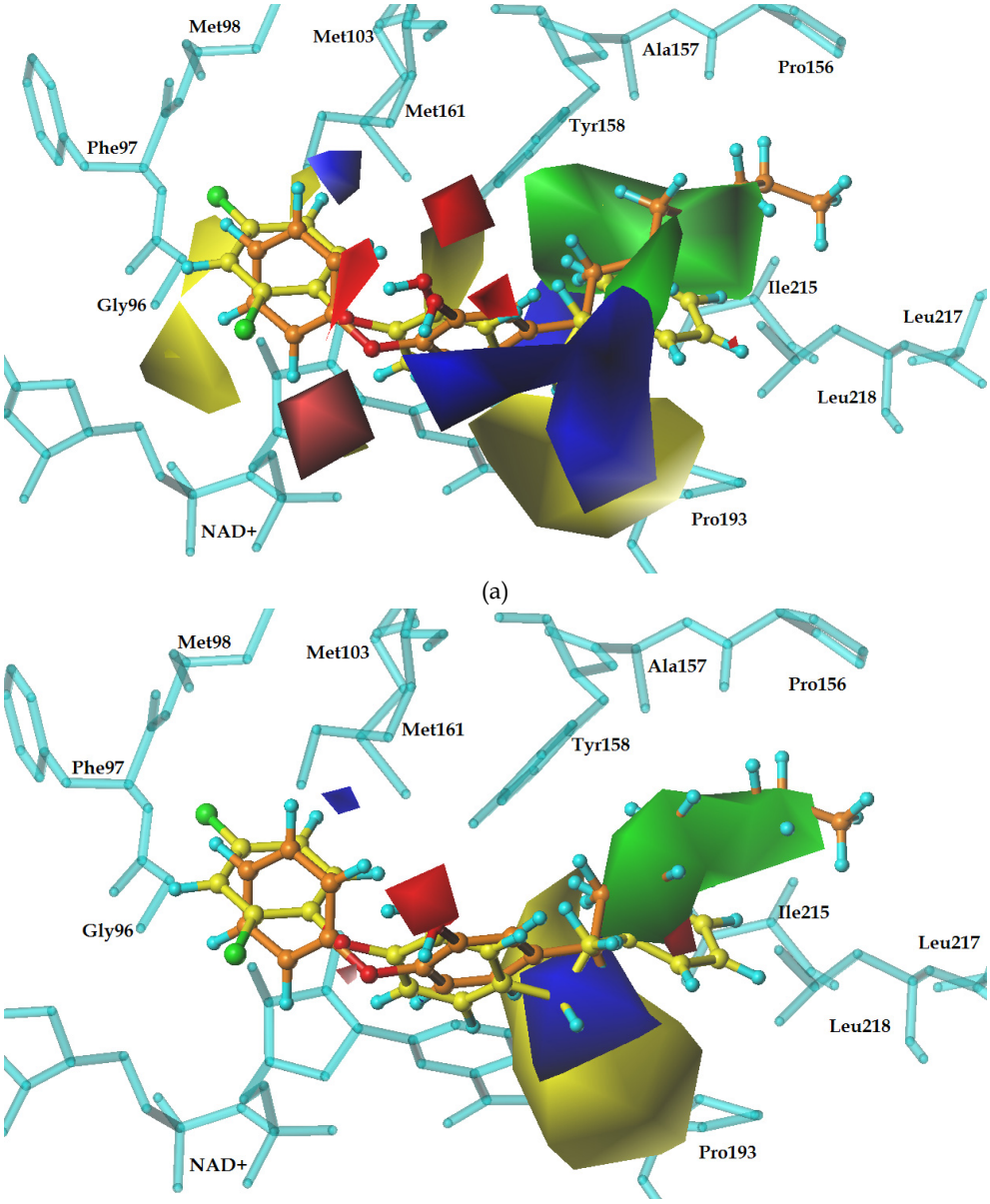


Fig. 8. Steric and electrostatic contours of CoMFA (a) and CoMSIA (b) models in combination with compounds 16 (yellow) and 22 (orange) in InhA binding pocket (cyan). Green and yellow contours represent favourable and unfavourable steric regions, respectively. Blue and red contours are favoured for electropositive and electronegative groups, respectively.

inhibitory activity of 5-substituted triclosan and alkyl diphenyl ether derivatives. Moreover, this structural requirement is preferable for the interaction of the B ring with the rigid moiety consisting of residues Gly96, Phe97, Met98, Met161 and NAD+. In addition, the B rings of compounds 16 and 22 are covered by a large purple contour and immediately flanked by two white contours as shown in Fig. 9. A large purple region conforms to the aromatic B rings of 5-substituted triclosan and alkyl diphenyl ether derivatives which are crucial for the forming of the methyl-π interaction with the methyl side chain of Met161. Furthermore, the presence of hydrophilic substituent with a small size at both sides of the B ring should enhance the activity against InhA of 5-substituted triclosan and alkyl diphenyl ether derivatives.

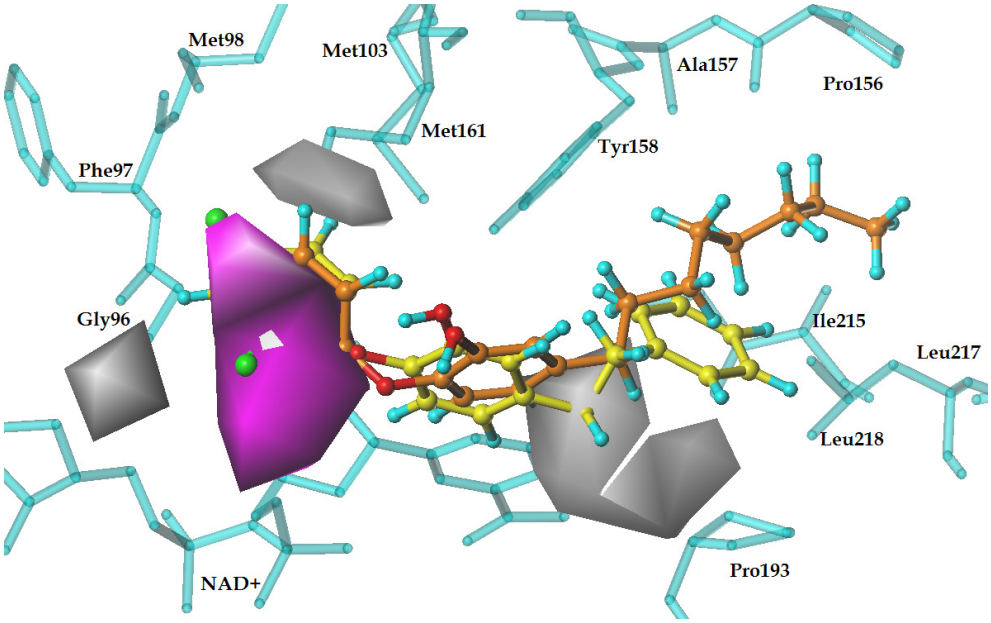


Fig. 9. CoMSIA hydrophobic contours in combination with compounds 16 (yellow) and 22 (orange) in InhA binding pocket (cyan). Purple and white contours show favourable and unfavourable hydrophobic regions, respectively.

With regard to the substituent R_1 of the A ring, CoMFA and CoMSIA models in Fig. 8 and 9 present the large blue, white and yellow contours near the ethyl linker of compound 16 and the first two carbons of the octyl side chain of compound 22 which are surrounded by the rigid residues 148-168. These contours suggest that the blue, white and yellow regions near those of compounds 16 and 22 favor the small moiety with the electropositive and hydrophilic properties. It is important to note that this favored small moiety could be helpful not only for the activities against InhA of 5-substituted triclosan and alkyl diphenyl ether derivatives but also for the binding of these compounds at a position where rigid residues 148-168 are presented. This finding agrees well with the experimental data that compounds with no linker and the methyl linker between the A ring and the analogues of phenyl and pyridyl at substituent R_1, compounds 10-15, show IC_{50} values higher than that of

compound 16 containing the ethyl linker. Based on the obtained molecular docking results, the analogues of bulky phenyl and pyridyl at substituent R_1 of compounds 10-15 overlap with the ethyl linker of compound 16 located near the blue, white and yellow regions. Another interesting contour of CoMFA and CoMSIA models is the large green contour at the phenyl substituent of compound 16 and the last six carbons of the octyl chain of compound 22. The green contour indicates that the presence of the bulky substituent at this region should increase the InhA inhibitory activities of 5-substituted triclosan and alkyl diphenyl ether derivatives. This result is in line with the experimental results that compounds 1-2, 4-9, 19-21 presenting the shorter chain of substituent R_1 display lower activities as compared with that of compound 22 bearing the longer octyl chain. Remarkably, based on the large green area, the combination between substituents R_1 of 5-substituted triclosan derivatives and alkyl diphenyl ether derivatives, the phenyl substituent of compound 16 and the last six carbons of the octyl chain of compound 22, may generate the optimal substituent R_1 such as the phenyl incorporated with alkyl chain. Therefore, the designed compounds should display the better profile against InhA.

3.6 HQSAR contribution maps

HQSAR contribution maps are helpful to visualize the contributions of molecular fragments to the activities against InhA of 5-substituted triclosan and alkyl diphenyl ether derivatives in the present data. The color codes indicate the different contributions of all atoms in each compound to the biological activity. An atom with negative contributions is represented at the red end of the spectrum, whereas an atom with positive contributions is represented at the green end of the spectrum. The white colored atoms stand for intermediate contributions. Fig. 10 depicts the individual atomic contributions to the activity against InhA of compounds 13, 16 and 22. There are green and yellow atoms at the A ring of compounds 16 and 22 indicating the positive contributions of the A ring to the activity against InhA of these compounds. Moreover, the positive contributing fragments are presented at the ethyl linker of compound 16 and the first carbon of the octyl chain of compound 22 emphasizing the importance of these fragments. As previously shown by CoMFA and CoMSIA contours, these positively contributing moieties of compounds 16 and 22 are surrounded by yellow, blue and white contours implying that these fragments are optimal for steric, electrostatic and hydrophobic requirements. Obviously supporting this finding, the omission of the ethyl linker resulting in compound 13 induces the appearance of the negative contributing fragments at the A ring and the phenyl substituent leading to the lower activity of this compound as compared with that of compound 16. Considering the phenyl substituent of compound 16 and the last six carbons of the octyl chain of compound 22 buried in green CoMFA and CoMSIA contours as shown in Fig. 8, these fragments are colored by white indicating that these atoms have no contributions to the activity. Therefore, the modification of these white parts following the CoMFA and CoMSIA suggestions may improve the activity against InhA of 5-substituted triclosan and alkyl diphenyl ether derivatives. In case of the B rings of compounds 16 and 22, most atoms in the B ring and Cl substituents of compound 16 are colored by red and orange, suggesting the negative contributions of these fragments, whereas those of compound 22 are colored by white indicating no contribution of these fragments. This result may be accounted for the higher activity of compound 22 as compared with that of compound 16. Based on the obtained CoMFA and CoMSIA models, the B ring of compounds 16 and 22 are covered by a large purple contour and immediately flanked by two white contours. Therefore, the adjustment of the substituent at the B ring

based on the structural requirement suggested by CoMFA and CoMSIA models may induce the occurrence of the positive contribution on the B ring and its substituents resulting in the better activity against InhA. Noticeably, about the chlorine substituents at the B ring of 5-substituted triclosan derivatives, the precence of a positively contributing fragment at the substituent R_1 of the A ring induces the negative contribution on the chlorine substituents as shown by HQSAR contribution maps of compounds 13 and 16 in Fig. 10. This result implies that more interactions of substituent R_1 of the A ring reduce the role of the B ring chlorines on the activities. Consistent with this finding, alkyl diphenyl ether derivatives without the B ring chlorines, compounds 18, 21 and 22, display activities against InhA higher than those of triclosan and its derivatives bearing the B ring chlorines, compounds 1-17. On the other hand, the B ring chlorines can be more preferable for the activities in case of compounds with less interactions of substituent R_1 to InhA. As exemplified by compounds with shorter lenght of the alkyl chain (C2 and C4 chains at the substituent R_1), compounds 19 and 20 possess lower activites than those of compounds 4 and 6 containing the B ring chlorines. It is important to note that the influence of B ring chlorines on inhibitory activity of triclosan derivatives which is argued in Freundlich's work (Freundlich et al. 2009) and Sullivan's work (Sullivan et al., 2006) could be evaluated by our HQSAR model.

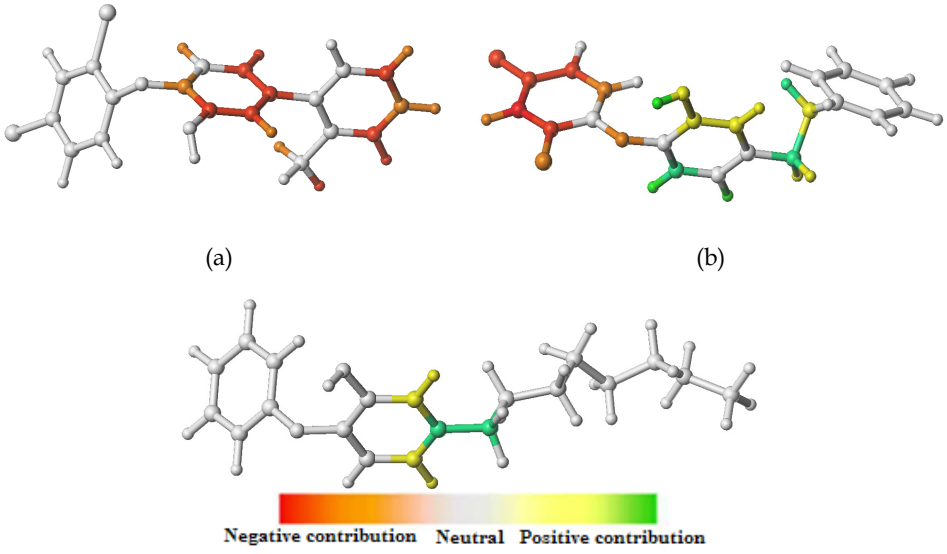


Fig. 10. The final HQSAR contribution maps of compounds 13 (a), 16 (b) and 22 (c)

4. Conclusion

Molecular docking calculations were successfully applied to determine the potential binding modes of 5-substituted triclosan and alkyl diphenyl ether derivatives in the InhA binding pocket. The B rings of these compounds are occupied in the rigid pocket consisting of residues 96-105 and 148-168, whereas the A rings and their substituents are buried in the

flexible residues 195-225. The B ring substituents that could perturb the methyl-π interaction of the B ring with Met161 produce the poor activities of alkyl diphenyl ether derivatives. On the other hand, the substituent R_1 at the A ring that could interact more with the flexible residues 195-225 and avoid the steric conflict with the rigid residues 148-168 might result in the better activities of 5-substituted triclosan and alkyl diphenyl ether derivatives against InhA. Besides, the key structural elements for a good activity against InhA of these compounds based on CoMFA, CoMSIA and HQSAR were clearly elucidated in the present study. Based on CoMFA and CoMSIA guidelines, compounds with the combination of substituents R_1 of 5-substituted triclosan derivatives and alkyl diphenyl ether derivatives should display the better profile against InhA. In agreement with CoMFA and CoMSIA results, the HQSAR contribution maps show the individual contribution of the atoms to IC_{50} values of 5-substituted triclosan and alkyl diphenyl ether derivatives. Moreover, HQSAR contribution maps could reveal the contribution of two chlorine atoms on the B ring of 5-substituted triclosan derivatives to their IC_{50} values. Consequently, the integrated results from the structure-based design using molecular docking calculations and the ligand-based design using various QSAR approaches provide insights into key structural features that can be utilized for designing novel and more active InhA inhibitors in the series of 5-substituted triclosan and alkyl diphenyl ether derivatives. Particularly, the modified compound suggested in the present study might be a member of a next drug generation of InhA inhibitor. These results demonstrate that computer aided molecular design approaches are fruitful for rational design and for possible syntheses of novel and more active InhA inhibitors that might be next generation of antitubercular agents.

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Part 3

Novel Targets and Technologies Leading to Improved Treatment Options

11

RNA Interference-Based Therapeutics: Harnessing the Powers of Nature

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1. Introduction

The central dogma of biology describes the transfer of biological information from DNA through to protein (Crick, 1970). In the first phase, known as transcription, DNA is converted into a complementary sequence of messenger RNA (mRNA). This mRNA allows the genetic message to be communicated outside of the nucleus, to other areas of the cell, where it is then translated into protein by ribosomes.

Most human diseases arise from increased function or dysfunction of proteins within the body. Since these proteins are generated from DNA via mRNA, modulation of this flow of genetic information can convey a therapeutic effect on the disease state. Mammalian cells possess the genetic instruction to make 50,000 to 100,000 different proteins but only 10-20% of these are found in any single cell. Therefore, a gene must contain instructions for the regulation of the production of protein in correct amounts and at the correct time for each cell type. Gene regulation is one of the most complex molecular processes known, involving up to 10% of the proteins that cells produce.

In 1998, Andrew Fire and Craig Mello described RNAi as an endogenous gene expression pathway activated by double-stranded RNA (dsRNA) in the worm *Caenorhabditis elegans*. For this pioneering work, Fire and Mello were awarded the 2006 Nobel Prize in Physiology or Medicine. The discovery of the natural RNAi mechanism for sequence-specific gene silencing launched a new era in antisense technology. During the 1990s, a number of gene-silencing phenomena that occurred at the posttranscriptional level were discovered in plants, fungi, animals and ciliates, introducing the concept of post-transcriptional gene silencing (PTGS) or RNA silencing (Baulcombe, 2000; Matzke et al., 2001).

The most important technologies for gene suppression are: antisense oligonucleotides, aptamers, ribozymes and RNA interference (RNAi). The first report that gene expression could be modulated by the use of reverse complementary (antisense) oligonucleotides was made in 1978. Antisense molecules are synthetic segments of DNA or RNA, designed to mirror specific mRNA sequences and block protein production, these molecules are designed to inhibit translation of a target gene to protein via interaction with mRNA. Aptamers are single-strand DNA or RNA oligomers, which can bind to a given ligand with high affinity and specificity due to their particular 3-D structures and thereby antagonize the biologic function of the ligand. Recent developments demonstrate that aptamers are valuable tools for diagnostics, purification processes, target validation, drug discovery and therapeutics. Ribozymes are

enzymes that are generally considered to be comprised of RNA, which can act as catalysts as well as genetic molecules. They cleave a target RNA, inhibiting the translation of RNA into protein, thus stopping the expression of a specific gene. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. RNAi is a naturally occurring regulatory mechanism present in most eukaryotic cells that uses small double-stranded RNA (dsRNA) molecules to direct homology-dependent gene silencing. Two types of small RNA molecules -microRNA (miRNA) and small interfering RNA (siRNA)- are central to RNA interference. RNAs are direct gene products, and these small RNAs can bind to other specific mRNA and either increase or decrease their activity, for example by preventing a messenger RNA from producing a protein.

2. Mechanism of RNA interference

The mechanism of RNAi is initiated when long dsRNA are processed to siRNAs by the action of an RNase III-like protein known as Dicer (Bernstein et al., 2001; Hammond et al., 2000) (Figure 1). The resultant siRNA are 21 to 24 nucleotides in length, double stranded and have 3′ overhangs of 2 nucleotides (Stevenson, 2004). Exogenous synthetic siRNA or endogenous expressed siRNAs are incorporated into the effector complex known as RNA-induced silencing complex (RISC), where the antisense or guide strand of the siRNA guides RISC to recognize and cleave target mRNA sequences (Elbashir et al., 2001) upon adenosine-triphosphate (ATP)-dependent unwinding of the double-stranded siRNA molecule through an RNA helicase activity (Nykanen et al., 2001). The catalytic activity of RISC, which leads to mRNA degradation, is mediated by the endonuclease Argonaute 2 (AGO2) (Liu et al., 2004; Song et al., 2004). AGO2 belongs to the highly conserved Argonaute family of proteins. Argonaute proteins are ~100 KDa highly basic proteins that contain two common domains, namely PIWI and PAZ domains (Cerutti et al., 2000). The PIWI domain is crucial for the interaction with Dicer and contains the nuclease activity responsible for the cleavage of mRNAs (Song, et al., 2004). AGO2 uses one strand of the siRNA duplex as a guide to find messenger RNAs containing complementary sequences and cleaves the phosphodiester backbone between bases 10 and 11 relative to the 5' end of the guide strand (Elbashir, et al., 2001). An important step during the activation of RISC is cleavage of the sense or passenger strand by AGO2, removing this strand from the complex (Rand et al., 2005). Once the mRNA has been cleaved, and due to the presence of unprotected RNA ends in the fragments, the mRNA is further cleaved and degraded by intracellular nucleases and will no longer be translated into protein (Orban & Izaurralde, 2005) while RISC will be recycled for subsequent rounds (Hutvagner & Zamore, 2002). This constitutes a catalytic process leading to the selective reduction of specific mRNA molecules and of the corresponding proteins. It is possible to exploit this native mechanism for gene silencing with the purpose of regulating any gene(s) of choice by directly delivering siRNA effectors into the cells or tissues, where they will activate RISC and produce a potent and specific silencing of the targeted mRNA.

PTGS cannot only be induced by siRNA through sequence specific cleavage of perfectly complementary mRNA. Recent discoveries have reported the existence of other endogenous post-transcriptional regulatory mechanisms. One of these mechanisms is that mediated by miRNAs, which are functional naturally occurring small non coding RNAs only require partially complementary targets to bind to their target mRNAs through their 3′ untranslated regions (3′ UTRs) (Lee et al., 1993; Wightman et al., 1993). miRNAs act as guide sequences to

regulate the expression of multiple genes that are often functionally related. Furthermore, the translation of many mRNAs is regulated by multiple different miRNAs. They are critical factors in coordinating the development, differentiation and function of cells and tissues and it is estimated there are hundreds of these molecules in humans. There are approximately 1400 miRNAs that have been identified in the human genome and they are believed to regulate the expression of up to 30% of all human genes by preventing translation of mRNAs into proteins. For further details on miRNA biology and therapeutic potential refer to section 6.

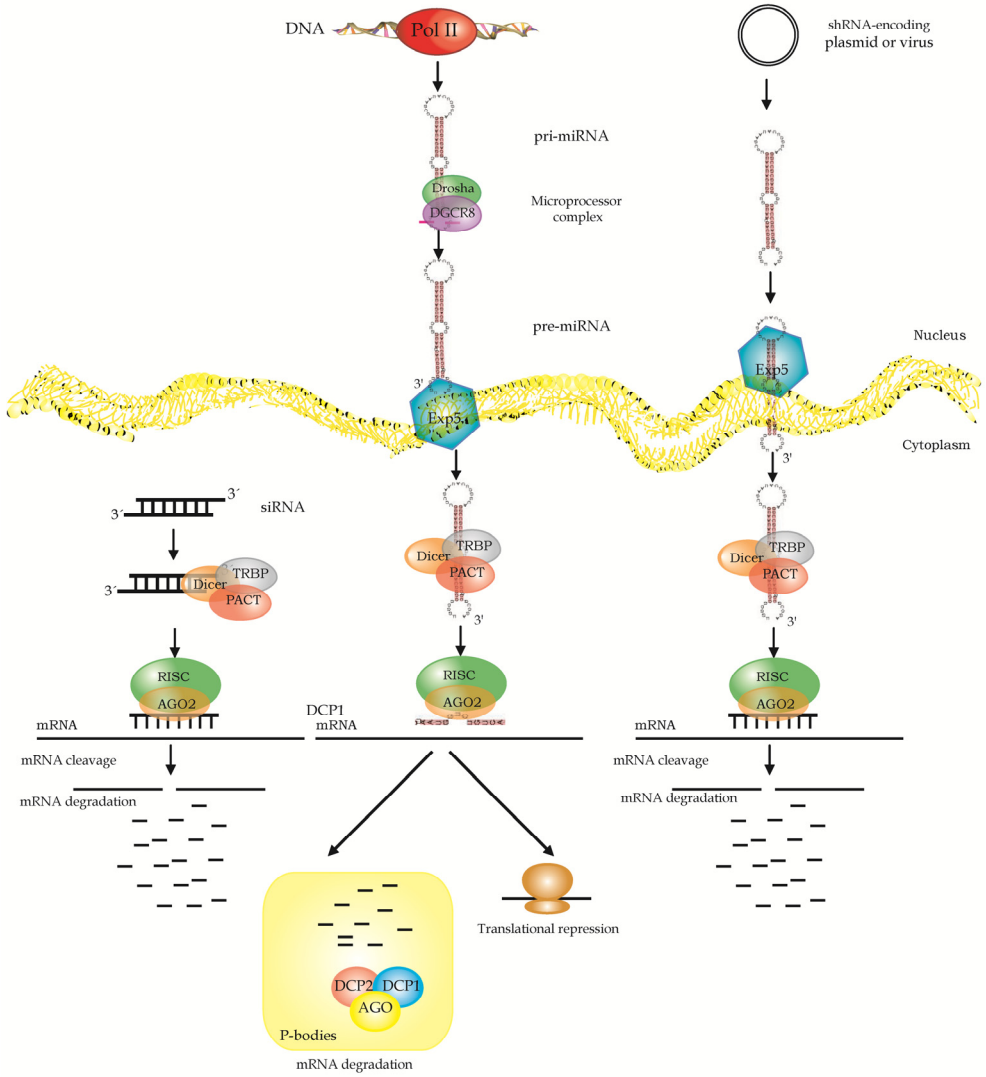

Fig. 1. Mechanism of RNA interference in mammalian cells. RNA interference (RNAi) is an intracellular mechanism triggered through small RNAs that include small interfering RNAs

(siRNAs), microRNAs (miRNAs) and short hairpin RNAs (shRNAs). The siRNA pathway begins when double stranded RNAs (dsRNAs) are trimmed down by the Dicer complex into siRNAs. Alternatively, synthetic siRNAs can be introduced directly into the cell cytoplasm. These siRNAs are incorporated into the RNA-induced silencing complex (RISC), where they are unwound. If the siRNA has perfect sequence complementarity, the Argonaute 2 protein (AGO2) present in RISC cleaves the passenger (sense) strand so that active RISC containing the guide (antisense) strand can recognize target sites on the messenger RNA (mRNA) to direct mRNA cleavage. This cleavage is performed by the catalytic domain of AGO2. The miRNA pathway starts when primary miRNA (pri-miRNAs) are transcribed from RNA polymerase II (Pol II) promoters, forming hairpin-shaped structures. These are processed by the Drosha-containing microprocessor complex, giving rise to precursor miRNAs (pre-miRNAs), that are also stem-like structures with a 2-nucleotide 3′ overhang. Pre-miRNAs are transported into the cytoplasm by Exportin 5 (Exp5), where they are processed by a Dicer containing complex to ~21-25 nucleotide imperfect dsRNA duplexes that constitute the mature miRNAs. Once the miRNA duplex is processed, the guide sequence is loaded into RISC and then mediates binding to the target sequence in the 3′ UTR of cellular mRNAs. If the miRNA guide sequence is fully complementary to its target site, it triggers site-specific cleavage and degradation of the mRNA through the catalytic domain of AGO2. On the other hand, if the base pairing is incomplete but fully complementary in the seed region (nucleotides 2-8 of the miRNA), repression of protein expression occurs, often accompanied by mRNA degradation in cytoplasmic processing (P)-bodies. Mimicking the miRNA mechanism, synthetic DNA vector constructs or viral particles code for stable shRNAs, that are transcribed from a RNA polymerase II/III promoter and form haipin-like structures. These shRNAs are transported into the cytoplasm by Exp5 and recognized by Dicer, leading to the formation of siRNAs homologous to the target mRNA and, subsequently, to mRNA degradation.

Commercially available systems and other therapeutic initiatives aimed at mimicking the mechanism of RNAi make use of DNA vector constructs or viral particles coding for long term and stable short hairpin (shRNAs) expression that are transcribed from a RNA polymerase III promoter *in vivo* or shRNAs that are synthesized exogenously and transfected into the cells. The double stranded region of shRNAs is formed though a hairpin structure and intramolecular hybridization that resembles that of miRNA precursors (Brummelkamp et al., 2002a; Paddison et al., 2002) (Figure 1). These shRNAs molecules are recognized by Dicer, leading to the formation of siRNAs homologous to the target mRNA. The main difference with siRNAs is that while these mediate only transient silencing, virally expressed shRNAs mediate a very potent and stable silencing effect for as long as their transcription takes place. shRNAs also enter the endogenous silencing pathway at an earlier stage than siRNAs, having a higher chance of saturating the natural miRNA natural pathways (Grimm & Kay, 2006).

3. Efficacy and stability

3.1 Basic design rules

Bioinformatic tools play an important role in RNAi design. Several tools are available for this purpose and many papers have been published that describe algorithms for selecting RNA sites. Algorithms have been developed that can help select siRNA target sites that exhibit high target-specific activity and minimal off-target activity. Efficacy of siRNAs for

individual targets normally depends on different factors, such as thermodynamic stability (Khvorova et al., 2003), structural features (Castanotto et al., 2007), target mRNA accessibility (Patzel et al., 2005) and additional position specific determinants (Heale et al., 2005; Schwarz et al., 2003). Systematic studies on targeting efficacies have shown that optimal siRNAs should be between 19 and 25 nucleotides long, should have 3′ symmetric dinucleotide overhangs, low guanine-cytosine content (between 30 and 52%) (Schwarz, et al., 2003) and specific nucleotides at certain positions. For example, features that increase siRNA efficacy are the presence of an adenine or uracil in position 1, adenosine in position 3, a uracil in positions 7 and 11, a guanine in position 13, a uracil or adenine in position 10 (this is the site for RISC mediated cleavage), a guanine in position 21 and/or the absence of guanines or cytosine at position 19 of the sense strand (see (Amarzguioui & Prydz, 2004) for a full review of the topic). In general, enrichment in adenosines and uracils along the first 6-7 base pairs of the sequence, and consequently, weak hydrogen bonding, allows RISC to easily unravel the double stranded duplex and load the guide strand (Dykxhoorn & Lieberman, 2006).

As mentioned above, the siRNA duplex should also be thermodynamically flexible at its 3′ end, i.e., at positions 15-19 of the sense strand. This correlates with its silencing efficacy such that the presence of at least one adenosine-uracil pair in this region would decrease internal stability and increase silencing efficacy. On the contrary, internal repeats or palindrome sequences decrease the silencing potential of siRNAs.

Another consideration to be taken into account when designing an siRNA sequence is the nature of the target sequence. Under certain circumstances it will be preferable to include all splice variants and isoforms for the design of siRNAs whereas, in other instances, they should be specifically left out. Similarly, attention should be paid in order to choose sequences within the coding region of the target gene sequence, as gene silencing is an exclusively cytoplasmic process (Pei & Tuschl, 2006). Good news is that, taking all these established criteria into account, RNAi allows for an almost unrestricted choice of targets. Nevertheless, any theoretically optimal siRNA will require extensive testing to achieve high silencing efficacy without any unwanted side effects.

3.2 Fate of RNAi in biological fluids

Conventional siRNA is degraded within minutes in a serum-containing environment, which hampers their *in vivo* use. Depending on which delivery method is selected, stabilizing RNAi compounds by chemical modification may be critical for RNAi activity.

Various modifications improve siRNA stability in biological tissues and fluids. It is possible to modify siRNA molecules chemically without significant loss of activity. However, a fundamental requirement of siRNA function is that the antisense strand must either have a free hydroxyl or a phosphate at the 5′-terminus and therefore, this terminus cannot be modified. Of all the internal modifications, substitutions of the 2′OH of ribose remain the best studied and include –H,-OMe and –F. Positions 9 and 10 on the sense strand of the siRNA duplex are particularly sensitive to chemical modification, as cleavage at this position is necessary for removal of the sense strand and activation of RISC (Braasch et al., 2003; Martinez et al., 2002).

Locked Nucleic Acids (LNAs) are a family of conformationally nucleotide analogs, which provides very high affinity and high nuclease resistance to DNA and RNA oligonucleotides. This type of modification is compatible with siRNA machinery and has been reported to increase potency with minimal toxicity. Conjugating siRNA to large molecules, or

incorporating them into liposomes or nanoparticles, has been used to improve the pharmacokinetic properties of siRNAs by increasing the stability of the molecule to nuclease degradation and slowing the rate of renal clearance for small RNAs.

3.3 Tissue penetrance, intracellular delivery and targeting specific cells and/or tissues

One of the major problems in the development of RNAi-based therapies is the delivery of these molecules to the desired target cells, within their corresponding tissues and organs. The high therapeutic potential of RNAi compounds and their application in clinical settings is currently limited due to the lack of efficient delivery systems. For safe and effective delivery of RNAi to the mRNA target, many variables must be negotiated, e.g. size and diameter of delivery particles, toxicity, clearance of particle components and targeting to the appropriate points of action will be discussed.

Viral siRNA delivery has been used to specifically down-regulate the expression of genes of pathological relevance, especially for chronic diseases in which long-term gene silencing is desired, e.g. neurodegenerative disorders, cancer, heart failure and Human Immunodeficiency Virus (HIV) infections. This is achieved using gene therapy approaches in which an shRNA expression cassette is stably integrated into the host cell genome or expressed episomally. Subsequent sections of this chapter deal with specific developments based on expressed RNAi, the most notable being a treatment for HIV infection which has reached the clinic. Viral delivery systems have the advantage of achieving high transfection efficiencies due to the inherent ability of viruses to transport genetic material into cells. However, viral systems have a limited loading capacity, and pose severe safety risks because of their oncogenic potential via insertional mutagenesis (Lehrman, 1999; Sinn et al., 2005), their inflammatory and immunogenic effects (Donahue et al., 1992; Liu & Muruve, 2003) and the difficulties in controlling the timing and dose of interference. Therefore it is necessary to develop improved viral vectors that could target specific cell types or tissues after systemic *in vivo* administration in order to minimize toxicities associated with treatment.

Due to these diverse safety issues, non-viral delivery strategies have been extensively researched and more widely used. However, the issue of RNAi compound delivery has not yet been solved to a degree that allows their widespread use in therapy.

siRNAs are generally not taken up by mammalian cells, including those that actively sample their environment. However, certain tissues and cells in the lungs, mucosal environments, eyes, and even the central nervous system have been shown to efficiently take up siRNAs in the absence of transfection reagents (Bitko & Barik, 2007; Bitko et al., 2005; Luo et al., 2005; Nonobe et al., 2009; Thakker et al., 2004). As will be discussed later, some developments based on local delivery have reached the clinic, including those related to ocular, skin and lung diseases. Nevertheless, since uptake of naked siRNAs is not always possible, different options have been approached, such as bioconjugation and complex formation.

Bioconjugation strategies include conjugation with lipids, which may enhance siRNA uptake via receptor-mediated endocytosis or by increased membrane permeability of the negatively charged RNA. Although α-tocopherol conjugation to siRNA has also been described (Jeong et al., 2009), the most extensively used bioconjugate lipid has been cholesterol, that has proved to induce intracellular RNAi without any significant loss of gene silencing activity when compared to the unconjugated version, and with a good hepatic deposition after systemic administration *in vivo* (Cheng et al., 2006; Lorenz et al.,

2004; Soutschek et al., 2004). Cell penetrating peptides (CPPs) have also been proposed as an alternative to traditional methods of siRNA delivery. CPPs are short amino acid sequences, consisting mainly of positively charged amino acids that are able to interact with the plasma membrane, leading to a highly efficient uptake into the cytoplasm. Remarkable enhanced uptake effects have been shown by Davidson and coworkers in primary neuronal cells using a penetratin-coupled siRNA against several endogenous proteins (Davidson et al., 2004). siRNAs have also been conjugated to polyethylene glycol (PEG) showing increased resistance to serum degradation (Kim et al., 2006). These conjugates have also been further complexed with additional condensing agents to form colloidal nanoparticles (Jeong, et al., 2009). However, to date, none of these bioconjugates have reached the clinic without further encapsulation.

Regarding complex formation, several alternative developments have provided interesting results, some even having reached Phase I clinical trials, as discussed later on.

The flexibility in the design of cationic lipid structures and liposome composition, together with their *in vivo* efficiency, have promoted the notion that cationic lipids can be efficiently used for human gene transfer. Nucleic acids, including siRNAs, are able to electrostatically interact with cationic liposome-forming particles. However, due to their small size, they cannot condense into particles of nanometric dimensions (Spagnou et al., 2004). A development based on cationic lipids are stable nucleic acid lipid particles (SNALPs), which were developed by Tekmira Pharmaceuticals and have reached clinical trials for the treatment of hypercholesterolemia.

As with liposomes, the charged nature of siRNAs allows their complexation with various cationic polymers based on electrostatic interactions. Polymers used for delivery can be divided into two main categories: (i) those of synthetic origin, such as dendrimers, polyethyleneimine, and poly-L-Lysine; (ii) those of natural origin that are biodegradable and more easily degraded and excreted from the body, such as atelocollagen, gelatine, chitosan and cyclodextrin (Vorhies & Nemunaitis, 2009).

One of the most striking delivery methods, which has also reached clinical testing, was initially developed by Cequent Pharmaceuticals and was named transkingdom RNAi (tkRNAi). This technology uses non-pathogenic *E. coli* bacteria to produce and deliver therapeutic shRNA- into target cells to induce RNAi (Kruhn et al., 2009).

4. Safety issues related to the therapeutic use of RNAi technologies

4.1 Immune-mediated toxicities

The immune system has evolved cellular and molecular strategies to discriminate between foreign and self nucleic acids. It is activated by microbial RNA and DNA, leading to the production of type I interferon (IFN) and proinflammatory cytokines. Among the cytoplasmic sensors of long dsRNA is the dsRNA-dependent protein kinase (PKR), that phosphorylates translation initiation factor IF-2α leading to translation arrest, inhibition of protein synthesis and induction of apoptosis. This mechanism is an essential step in antiviral resistance (Akira & Takeda, 2004; Peters et al., 2006). Most human cells constitutively express low levels of PKR that remain inactive. Upon binding to a 30-80 nucleotide dsRNA fragment in a sequence-independent manner, PKR forms a homodimer, leading to its phosphorylation and activation. PKR can also activate the NF-κB signalling pathway via the phosphorylation of IKKβ (Sioud, 2010). A second protein that is stimulated by dsRNA is 2'-

5′ oligoadenylate synthetase (OAS), which is expressed constitutively and also upregulated by type I IFNs during antiviral responses (Samuel, 2001). This IFN–induced enzyme catalyzes the formation of 2′-5′-linked oligoadenylates from ATP that activate a latent ribonuclease, called RNAse L, that degrades both cellular and viral RNAs (Sioud, 2010). Although both OAS and PKR are involved in antiviral immunity, PKR and RNAse L are mainly IFN effectors and not absolutely required for IFN production. Therefore, other kinases may be involved. Two additional factors, intracellular cytosolic DExD/H box RNA-helicases retinoic-acid–inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5), have been identified as cytoplasmic sensors of viral dsRNA (Kato et al., 2006). Although RIG-I seems to be an important sensor of viral RNAs, microbial nucleic acids are also recognized by Toll Like Receptors (TLRs) , especially in immune cells (Sioud, 2006), making them crucial in sensing viral and bacterial nucleic acids. Whereas most TLRs are expressed on the plasma membrane, detecting extracellular bacterial components, TLR3, TLR7 , TLR8 and TLR9 are expressed in intracellular compartments (endosomes and lysosomes) (Takeda & Akira, 2005), allowing them to sense viral RNAs. While TLR3 is expressed on the cell surface and is believed to recognize viral dsRNA released during cell lysis (Alexopoulou et al., 2001), TLR4 and TLR8 recognize viral single-stranded RNA (ssRNA) (Heil et al., 2004). Recent studies have demonstrated how PKR and TLR3 are not the major pathways by which chemically synthesized siRNAs activate inmmunity (Hornung et al., 2005; Judge et al., 2006; Sioud, 2006). Indeed, certain siRNA sequences stimulate monocytes via TLR8 or dendritic cells via TLR7 to produce proinflammatory cytokines and IFN α (Sioud, 2005). Sometimes TLR7/8 induction is also sequence-dependent, as they recognize certain siRNA sequence motifs, such as 5′-UGUGU-3′ (Judge et al., 2005) and 5′-GUCCUUCAA-3′ (Hornung, et al., 2005). Many other sequences with high uridine content can also activate immune responses (Sioud, 2006).

In order to overcome siRNA immune activation siRNA design can be improved in many ways, such as:

- Avoiding sequence motifs and high uridine content
- By introducing modifications like 2′-deoxy. 2′-O-methyl, 2′-fluoro or selected LNA modifications in nucleotides and/or their backbone can abrogate immune response without compromising efficiency and silencing potency.(Hornung, et al., 2005; Judge, et al., 2006; Morrissey et al., 2005b; Sioud, 2006, 2010)
- By using naturally modified nucleotides such as 5′-methylcytidine, 5-methyluracil, N6-methyladenosine, 2-thiouridine or pseudo-uridine (Kariko et al., 2005)
- Staving off the activation of RIG-I, that recognises blunt end dsRNAs, by adding 2 nucleotide 3′-overhangs in the siRNA structure. These overhangs allow the siRNAs to escape recognition by mimicking endogenous small RNAs processed by Dicer (Marques et al., 2006)
- Using antibody-mediated receptor specific delivery techniques (Song et al., 2005) and agents, such as chloroquine or bafilomycin, which impede the activation of TLR7/8 by inhibiting siRNA retention in the endosomes (Sioud, 2005).

4.2 Non immune off target effects

The design of siRNAs includes the selection of siRNA sequences that are capable of knocking down the expression of their target genes. The cleavage of intended target RNAs by AGO2-RISC is highly sequence-specific and only a few mistmaches between the guide

strand and the target are tolerated (Dahlgren et al., 2008). Yet, siRNAs can trigger unintended silencing of hundreds of endogenous genes, resulting in toxic phenotypes and compromising the interpretation and outcome of the particular siRNA application (Birmingham et al., 2006). These siRNA off-target effects (OTEs) are primarily mediated by the interaction between the seed region of the RISC-associated guide strand (nucleotides 2 to 8 from the 5′ end) and complementary sites in the 3′UTR of the mRNA (Birmingham, et al., 2006; Jackson & Linsley, 2009; Jackson et al., 2006; Lin et al., 2005). Upon target binding, even partially complementary off targets are silenced through several mechanisms such as translational inhibition and mRNA destabilization (Doench & Sharp, 2004; Wu et al., 2006). Careful comparison of candidate guide strand sequence with the entire transcriptome, attempting to avoid long stretches of homology, might reduce the risk but it has been estimated that approximately 83% of the possible 21 mers within the coding sequences of the genome are unique, leaving one out of every five 21 nucleotide long siRNAs to display some homology compared with a given mRNA. Snove and Holen performed an independent investigation of 360 published siRNA sequences and found that almost 75% of the analysed oligonucleotides had the potential to trigger unwanted OTEs. They suggested that the use of inappropriate programs, such as basic local alignment (BLAST), to design silencing oligonucleotides lead to abundant OTEs because a precise homologous stretch of six or seven base pairs is necessary for detection of homology through BLAST (Snove & Holen, 2004).

The specificity of an siRNA sequence can be improved by taking into account some important designing parameters, such as thermodynamic stability of the duplex at the 5′ and 3′ ends, the Tm value of the seed sequence region (Ui-Tei et al., 2004) and the selection of the target position trying to avoid regions very close to the initiation codon (Yuan et al., 2004). As the siRNA silencing effect is concentration-dependent, success in reducing siRNA OTEs can be achieved by optimizing doses and using siRNA pools in order to minimize the contribution of individual siRNAs while preserving on-target activity (Bramsen & Kjems, 2011). An improvement on specificity could be also achieved by altering siRNA sequences and/or by introducing chemical modifications that are able to reduce off-target potentials. Several studies have aimed at reducing siRNA OTEs by chemically modifying the seed region of the guide strand with 2′O Me on position 2 (Jackson, et al., 2006) or by incorporating a strongly destabilizing unlocked nucleic acid (UNA) modification at position 7, which induces a position-specific destabilization of seed-target interactions (Bramsen & Kjems, 2011). Similarly, replacing of seed sequence nucleotides by deoxynucleotides results in a reduction of OTEs (Ui-Tei et al., 2008). These modifications avoid incorporation of the sense strand and promote incorporation of the antisense strand of the siRNA duplex into the RISC complex. Finally, another way of reducing the unwanted effects is by striving for very specific delivery, i.e., the more targeted the delivery of the siRNAs, the less the likelihood of suffering OTEs.

4.3 Oversaturation of endogenous RNAi-silencing complex

Bioactive drugs that rely on cellular processes to exert their functions face the risk of saturating endogenous pathways. This may be the case with RNAi-based drugs. Naturally occurring small RNAs exist in a perfect balance with their precursors and targets, as well as with the associated machinery involved in this process. Gene silencing is performed by

introducing artificially synthesized small RNAs into the cell or by inserting siRNAs/shRNAs within the cell, which enter the endogenous RNAi pathway at different levels. shRNAs and siRNAs are very similar to miRNA precursors before and after Dicer processing, respectively, relying on endogenous miRNA machinery to achieve target silencing. Therefore, miRNA pathways might get saturated by high doses of exogenous RNAs. One of the ways adenovirus avoid potential host RNAi antiviral activity is by expressing high amounts of a non-coding RNA stem-loop that interferes with transport from the nucleus to the cytoplasm by binding to the nuclear karyopherin Exp-5, inhibiting transport and subsequent processing of cellular pre-miRNAs (Fedorov et al., 2005). Similarly, some reports have described that *in vivo* adeno-associated virus (AAV)-encoded overexpression of liver-directed shRNAs can saturate Exp-5. This results in the inhibition of endogenous pre-miRNA nuclear export and, ultimately, death (Grimm et al., 2006). Strong expression of shRNAs has also been shown to induce cytotoxicity in primary lymphocytes, whereas the same shRNA expressed using a weaker promoter presents no toxic effects (Lu & Cullen, 2004). Similarly, robust levels of antisense RNAs emerging from shRNA expression systems cause toxicity in the mouse brain, regardless of the sequence (An et al., 2006). The export function mediated by Exp-5 is not required for the activity of synthetic siRNAs (McBride et al., 2008) but a recent report has shown that synthetic siRNAs and expressed shRNAs compete against each other and with endogenous miRNAs for transport and incorporation into RISC and that TRBP is one of the sensors for selection and incorporation of the guide sequence of interfering RNAs (Yi et al., 2003). If the siRNA design parameters are not optimal they might cause imbalance of the endogenous small RNA mediated pathways resulting in various and deleterious unwanted effects in the cells. Thus, a number of factors altering endogenous cellular processes can result in toxicity. It becomes crucial to optimize the siRNA/shRNA design parameters and work at the lowest possible concentrations to mitigate the potential of unwanted side effects.

5. Targets addressable by RNAi: Therapeutic application of RNAi

Since the first description of RNAi in 1998 by Tuschl and coworkers (Tuschl et al., 1998), this mechanism has rapidly been exploited for therapeutic applications. To date, several RNAi-based drugs against a variety of targets in humans have been developed. Antisense strategies offer very high target specificity, having the potential to lead a revolution in the field of drug development. Additionally, the relatively short turnaround for efficacy testing of potential RNAi molecules and the fact that any target is theoretically amenable to targeting, makes them invaluable tools to treat a wide range of diseases.

5.1 Infectious diseases

5.1.1 Virus infections

Viral infections are usually difficult to treat with conventional drugs and, in the cases where success is achieved, drug resistance may rapidly become an issue. This latter characteristic, together with the fact that viral genes are substantially different from human genes, makes viral infections obvious candidates for RNAi therapy. In order to obtain durable and effective antiviral therapies, viral proteins that can be disabled need to be identified. Ideally, these targets should be essential factors that share conserved sequences across many

different strains or even among different species of virus of the same family, so that a single target will have broad effectiveness (Das et al., 2004; Wilson & Richardson, 2005). In addition, target sequences should be as different as possible from any human protein to reduce the likelihood of side effects. Alternatively, host factors essential for virus replication could also be targeted, reducing the risk of viral escape, although the chances of affecting vital cell processes by this approach are considerable. The main caveat of targeting viral infection with RNAi is the ability of most viruses to mutate their target sequences in order to escape RNAi attack.

Several reports have indicated the promise of siRNAs for treatment of viral infections, showing a cessation of viral proliferation in human cell cultures. Upon exposure to siRNA, cells shut down production of the proteins needed for pathogen reproduction. Research is ongoing to find antiviral compounds for the following viral infections: hepatitis C and B virus (McCaffrey et al., 2003; Morrissey et al., 2005a), HIV type 1 (DiGiusto et al., 2010), human papillomavirus type (HPV) 16 (Jung et al., 2010; Palanichamy et al., 2010), influenza viruses A (Seth et al., 2010; Zhiqiang et al., 2010), respiratory syncytial virus (RSV) (Bitko & Barik, 2001; Bitko, et al., 2005; DeVincenzo, J. et al., 2010; DeVincenzo, J.P. et al., 2010; Zamora et al., 2010), herpes simplex virus 2 (Palliser et al., 2006), West Nile virus (WNV) (Anthony et al., 2009; Ye et al., 2011), severe acute respiratory syndrome (SARS) virus (Li et al., 2005; Wu & Chan, 2006) and cytomegalovirus (CMV) (Wiebusch et al., 2004).

Sereval R&D programs are currently focusing their efforts on infection diseases, HIV is one of these examples. Introduction of synthetic siRNA into cells or its stable endogenous production using vector-driven shRNA have been shown to suppress HIV replication *in vitro* and, in some instances, *in vivo* (Subramanya et al., 2010). RNAi can specifically degrade the HIV-1 genome in infected T cells very early in the viral replication cycle. This suggests the possibility of a therapeutic strategy that targets the virus before it has a chance to develop escape mutations. To date numerous siRNAs targeted to a number of HIV-1 or host dependency factors (HDF) transcripts have been demonstrated to achieve viral inhibition both *in vitro* and *in vivo*. Indeed, HIV-1 encoded genes tat, rev, gag, pol, nef, vif, env, vpr and the long terminal repeat (LTR) are all susceptible to RNAi–induced gene silencing in cell lines (Tsygankov, 2009). Several researchers have described the ability of siRNAs to interfere with HIV viral production after infection before mutations can occur. However, after a provirus is established, many thousands of viral transcripts are generated *de novo* in the infected cell and degradation of these is a far greater task for the RNAi machinery. For the development of a durable gene therapy that prevents viral escape, a multiple shRNA approach has been proposed against conserved HIV-1 regions (Schopman et al., 2010; ter Brake et al., 2006). Expression of three different shRNAs from a single lentiviral vector resulted in high levels of inhibition. Thus, their combined expression induced a much stronger inhibition of virus production.

Scientists at Benitec Ltd in collaboration with the City of Hope Medical Center in California are investigating a method to fight HIV infection in lymphoma patients with genetically modified stem cells, eradicating both the lymphoma and the HIV infection at the same time. The treatment is the first to use specially engineered anti-HIV genes inserted into the patients' own harvested stem cells. The cells are then reinfused into the HIV-infected patient. If successful, the new treatment would allow the organism of the patient to produce HIV-resistant white blood cells indefinitely. A triple combination lentiviral construct is used

to carry the gene segments into the stem cells. The lentivirus vector encodes 3 forms of anti-HIV RNA: (1) RNAi as shRNA targeted to HIV-1; (2) a decoy for the HIV TAT-reactive element (TAR); and (3) a ribozyme that targets the host cell CCR5 chemokine receptor. This compound received FDA approval and entered phase I clinical trial in 2007 (DiGiusto, et al., 2010).

Several well-known HDF, including NFkappaB, CD4 receptor and coreceptors CCR5 and CXCR4, have been successfully targeted by siRNAs, thereby suppressing HIV replication or entry (Singh & Gaur, 2009). A potentially promising strategy is to exploit siRNAs to prevent viral entry at the cell surface by downregulating essential cell surface HIV-1 co-receptors. This approach has been used by two groups, the first one targeted the CXCR4 co-receptor with siRNA (Anderson et al., 2003); while the second group transduced T lymphocytes with a CC5-siRNA reducing cell membrane CCR5, and protecting the cells from infection with R5-tropic HIV (Cordelier et al., 2003). Transduction with CCR5-siRNAs substantially reduced CCR5 mRNA in other cell lines such as CCR5 + cell lines, primary human macrophages and brain microglia. Scientists at the California Institute of Technology have reported success in constructing a lentivirus-based vector to introduce siRNAs against the HIV-1 coreceptor CCR5 into human peripheral blood T lymphocytes (Qin et al., 2003). This approach has not only successfully reduced CCR5 expression but also provided substantial protection from CCR5-tropic HIV-1 virus infection, dropping infected cells by 3- to 7-fold. Also bi-specific siRNA constructs, containing an 8 nucleotide intervening spacer targeted against CXCR4 and CD4 or CXCR4 and CCR5 have been shown to inhibit viral entry (Anderson & Akkina, 2005). Cleavage of the bispecific constructs yielding monospecific siRNAs was shown to occur in cell extracts showing significant downregulation of their respective coreceptors. These results demonstrated the practical utility of short hairpin shRNA bi-specific constructs synthesized as single transcripts. It is now possible to introduce promising multivalent shRNA constructs into retroviral and lentiviral vectors for *in vivo* gene therapeutic applications.

5.1.2 Parasitic diseases

Parasitic infections are especially endemic in low-income populations in developing regions of Africa, America and Asia. They include, among others, schistosomiasis infections, leishmaniasis, leprosy, tuberculosis and malaria. Although a few initial approaches have suggested RNAi may be a useful tool in the treatment of some of the neglected tropical diseases, it is the treatment of malaria that has received the most attention.

Despite intense efforts, malaria remains a leading cause of morbidity and mortality worldwide. Recent evidence strongly suggests that RNAi can play a key role in identifying the genetic factors that shape the vector parasite relationship and may be crucial to identifying new genetic means of controlling mosquito-borne diseases (Vlachou et al., 2005). The Institute of Molecular Medicine (IMM) in Lisbon has pioneered an *in vitro* assay system to monitor the crucial process of sporozoite infection using the malaria parasite *Plasmodium berghei* with cultured human liver cells. Cenix BioScience has collaborated with IMM to adapt and optimize the malarial infection assay for high throughput kinome-wide RNAi screening. The screen led to discovery of five targets clearly implicated in malaria, all of which resulted in significant reductions in infection *in vitro* when silenced with small interfering siRNAs (Prudencio et al., 2008). PKCζ, was selected for further evaluation *in vivo*. In mice given a systemically delivered, liposomally formulated anti-PKCζ RNAi

therapeutic, an inhibitory effect on infection was observed. In fact, loss of PKCζ function *in vivo* by RNAi-mediated silencing led to an 80% decreased infection rate. In addition, certain siRNAs also led to a delay in the appearance of parasites in blood and a significant reduction in average blood parasitemia. The identified genes will represent excellent candidates for the development of novel antimalarial therapeutics.

5.2 Neurological disorders

Neurodegenerative disorders are a group of neurological disorders characterized by selective dysfunction and eventual death of distinct subpopulations of neurons in the central nervous system, accompanied by a concomitant decline in specific neurological functions. Currently there are no effective therapies for most neurodegenerative diseases (DeKosky & Marek, 2003; Shaw et al., 2007). Whenever treatments are available, such as in the case of Alzheimer's disease (AD) or Parkinson's diseases (PD), these treatments provide only partial relief for some of the symptoms. In recent years, significant research effort has focused on understanding the underlying pathologies of diseases such as AD, PD, Huntington disease (HD) and amyotrophic lateral sclerosis (ALS).

The key therapeutic advantage of using RNAi for the treatment of neurodegenerative diseases is its ability to specifically and potently knock-down the expression of disease-causing genes of known sequence. In several neurodegenerative diseases, such as prion mediated diseases (Schwartz, 2009; White et al., 2008), polyQ-repeat diseases (HD and spinocerebellar ataxias) (Schwartz, 2009; Seyhan, 2011) PD and ALS (Maxwell, 2009), a single allele is mutated. This mutated allele leads to the production of a toxic protein, causative agent of the pathology.

AD is an example of neurological disorder addressable by RNAi technology. Tau and amyloid precursor protein (APP) are involved in the pathogenesis of sporadic and inherited AD. Certain dominantly inherited mutations have been associated with early onset forms of AD. Scientists at the University of Iowa have developed siRNAs that display optimal allele-specific silencing against a well-characterized Tau mutation (V337M) and the most widely studied APP mutation (APPsw) (Miller et al., 2004). Recent studies have shown that a reduction in BACE-1 expression using siRNA or lentiviral vectors expressing siRNAs targeting *BACE-1,* results in reduced Aβ production in primary neurons from wild-type and APPsw transgenic mice, showing a reduction in the number and size of plaques and protection from oxidative stress (Kao et al., 2004; Singer et al., 2005). Other potential therapeutic targets for siRNA-mediated gene silencing in AD are α- and β-secretases.

ALS is a progressive, devastating syndrome that affects both upper and motor neurons. ALS involves a multifactorial and interactive chain of pathogenic mechanisms that are probably common to several neurodegenerative disorders. Various therapies have been tried but none has proved to stop progression of the disease. In 25% of familial ALS cases, the disease is caused by dominantly acting point mutations in the gene superoxide dismutase (SOD1). RNAi has been evaluated for selective silencing of mutant SOD1 expression in cultured cells. In one study, siRNAs were capable of specifically inhibiting expression of ALS-linked mutant, but not wild-type SOD1, protecting from induced cell death (Maxwell et al., 2004). The main challenge of creating a potential RNAi-based therapeutic for ALS is in the large number of different SOD mutations that have been identified. In inducible mouse models of spinocerebellar ataxia type 1 (SCA1) and HD, repression of the mutant allele

expression improves disease phenotypes. Thus, therapies designed to inhibit expression of the mutant gene should be beneficial. Scientists at the University of Iowa College of Medicine in collaboration with the University of Minnesota and the NIH have shown that RNAi can inhibit polyglutamine-induced neurodegeneration caused by mutant ataxin-1 in a mouse model of SCA1. Mice suffering from SCA1 treated with RNAi-based compounds had normal movement and coordination, and their brain cells were protected from the destruction normally caused by the disease.

HD gene has been cloned and the mutation contains an unstable trinucleotide repeat (CAG) that expands the length of a repeated stretch of amino acid glutamine in the gene product, a protein called huntingtin. Currently there is no cure for HD and treatments can only alleviate disease symptoms (Harper, 2009) RNAi directed against mutant human *hungtingtin* reduces hungtingtin mRNA and huntingtin protein expression in cell culture and in the brain of a HD mouse model after intrastriatal treatment with AVV-expressing shRNA against human huntingtin (Harper et al., 2005). Detailed examination of the protein levels in the treated mice showed that levels of the toxic protein were reduced to about 40% of the levels found in untreated mice. Behavioural and neuropathological abnormalities associated with HD improved following *hungtingtin* gene silencing.

5.3 Respiratory diseases

Targeted, local delivery of RNAi to the lungs via inhalation offers a unique opportunity to treat a wide range of untreatable respiratory conditions. Delivering the drug to the lungs by instillation allows for direct access to the lung epithelial cells. This direct targeting to the lung cells allows for a reduction in the dosage of the siRNA required for achieving efficacy and simultaneously diminishes the likelihood of off target effects. Diseases treatable with this approach include viral lung diseases, cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), allergy, asthma and lung cancer among others.

CF is the most common autosomal recessive disorder in Caucasians. A defective gene causes the body to produce abnormally thick, sticky mucus that obstructs the lungs, leading to life-threatening lung infections, and the pancreas, causing difficulty absorbing food. This genetic disease is marked by defects in a protein, known as the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein, required for proper transport of salt and water across the cell membrane. It has been hypothesized that the missing function of CTFR causes an increased ENaC-mediated sodium uptake from the luminal secretion of the airways, deplets the airway surface liquid and leads to defective mucociliary clearance (Chambers et al., 2007) and to the production of a thick and sticky mucus that clogs the lungs and the digestive system. This promotes bacterial infections, resulting in a slow progressive destruction of the airway tissue and difficulties in absorbing food. Due to the central role of ENaC-mediated sodium uptake by the respiratory epithelium for the pathogenesis of CF, it has been postulated that a reduction in ENaC activity could attenuate CF lung disease (Yueksekdag et al., 2010). Yueksekdag and co-workers have designed, synthesized and first used ENaC-specific small interfering RNAs in M-1 cell cultures and in a mouse model (Yueksekdag, et al., 2010). Their results showed that a single *in vitro* application was sufficient to decrease ENaC mRNA levels, whereas repeated administrations were necessary to obtain a significant reduction for ENaC mRNA level in the *in vivo* application.

Asthma is marked by inflammation of the airways and Syk kinase has been described to play a critical role in the regulation of such immune and inflammatory responses. Initial

studies used aerolised Syk-specific antisense oligonucleotides in liposome complexes to significantly decrease lung inflammatory responses in asthma and acute lung injury models (Ulanova et al., 2005). This approach has been further developed by Canadian biotech Zabecor, who is developing an siRNA against Syk for asthma treatment. It has also been suggested that IL-5 is involved in the development of airway hyper-responsiveness (AHR). siRNAs targeting IL-5 were characterized *in vitro*, and administered intratracheally to OVA-induced murine model of asthma (Huang et al., 2008). siRNA targeting IL-5, efficiently moderated the characteristics of asthma, including AHR, cellular infiltration of lung tissues, and IL-5 mRNA levels in lungs in the mouse model of asthma.

5.4 Skin disorders

The skin has a high turnover rate and can be removed without complicated surgical procedures if required; hence, it represents a good model for monitoring of treatment. Applications for cutaneous delivery of therapeutic siRNA are emerging owing to a strong demand for effective treatments of various skin disorders. Although successful studies have been performed using several different delivery techniques, most of these techniques encounter limitations for translation into the clinic with regards to patient compliance.

Pachyonychia congenita (PC) is an extremely rare, highly disabling autosomal dominant inherited disorder with less than a few thousand cases worldwide (Kaspar, 2005). This disorder which affects nails, skin, oral mucosa, hair and teeth (Smith et al., 2005), causes painful plantar calluses for which no satisfactory treatment is currently available. PC is caused by mutations in either keratin K6, K16 or K17, including small deletions and single nucleotide changes. Selective depletion of the mutated keratin with siRNAs has the potential to directly target the molecular aetiology of the disease (Leachman et al., 2010). The lack of effective PC therapies, the accessibility of skin disease lesions and the ability to visually observe changes during treatment make PC a good human skin disease model for testing siRNA in a proof-of-concept trial for genetic disorders. TransDerm Inc. has developed an siRNA-based therapy for PC. Their compound, TD101 has been shown to specifically target the cytosine-to adenine single nucleotide K6a mutation (Leachman et al., 2008) and to reverse the mutant phenotype of cells in a dominant-negative tissue culture model by restoring their ability to form a structurally intact keratin intermediate filament network (Leachman, et al., 2010).

5.5 Cardiovascular pathologies

During the past few years many conceptual and technical advances have been made towards the therapeutic modulation of cardiac gene expression, leading to the identification of new therapeutically relevant targets.

One of the most important risk factors for cardiovascular disorders is hypercholesterolemia. RNAi approaches are under investigation for this disorder. Hepatic ABCA1 contributes to HDL plasma levels and influences lipemia. An adenovirus-mediated RNAi approach has been used to test the efficiency of plasmid-based siRNA-induced knockdown of cotransfected murine ATP binding cassette transporter A1 (ABCA1) (Ragozin et al., 2005). Compared to controls, Ad-anti-ABCA1 infected mice showed 50% reduction of endogenous ABCA1 and a clear upregulation of apolipoprotein E (ApoE). Similarly, in 2004 Alnylam scientists demonstrated that chemically modified siRNAs can silence an endogenous gene encoding apolipoprotein B (ApoB) after intravenous injection in mice resulting in reduction of total cholesterol (Soutschek, et al., 2004). These siRNAs were also shown to silence human

ApoB in a transgenic mouse model. Additionaly, ApoB specific siRNAs encapsulated in SNALPs, and administered intravenously, were shown to silence ApoB in non-human primates, and resulted in dose-dependent silencing of ApoB mRNA in the liver, with >65% lowering of cholesterol and >85% lowering of LDL. Significant reductions in ApoB protein, serum cholesterol and low-density lipoprotein levels were observed as early as 24 h after treatment and lasted for 11 days at the highest siRNA dose, thus demonstrating an immediate, potent and lasting biological effect of siRNA treatment. These findings show clinically relevant RNAi-mediated gene silencing in non-human primates (Zimmermann et al., 2006). Similarly intravenous administration of an ApoB-specific siRNA modified with a dendritic poly-L-lysine (KG6) resulted in knock-down of ApoB in healthy C57BL/6 mice without hepatotoxicity, and a significant reduction of serum lipoprotein cholesterol in apoliporpotein E-deficient mice (Watanabe et al., 2009). In 2006, Alnylam scientists presented data on a new target for the treatment of hypercholesterolemia. They showed that silencing the proprotein convertase subtilisn/kexin type 9 (PCSK9) a protein involved in the regulation of LDL cholesterol in mice resulted in meaningful reductions in cholesterol levels, yielding the first *in vivo* evidence that pharmacologic targeting of PCSK9 can result in potential therapeutic benefit (Duff & Hooper, 2011).

5.6 Ophthalmology

Perhaps the greatest success of siRNA technology has come from local administration of siRNAs to the eye. The eye is a relatively isolated compartment with low content in RNAses and limited access to the immune system. This relative isolation of the eye allows for a reduced systemic exposure to the drug and thus a reduction in possible toxic side effects. Several eye diseases have been approached with siRNA-based therapeutics, including wet age-related macular degeneration (AMD) and diabetic retinopathy (DR). These diseases are the two main causes of irreversible vision loss in the developed world. in fact, the first approaches to target human diseases with siRNAs aimed at the treatment of AMD and, currently, the eye is the focus of several R&D programs (see section 8 for more information on this topic).

Dry eye syndrome is a prevalent disease that affects visual acuity, activities of daily living and quality of life. Symptoms accompanying dry eye syndromes include itching, burning and irritation of the ocular tissues. Its severity can range from very mild disease to extremely severe cases with vision-threatening consequences. A more severe form of dry eye occurs in patients with Sjogren's syndrome. Further taking advantage of the ocular environment, Sylentis is developing an anti-TRPV1 siRNA compound for the treatment of ocular discomfort associated to dry eye syndrome.

Retinitis pigmentosa (RP) is a leading cause of inherited blindness. This disorder involves photoreceptor-cell degeneration and affects ~ 1 in 3000 people (Farrar et al., 2002). Mutated forms of the rhodopsin (RHO) produce a toxic protein in the retina that kills cells that receive light. Although great efforts have been made to explore new gene therapies for RP, inter and intragenic heterogeneity represents significant barriers to a therapeutic development. For example, more than 100 mutations in the human *RHO* gene, which encodes the photosensitive pigment in rod photoreceptors, have been identified in autosomal dominantly inherited RP (adRP) (O'Reilly et al., 2007). Development of therapies for each individual mutation would be technically difficult to achieve and not economically viable; thus a therapeutic approach that circumvents mutational diversity would be of great value. Several approaches have been made with this aim but none of them have successfully passed beyond R&D so far.

5.7 Cancer

In general, there are three potential targets for cancer therapies: genes that are part of cancer–associated pathways, genes involved in tumor-host interaction or those that are part of chemo-or radiotherapy resistance (Gartel & Kandel, 2006; Grimm & Kay, 2007; Takeshita & Ochiya, 2006). The most promising oncogene targets are those associated with malignant transformation. These genes are usually amplified, mutated and amplified as a result of chromosome or gene rearrangement or exogenously introduced by transforming viruses in human tumours. Although multiple genetic mutations have been identified in most human tumours it is often not clear which of these mutant oncogenes are the actual cause of tumorogenesis (Brummelkamp et al., 2002b). Oncogenes found in human cancer often differ by only a single base mutation from their wild-type counterparts required for viability of normal cells. No treatments are available to date to specifically inactivate just the mutant version of such oncogenes. Hence, RNAi has become a logical strategy to selectively target each of these mutant alleles that define oncogenesis, including novel fusion proteins that define many types of cancer (Ameyar-Zazoua et al., 2005). There is emerging evidence that siRNA may represent a novel therapeutic modality for cancer treatment when optimized local and systemic delivery systems are available. Researchers at the Netherlands Cancer Institute reported successfull retroviral delivery of siRNAs that leave the wild-type K-RAS allele untouched while specifically inhibiting the mutant K-RASV12 allele in human pancreatic carcinoma, leading to loss of tumorigenicity and persistent loss of oncogenic phenotype (Brummelkamp, et al., 2002b). This is the first evidence in living animals that cancer can be controlled by blocking the expression of a single mutant protein using RNAi. HPV plays an important role in causing cervical cancer. Specific knockdown of HPV18 E6 and E7 oncogenes has been achieved resulting in efficient inhibition of the growth of HPV-positive cervical cancer cells (Wise-Draper et al., 2005). Different lentiviral RNAi approaches have been used to target different oncogenes. For example, researchers introduced a lentiviral vector into glioma cells targeting bcl-2, whose overexpression mediates protection from apoptosis. Delivered alone or in combination with another vector expressing a secreted form of TRAIL, a TNF family member, a significant reduction of tumours was achieved after their transplantation into nude mice (Kock et al., 2007). Similarly, others used a lentiviral RNAi to inhibit BRAF and Skp-2 genes, that are frequently up-regulated and mutated in melanoma cells, showing significant anti-tumour efficacy (Sumimoto et al., 2005). Fusion oncogenes, especially prevalent in lymphoproliferative cancers are attractive targets for RNAi, as chimeric mRNA is unique to tumour cells. The bcr-abl fusion oncogene in chronic myelogenous leukemias resulting from translocations t(9;22) is an example. Sengupta and collaborators targeted the bcr-abl junction in primary CD34+ CML cells using an Epstein-Barr virus-encoded shRNA in primary CD34+ CML cells which led to apoptosis (Sengupta et al., 2006). RNAi in combination with anticancer drugs has also been investigated in mice to test whether a combination therapy can promote apoptosis and reduce tumour burden. Small molecule inhibitors, such as Imatinib, are effective therapies for tyrosine kinase fusions BCR-ABL-TEL-PDGFβR-mediated human leukemias, but resistances are often developed. The unique fusion junctions of these molecules are attractive candidates for RNAi. Chen and co-workers have developed a retroviral system for stable expression of siRNA directed to the unique fusion junction sequence of TEL-PDGFβR oncogene (leads to leukemia in humans) in transformed hematopoietic cells. Stable expression of the siRNA resulted in approximately 90% inhibition of TEL-PDGFβR expression and its downstream effectors. Inhibition of this gene extended disease latency and survival of mice that received

transplants, but also mediated synergism with Imatinib (Chen et al., 2004). Similarly Landen and collaborators demonstrated that inhibition of EphA2 by siRNA resulted in a 50% of reduction of tumor size and its combination with paclitaxel resulted in a 50% reduction of tumor size in ovarian cancer cells (Landen et al., 2005). RNAi strategy has also been described to effectively silence other cancer related genes such as mutant forms of P53, KRas (Berns, 2010) to increase chemosensitivity by RNAi, silencing thymidylate synthase (TS) mRNA (Schmitz et al., 2004) or drug sensitivity and resistance genes (McCarroll et al., 2010), or to induce cancer immunity by the RNAi-induction of potent antigenic determinants and the immune-mediated rejection (Pastor et al., 2010).

6. Targeting microRNAs: A new paradigm and emerging field in drug development

6.1 Mechanism of action of miRNAs

MicroRNAs are small non-coding RNA molecules of approximately 22 nucleotides that regulate gene expression of their target genes usually at the post-transcriptional level (Bartel, 2004). miRNAs are synthesized as pri-miRNAs by polymerase II (Figure 1). Pri-miRNAs are processed in the nucleus by an RNase III enzyme named Drosha to generate the so called pre-miRNAs (Lee et al., 2003). Pre-miRNAs are transported from the cell nucleus to the cytoplasm by exportin-5 where the final cleavage takes place (Lund et al., 2004). The final cleavage is performed by another RNase III enzyme called Dicer, which yields the mature functional miRNAs. The mature double-stranded miRNAs are dissociated and one of the strands is loaded into AGO proteins resulting in the assembly of the miRNA ribonucleoprotein complex (miRNP). This strand then guides the miRNP complex to the target. In contrast to siRNAs, miRNAs are not in general perfectly complementary to their target mRNA. miRNA interact with their target mRNA by small "seed sites"; which are base pairings between 2 and 8 nucleotides of the miRNA with the 3′ UTR region of the target mRNA. Binding of the miRNP to its target leads to repression of the target gene by translational inhibition of the target mRNA (Wightman, et al., 1993). Depending on the degree of miRNA-mRNA complementarity, miRNA can induce an AGO-mediated degradation of the target mRNA, thus regulating gene expression by reducing mRNA levels (Hutvagner et al., 2001). Although miRNAs usually silence gene expression, recent work has shown that a few miRNAs are able to enhance the expression of their target genes (Orom et al., 2008). Enhancement of gene expression is achieved by using miRNA binding sites located in the 5′ UTR of the target mRNA.

The seed region of a miRNA is of particular interest in targeting and is also the most evolutionary conserved region of miRNAs. According to the 17th version of the miRBase released in April 2011, 1424 miRNAs have been identified in humans and 720 in mice. Because miRNAs do not require being perfectly complementary to their target sequence one microRNA is potentially able to affect hundreds or even thousands of targets. This mechanism of regulation indicates that miRNAs could regulate a given phenotype by regulating the expression of a single upstream key target or by regulating several targets in the same biological route. It should be mentioned however that miRNAs do not usually robustly modulate a single mRNA, but it is its ability to affect multiple targets that allows them to be key regulators of most biological processes. The activity of miRNAs acts as an amplification mechanism in which the actual number of identified miRNAs could potentially regulate most of the human genes. Given the prominent role miRNAs have on

gene expression it is not surprising that alterations in their expression profile can lead to disease and to alterations in the mechanisms of drug action (Rukov & Shomron, 2011). As a consequence, multiple pharmaceutical and biotechnology companies have focused their efforts on miRNAs as therapeutic targets and diagnostic biomarkers.

6.2 Role of miRNAs in disease

The ability of miRNAs to regulate gene expression at a very upstream level point out that dysregulation of miRNAs can lead to multiple pathologies. Concomitantly, the machinery that leads to the biogenesis of miRNAs has been profoundly studied, especially at the level of Dicer, and its alteration has been shown to have devastating consequences. Loss of Dicer1 leads to lethality early in development, with Dicer1-null embryos depleted of stem cells (Bernstein et al., 2003). Because Dicer1 knock-out mice are lethal, Dicer1 has been selectively knocked-out in most tissues. The consequences of deleting Dicer1 are multiple and reflect the key role of miRNAs in gene regulation. Targeted deletion of Dicer1 from specific tissues or cells has been shown to alter cell cycle regulation and enhance tumor susceptibility (Sekine et al., 2009), dysregulate immune cell differentiation (Koralov et al., 2008; Muljo et al., 2005), cause autoimmunity (Zhou et al., 2008), impair neural development (Davis et al., 2008; Huang et al., 2010), alter central nervous system function and promote neurodegeneration (Damiani et al., 2008; Hebert et al., 2010), impair pancreatic islet cell genesis (Lynn et al., 2007), cause dilated cardiomyopathy and heart failure (Chen, 2008), impair spermatogenesis (Hayashi et al., 2008), dysregulate skin development (Yi et al., 2009), prematurely stop inner ear development (Soukup et al., 2009), alter smooth muscle function (Albinsson et al., 2010) and impair prostate development (Zhang et al., 2010).

One of the most critical observations in miRNA pathobiology is the recognition that miRNAs modulate the transcriptome to promote or to attenuate cancer predisposition. miRNAs have a critical role in regulation of cellular differentiation, angiogenesis, survival and growth. Additionally, global repression of miRNA seems to be a common feature in most cancers, including lung cancer, breast cancer, colon cancer, pancreatic cancer and leukemia (Croce, 2009). Remarkable changes have been observed in the miRNA expression pattern between tumours and normal tissue (Volinia et al., 2006; Yanaihara et al., 2006) and approximately 10% of all the up to date described miRNA have been shown to be altered in pulmonary fibrosis (Pandit et al., 2011).

Myocardial miRNA profiling has led to the identification of more than 200 miRNAs that are consistently expressed in heart tissue. Many of these miRNAs are deregulated in heart disease. Several miRNAs that control the switch between foetal and adult enzymes have been identified. Deregulation of these miRNAs has been linked to cardiomyocyte hypertrophy, ventricular septal defect and heart failure (van Rooij et al., 2009).

During the past several years miRNAs have emerged as key regulators of immune cell lineage commitment, differentiation and maintenance of both innate and adaptive immune response (Baltimore et al., 2008). Dysregulation of the checkpoints that maintain immune tolerance is one of the hallmarks of autoimmunity. Regulatory T cells (Tregs) suppress the activation of effector T cells to maintain immune system homeostasis and tolerance to self-antigens. Ablation of miR-14 in Treg cells and overexpression of miR17-92 in lymphocytes have shown to promote autoimmunity in mouse experimental models (Kohlhaas et al., 2009). Additionally, deregulation of the normal pattern of miRNA expression has been identified in patients of systemic lupus erithematosus, rheumatoid arthritis and multiple

sclerosis (Dai & Ahmed, 2011). In addition to autoimmunity, miRNAs have been found to have a role in other alterations in immunity such as psoriasis, a chronic inflammatory disease.

Several miRNAs have been found to be differentially expressed in the central nervous system (CNS) (Fiore et al., 2008). These CNS-restricted miRNAs have a role in neuronal development and function. As seen in other organs, deregulation of the normal pattern of expression of these miRNAs can lead to disease, in the case of the central nervous system, to neurodegenerative diseases. Although miRNA expression profiling has been achieved with different degrees of success for some neurodegenerative diseases such as AD, PD and HD, it should be mentioned that miRNA profiling is particularly challenging in the central nervous system. Firstly, some of the miRNAs are only expressed in very small populations of neurons or non-neuron cells in specific brain areas; secondly due to the particularities of neurodegenerative diseases, it is difficult to obtain healthy and diseased tissue from the same patient. This latter reason has raised issues when addressing the role of miRNAs in initiating the disease rather than being a secondary factor caused by deregulation of biological pathways. Conditional inactivation of Dicer in neurons has shed some light to this issue. Selective deletion of Dicer in dopaminergic neurons in mice leads to ataxia, front and hind limb clasping, reduced brain size and smaller neurons (Cuellar et al., 2008). Deletion of Dicer in other brain regions such as the hippocampus and in Purkinje cells in the cerebellum cause alterations that indicate that dysregulation of miRNA expression could be contributing to neurodegeneration (Schaefer et al., 2007), however effort should be made in the near future in elucidating the role of individual miRNAs in neurodegeneration in order to identify potential biomarkers and targets for the development of new drugs.

As summarized above, miRNAs have a crucial role in development of diseases. Dysregulation of gene expression is a common feature of most pathologies, thus it shouldn't be surprising that in the coming years alteration in the miRNA profiles are found for virtually every disease. Addressing the impact of these alterations on pathobiology is essential in order to develop clinically useful miRNA based therapies.

Some examples of the alteration of miRNAs in disease are shown in Table 1.

6.3 miRNAs as diagnostic biomarkers

Because of the crucial role of miRNAs in a plethora of diseases considerable effort has been made to identify specific miRNA profiles that could serve as biomarkers to help in the diagnosis of particular diseases or indicate the eventual course of a given disease. Some promising data has been obtained in the diagnosis of cancer where Rosenfeld and coworkers successfully profiled over 130 metastases of unknown tissue origin and were able to identify the tissue origin of the primary tumour with a 90% accuracy studying the miRNA profile (Rosenfeld et al., 2008). miRNA profiling has also proven useful in predicting the survival rate of cancer patients. Poor survival rate has been linked to high levels of miR-21 in colon adenocarcinoma (Schetter et al., 2008) and a combination of low levels of let-7a and high levels of miR-055 correlate with poor prognosis in lung adenocarcinoma (Yanaihara, et al., 2006). Several studies have also addressed the potential role of circulating miRNA profiling. miRNAs are stable in serum and plasma and their expression in blood is reproducible and, in some cases, indicative of the diseased state (Mitchell et al., 2008). miRNA serve as diagnostic markers in plasma and serum isolated from human patients with distinct forms of cancer (Kosaka et al., 2010). Correlation between levels of certain miRNAs and cancer has been found for lung cancer, hepatocellular carcinoma, pancreatic cancer, gastric cancer and

CLL among others (Ceman & Saugstad, 2011). The sensitivity of some of these methods has shown to be greater than traditional and more invasive diagnostic methods (Xie et al., 2009). Specific patterns of miRNA expression have been identified in plasma samples of rats following tissue injury (Laterza et al., 2009). Plasma levels of miR124 have arisen as a promising candidate biomarker for cerebral infarction (Wang et al., 2011) and correlation has been found between the plasma levels of miR17 and miR20 and multiple sclerosis (Cox et al., 2010). Additionally, modifications of the pattern of expression of miRNAs could eventually anticipate how a certain individual would respond to a particular treatment. This kind of approach has yielded some success in cancer, where miR-21 has been identified as a biomarker for early detection on non-small cell lung cancer and its levels are related to the sensitivity to platinum-base chemotherapy (Wei et al., 2011). It should be noted that, if successful, this approach is especially attractive due to the low invasiveness of the technique.

miRNA	**Disease**	**Change in disease**
miR-15a/16-1, miR-29b, miR-181b, miR-34	Chronic lymphocytic leukemia	Downregulated
miR-21, miR-155	Pancreatic cancer	Upregulated
let-7, miR-1, miR-29, miR126	Lung cancer	Downregulated
miR-17-92, miR-21, miR-155, miR-221/222	Lung cancer	Upregulated
let-7, miR-29, miR-30	Idiopathic pulmonary fibrosis	Downregulated
miR-126, miR-210	Ischemic myocardium infarct	Upregulated
miR-203, miR-146	Psoriasis	Upregulated in skin (miR-203), upregulated in immune cells (miR-146)
miR-125a	Lupus erithematosus	Downregulated in T cells
miR-148a , miR-21	Lupus erithematosus	Upregulated in CD4+ T cells
miR-146a	Rheumatoid arthritis	Upregulated in selected cells
miR-124[a]	Rheumatoid arthritis	Downregulated in synoviocytes
miR-326	Multiple sclerosis	Upregulated in CD4 T cells
mi-R34a, miR-155 , miR-326	Multiple sclerosis	Upregulated in MS lesion
miR-9, miR-25b, miR-128	Alzheimer's disease	Upregulated in hippocampus
miR-124[a]	Alzheimer's disease	Downregulated in hippocampus
miR-133b	Parkinson's desease	Downregulated in midbrain

Table 1. Some examples of miRNAs dysregulated in disease

6.4 miRNA therapeutics

Deregulation of miRNAs significantly modulates biological pathways. Alteration in these pathways cannot only lead to disease but also alter the way an organism responds to a drug.

miRNA expression profile can successfully be manipulated in order to modulate genes that cause disease or to alter the natural response to a given drug. For diseases in which a reduction in certain miRNAs underlies the diseased condition, re-introduction of the miRNA could be of therapeutic benefit (miRNA mimics). For those diseases in which overexpression of miRNAs are found, inhibition of the miRNA function could restore the proper pattern of gene expression (miRNA inhibitors).

6.4.1 Target validation

One of the first challenges in miRNA therapeutics is identification and validation of miRNA targets. Validation strategies usually imply proving the interaction between miRNAs and the predicted mRNA targets, demonstration of co-expression of the miRNAs and the target mRNAs in the same cell population, proving a predictable effect of the miRNA on target protein expression and finally demonstrating that miRNA mediated regulation of its target mRNAs leads to changes in biological function (Kuhn et al., 2008). In silico analysis largely facilitates the prediction of possible miRNA-mRNA interactions, thus predicting potential targets. Multiple algorithms have been developed in order to help predict the interaction between miRNAs and their targets (Lewis et al., 2003; Rajewsky, 2006). These analysis are generally based on complementary analysis between the seed region of the miRNA and the 5'UTR of the potential target mRNA. However, in order to demonstrate that a given mRNA is indeed target of a certain miRNA, reporter assays have to be carried out (Nicolas, 2011). To further validate miRNA targets *in vitro* experiments are usually performed in which mRNA profiles of cells or tissues either overexpressing or lacking a given miRNA are compared with wild-type cells or tissues. An alternative approach to demonstrate miRNA-mRNA interaction is to take advantage of the miRNA-mRNA-AGO interaction to form the miRNP complexes. miRNP complexes can be immunoprecipitated followed by generation of cDNA libraries which can then be analysed by microarrays (Beitzinger et al., 2007). Finally, gain or loss of function *in vivo* studies are needed to assess the biological function of miRNAs in health and disease (Kuhn, et al., 2008).

6.4.2 Therapeutic options, chemistry and delivery

Several approaches can be used when aiming to restore the normal function of miRNA mediated gene modulation. Increasing the function of a given miRNA can be achieved by re-introducing the miRNAs in the target cell population or by reactivation of its biogenesis by manipulating gene expression using a conventional small molecule strategy. Reduction or even inhibiting the action of miRNAs can be approached by inhibiting its expression using either conventional strategies, that target transcription factors, which would in turn decrease its gene expression, or innovative RNAi based technologies. Introduction of miRNA mimics or other types of oligonucleotides in order to decrease the expression of miRNAs using the cellular RNAi machinery requires delivering stable oligonucleotides into the target cells or tissues. Biological cell membranes are natural barriers for oligonucleotides, which are high molecular weight, highly charged polyanionic molecules. By introducing chemical modifications in the oligonucleotides, some of the hurdles these molecules face when trying to reach their target organs have been addressed. Some of the nucleotide modifications developed in order to increase stability of the molecules have collaterally increased their delivery rate as well. Some of these modifications include 2'-O-methyl modification of RNA, phosphorothioate linkage, modifications in the 2' ribose and LNAs.

Oligonucleotides carrying these modifications have been found to be effective in both cell cultures and mice (Seto el al., 2010). The success of these modifications has allowed Santaris Pharma to develop one compound, carrying LNA modifications in its structure, to phase II clinical trials. Oligonucleotide analogs are also being used hoping to improve stability of miRNA inhibitors and to increase the affinity of the miRNA-mRNA target interaction; these analogs include peptide nucleic acids (PNA) and morpholinos. Although morpholinos have demonstrated their efficacy when injected in zebrafish embryos (Moulton & Jiang, 2009) and PNA oligonucleotides have been used to inhibit the expression of miRNAs in cell culture (Oh et al., 2011), *in vivo* validation in small animal models has yet to be carried out.

Depending on the therapeutic goal and the target tissue viral vectors may be used to introduce miRNAs into cells. The obvious advantages of using viral vectors for delivery of miRNAs are efficient delivery to the target organs and increased over time production of the transgene. Retroviruses, adenoviral vectors, AVV and lentiviral vectors have been widely used to express miRNA mimics in cells (for a recent review see (Liu & Berkhout, 2011)). Viral vectors have also been developed to inhibit miRNA function, these vectors express miRNA target sites to inhibit specific miRNAs or a miRNA family from regulating their natural targets, they are the so called "antagomirs". These novel approaches have been used to target multiple miRNAs and have already demonstrated efficacy in mouse models (Krutzfeldt et al., 2005).

miRNA-targeted therapies can be systemically or locally administered, and recent efforts have been aimed towards targeted delivery of drugs. Systemic delivery of oligonucleotides is challenging due to the poor biodistribution achieved, different strategies have been used to improve this drawback. Recently polyethilenimine (PEI)-mediated delivery of miRNA has shown promising results in mouse models of cancer (Ibrahim et al., 2011), in this study miR-145/PEI complexes were administered systemically resulting in unmodified miRNA molecules being delivered into the tumors where the miRNA was shown to exert its biological activity. Encapsulation of the nucleic acid with cationic lipids or polymers is an attractive field of research. Such an approach has recently been validated *in vivo* for the delivery of miR-122 in liver where it has been shown to modify gene expression resulting in lowering plasma cholesterol levels (Su et al., 2011). Some of the oligonucleotide modifications developed to improve stability of the nucleic acids have also improved the delivery of miRNAs to tissues where systemic delivery is most effective such as liver, jejunum, lung and liver (Seto, 2011). Some of these modifications include cholesterol-conjugation of miRNAs and LNA-modified oligonucleotides.

Local delivery is very attractive in organs that are easily accessible such as the eye or the lungs. Local delivery ensures delivery to the correct organ and diminishes the eventual off-targets but can only be used in cases where accessibility is not an issue. In a recent publication McArthur and colleagues demonstrate that intravitreal administration of miR-200b prevented diabetes-induced increased vascular endothelial growth factor (VEGF) mRNA and protein in a rat model of diabetic neuropathy (McArthur et al., 2011).

Targeted delivery of miRNAs implies physically steering the microRNA molecule to a given tissue. Targeted delivery can be achieved by using viral vectors with specific tropism for a tissue. Some serotypes of AVV virus show natural tropism for the heart. These viral vectors have been reengineered with therapeutically desirable properties while maintaining the tropism for the heart. These vectors combined with cardiac tissue specific promoters have been used to transfer RNA-based therapies with exceptional outcomes and are already

undergoing translational clinical trials (Poller et al., 2010). Further approaches used for target delivery of siRNAs can be applied to miRNAs.

6.4.3 Specificity and off targets

miRNA based therapies are potentially very powerful since targeting miRNAs can affect a biological pathway at different levels regulating multiple gene targets simultaneously. At the same time, precisely the fact that makes miRNAs promising therapeutic targets is the source of their major drawback for these kind of approaches: the unanticipated OTEs leading to safety issues. It should be noted that when introducing miRNA mimics sequence optimization and off targets are not usually big concerns since miRNAs have naturally evolved for optimal targeting. However when high doses of the miRNA mimics are administered or when miRNA mimics are introduced into cell populations or tissues that do not naturally express the miRNA, unforeseen toxic effects of these molecules could arise and should be taken into consideration when assessing these strategies (Seto, 2011).

6.5 Current status of miRNA-targeted compounds

In May 2008, Santaris Pharma, was the first company to receive approval to start clinical trials with a miRNA targeting drug. The compound, named miravirsen, targets miR-122, a liver specific miRNA involved in hepatitis C replication. Currently miravirsen is undergoing Phase II trials and is the most advanced miRNA targeted compound.

miRagen is a pharmaceutical company currently developing products targeting miRNAs. Four compounds with two different indications in their pipeline are currently in pre-clinical stages. The first two target miR-208 and miR-499 respectively, and are for chronic heart failure whereas the other two target miR-15 and miR-195 for the treatment of post-myocardial infarction remodelling. Other compounds in miRagen pipeline are focused on cardiovascular disease and ALS.

Regulus is advancing miRNA therapeutics in several areas. Its fibrosis program is developing compounds that target miR-21, pre-clinical studies have demonstrated a role for this miRNA in different fibrosis models including heart, lung, kidney and liver, miR-21 is also under study in the company's oncology program. Other miRNAs being targeted include miR-122 for hepatitis C, miR-155 for anti-inflammatory diseases and miR-33a and b for metabolic diseases.

Mirna Therapeutics Inc. is also developing eight lead candidates as part of their oncology program. Some of the miRNAs targeted are let-7, miR-34 and miR-16.

Rosetta Genomics main focus is on developing diagnostic tests for cancer. Several diagnostic kits are currently in their pipeline, e.g., miRview bladder will be able to predict the risk of superficial bladder cancer to become invasive and miRview lung is aimed towards the differentiation of neuroendocrine tumors from non small cell lung cancer tumors using small preoperative biopsies. In the field of therapeutics, Rosetta genomic's is putting together efforts with Regulus in miRNA targeted therapeutics targeting miRNAs that have a potential role in hepatocellular carcinoma.

6.6 Future prospects of miRNA targeted therapeutics

Targeting human disease with miRNA-targeted therapies represents a very powerful therapeutic approach. Enormous advances in understanding the biology of miRNAs have

been made in the past decade. These lessons are a fundamental basis to approach miRNA targeting with rational design, focused on enhancing target affinity, stability and tissue uptake. Despite the huge therapeutic potential of miRNA regulation, this novel therapy still faces some major challenges; addressing OTEs is at the top of the list. However, the impact of off targets will be greatly reduced with improved mechanisms of delivery. In this regard, targeted delivery represents some very interesting features including local delivery to the target sites combined with reduced systemic exposure. Improvements in *in silico* programming are also needed in order to increase the accuracy of miRNA target identification. With one compound already in clinical trials, the future of miRNA-targeted therapies depends on our ability to translate the accumulated knowledge on miRNA biology into the clinic.

7. Advantages of RNAi-based therapeutics and challenges ahead

RNAi technology presents many advantages for therapeutic development with respect to classical compounds, which are centred on the following points:

- They are based on an endogenous mechanism and involve the administration of a type of molecule already present in the cells. Hence, in principle, they may be less hazardous: cells should have the capacity to handle resulting breakdown products.
- They can potentially address any pathological target, which means that even diverse intracellular molecules could be addressed.
- RNAi-based therapeutics are considerably more potent than antisense molecules before them (Bertrand et al., 2002) and, consequently, may be used at lower concentrations. They are believed to have a more persistent pharmacological action than traditional drugs because they block protein synthesis and, hence, the cell will have to re-synthesise the protein from scratch to return to its previous state. Thus allowing the use of lower or less frequent doses.
- Given the high selectivity and specificity of these compounds, they may potentially have less harmful side effects.
- Another main advantage from an industrial perspective is their much shorter pharmacological development, ~2-3 years from proof of concept vs ~4-6 years for traditional compounds. This can be attributed, among other things, to the fact that the compounds can be designed against homologous regions between the human gene sequence and the different animal models used for preclinical studies, thus simplifying toxicology studies.
- Finally, despite being novel entities, they are easy to manufacture using a nucleotide synthesiser, which simplifies large scale production. Since these compounds are chemical entities rather than biological products, the regulatory process for approval of these medicaments is greatly simplifyed.

Nevertheless, RNAi still has certain hurdles to overcome before its full potential can be exploited. The main obstacle is delivery to the desired tissues. Although many advances have been made in this area, much work still remains for the complete therapeutic possibilities to be fully unveiled. On the other hand, in a clinical setting, RNAi can only be used to treat pathologies caused by expression or overexpression of a given protein or by the presence of exogenous organisms, as its mechanism works through suppression of protein expression, i.e., it is only of use when the therapeutic option requires a loss of function. Furthermore, although any gene is a potential target for RNAi, in practice not all

mRNAs are as easily silenced and new more sophisticated algorithms will need to be developed to overcome this issue. Last, but not least, the issue of OTEs resulting from siRNAs silencing unwanted genes can lead to important safety considerations when developing new medicaments.

8. RNA interference in the clinic: Current state and prospects for siRNA-based strategies

Considering RNAi was discovered just over a decade ago, this technology has advanced towards clinical trials with amazing speed. Initial most advanced compounds were designed to treat wet AMD taking advantage of the relatively RNAse free environment of the interior of the eye.

This disease causes loss of vision due to abnormal blood vessel growth (choroidal neovascularisation) behind the retina and macula. Bleeding or leaking of fluids from these newly formed blood vessels causes the macula to bulge or lift, resulting in irreversible damage and loss of vision if left untreated. Anti-VEGF agents cause regression of abnormal blood vessel growth and improvement of vision. In 2004 the FDA approved Pegaptanib (Macugen®, OSI Pharmaceuticals, Pfizer), a VEGF targeting RNA-aptamer to be administered by intravitreal injection and which was the first antiangiogenic therapy to be approved. Later, Lucentis was approved, a monoclonal antibody also against VEGF, which is again administered through intravitreal injection and which is more effective at maintaining and restoring vision.

Based on RNAi, Bevasiranib (a VEGF siRNA developed by Acuity Pharmaceuticals and later by Opko Health) and Sirna-027 or later AGN211745, developed initially by Sirna Therapeutics and licensed to Allergan, targeting VEGF-receptor, were developed with the same therapeutic objective (AMD). Both advanced through phase II clinical trials and Bevasiranib initiated a Phase III study, having shown very promising results in animal models. However, in 2009 both compounds were halted within a few months of each other, due to not improving efficacy over Lucentis, the reference treatment. Also, Quark Pharmaceuticals developed an siRNA for the treatment of AMD targeting the hypoxia-inducible RTP801 gene involved in disease progression, which was later licensed to Pfizer. This compound has reached Phase II, and Quark announced recently their intention to initiate a Phase 2b study to determine the correct dose necessary to produce a therapeutic effect sufficiently superior to the current standard of care to benefit patients over the long term given new emerging therapeutic modalities.

Quark Pharmaceuticals has another two compounds undergoing clinical development. Firstly QPI-1002, an siRNA which targets p53, thus inhibiting apoptosis, is under development for the treatment of acute kidney injury and also for delayed graft function following renal transplantation. Both indications are currently in phase II clinical trials and in August 2010 Quark granted Novartis an option for a worldwide exclusive license for all indications of this compound. Furthermore, QPI-1007, an anti-caspase 2 siRNA, has recently initiated a phase I trial for the treatment of non-arteritic anterior ischemic optic neuropathy. This compound is expected to inhibit apoptosis of retinal ganglion cells thus preventing vision loss ensuing in these patients. QPI-1007 is also under earlier stages of development for the treatment of glaucoma, which also leads to blindness through damage of the optic nerve, in this case as a consequence of elevated intraocular pressure.

Also taking advantage of the peculiarities of the eye's environment, Sylentis has developed a compound, SYL040012, for the treatment of ocular hypertension and glaucoma presently undergoing a Phase Ib clinical trial. This compound inhibits expression of adrenergic receptor β2 in tissues of the ocular anterior chamber, resulting in a decrease of aqueous humour production and consequent reduction of the pressure inside the eye. Just one step behind in its product pipeline is SYL1001 for the treatment of ocular pain associated to dry eye syndrome, poised to enter the clinic this year.

Another of the most advanced siRNA compounds in the clinic, again in Phase II, is Alnylam Pharmaceutical's compound for the treatment of RSV infection. This compound, ALN-RSV01, targets the nucleocapside encoding gene of the virus and therefore inhibits viral replication in the lung. This compound is administered intranasally directly to the lung and is partnered with Kyowa Hakko Kirin in Asia, and with Cubist Pharmaceuticals for the rest of the world.

Further programs in the clinic developed by Alnylam include their compound ALN-VSP, for the treatment of liver cancers and potentially other solid tumors, currently in Phase I. This RNAi therapeutic targets two key genes each involved in the disease pathway of liver cancer: kinesin spindle protein, or KSP, involved in cancer proliferation, and vascular endothelial growth factor, VEGF, involved in the growth of new blood vessels that feed tumors. Also, as part of their new 5x15 programs, ALN-TTR, an RNAi therapeutic targeting transthyretin (TTR) for the treatment of TTR amyloidosis (an orphan hereditary disease which results in toxic protein deposits in several tissues) entered Phase I trials in early July 2010.

As previously mentioned in section 5.4, TransDerm Inc. has developed an siRNA for Pachyonychia congenital disorder which underwent a phase I clinical trial. However, upon completion the company announced it was directing its resources at developing an enhanced delivery system that was less painful than the intradermal injections used in this trial.

A still unsolved problem in medicine is the treatment of HIV infection, and one of the pioneering RNAi compounds in clinical trials was termed pHIV7-shl-TAR-CCR5RZ, initially developed by Benitec Inc. This compound was in a Phase I/II pilot study from 2007 to 2010 in collaboration with City of Hope Research Hospital. It is based on expression of a construct producing specific shRNA specific for the above targets introduced into CD34+ blood progenitor cells. According to Benitec's website, this study is now being progressed by the research and clinical team at the City of Hope Research Hospital.

A further clinical setting with multiple participants is the treatment of cancer, with different compounds in Phase I clinical trials. This includes Atu027 developed by Silence Therapeutics for the treatment of advanced, recurrent or metastatic solid malignancies. This compound, which targets PKN3 (a protein kinase C-related molecule believed to be an effector mediating malignant cell growth), was one of the first to advance in the RNAi landscape. However, it has suffered numerous delays in its move into the clinic due to the difficulties associated to delivery of these compounds. The company then developed a proprietary lipid delivery technology called AtuPlex. This platform is based on lipid components that embed siRNA into multiple lipid bi-layer structures. The resulting nanoparticle structure consists of the siRNA combined with a cationic lipid, fusiogenic lipid and PEG. This system was used to formulate Atu027, which was then administered in the clinical trial and produced positive results according to the company's abstract at ASCO meeting this year.

Further RNAi for the treatment of cancer include CALAA-01 for the treatment of solid tumours developed by Calando Pharmaceuticals. CALAA-01 is a siRNA which targets RRM2 (M2 subunit of ribonucleotide reductase) and is formulated in cyclodextrin-based particles. CEQ508 has been designed for the treatment of familial adenomatous polyposis (FAP) by targeting beta-catenin using tkRNAi. This technology, developed by Cequent Pharmaceuticals (now Marina Biotech), uses modified *E. coli* to deliver RNAi compounds to the intestine.

Another indication of interest to the pharmaceutical industry is the treatment of hypercholesterolaemia, with different companies having compounds under development. In clinical trials, Tekmira Pharmaceuticals has a compound TKM-ApoB (previously PRO-040.201) which targets expression of Apolipoprotein B that has completed a phase I trial.

The pioneer in the field of miRNA silencing is Santaris Pharma with its LNA technology. The most advanced compound is SPC3649, which targets miR-122, for the treatment of hepatitis C virus infection and currently in Phase II.

9. Navigating the patent thicket

At the turn of the century a revolutionary discovery was made in the field of molecular biology: the existence of RNA interference. Firstly in flatworms by original finders and Nobel laureates Andrew Fire and Craig Mello, and later its existence in mammalian cells by Tuschl and coworkers, opened the door to this truly limitless (or so it seemed) technology. Following the expected course of events characteristic of science in our era, the parties involved rapidly filed patent applications on their discovery. These initial filings were done by the academic institutions where discoveries were made, such as Carnegie Institution or Max Planck Institute; and were rapidly out-licensed to industry.

Fire and Mello's finding in flatworms generated a seminal patent—the Carnegie patent—which is available for licensing to any interested party and describes how double-stranded RNA silences target genes in a cell. The initial filing has given rise to a complicated patent family with many sister applications both in Europe and in the US, including different limitations such as length of the molecules, *in vitro* or therapeutic use, *in vivo* use in plants, in flatworms, etc.

Initial discoveries used molecules of 25 nucleotides or more in length, and at that size, the molecules can trigger dangerous immune responses, making them unsuitable for clinical use. However, it was later found that making the molecules smaller could avoid immune reactions while disabling mRNA. This discovery was made by 4 scientists—Thomas Tuschl at Max Planck Institute, Philip Sharp at MIT, Philip Zamore at UMass Medical School and David Bartel at the Whitehead Institute—and produced the fundamental patent known as Tuschl I, which covers both modified and unmodified siRNA molecules, 19 to 25 nucleotides long. In 2002, three of the four schools involved in the discovery agreed to license Tuschl I exclusively to Alnylam. UMass Medical School chose not to, and granted a nonexclusive license to CytRx (now RXi) for limited therapeutic rights. Then in 2003, Sirna took a coexclusive license to Tuschl I from UMass Medical School, the end result being that Sirna, Alnylam and CytRx (to a limited degree) can operate under Tuschl I. Further research on Tuschl's part then led to the finding that siRNA structures containing three prime overhangs were particularly advantageous. These results led to filing of the Tuschl II patent, which was licensed exclusively to Alnylam. Again, both of these patents have evolved into

complicated patent families with multiple sister applications both in Europe and the US covering specific features.

These filings led to an initial competition for dominance between two major companies: Alnylam Pharmaceuticals and Sirna Therapeutics. There was some controversy about the value of Tuschl I and Tuschl II over each other. While Alnylam assured the supremacy of Tuschl II, Sirna argued that Tuschl I contains most of the major features found in Tuschl II anyway. Both companies invested significant resources in building up their patent portfolio either through further licenses or internal filings on further chemical architecture and/or use of modified nucleotides in siRNA compounds. Consequently, most subsequent players in the field will have to negotiate licenses (or have already done so) with these companies for the development of their compounds, thus contributing to maintaining their dominant position.

In any case, all three of these families there have been some granted patents, some of these undergoing complicated opposition procedures, and there are also still applications undergoing prosecution despite the fact they were filed in 1999 and 2000. This makes the outcome of what will eventually be covered somewhat uncertain.

Despite the many licensing deals reported by the press during this period, and the active patenting endeavours of all parties, there has been relatively little litigation of the patents involved. One notable case was made by two companies Nucleonics and Benitec, arch rivals working on expressed RNA interfering compounds to treat viral infections. The costs of their legal battle led to the dissolution of Nucleonics in mid 2008, and to Benitec withdrawing its international operations and reducing costs in its native Australia.

The next visit to the courts was made in mid 2009 by Alnylam Pharmaceuticals, who originally licensed the Tuschl I patent family from co-applicants Max-Planck Institute, University of Massachusetts, the Massachusetts Institute of Technology and the Whitehead Institute for Biomedical Research. Alnylam shares therapeutic rights to the Tuschl I estate with Sirna Therapeutics (now Merck) and to a limited degree with RXi. In this case Alnylam and Max-Planck were accusing the other co-applicants of misappropriating features relating to 3'-overhangs, which are object of the Tuschl II patent family, licensed exclusively to Alnylam. This case would have been of great interest to all players in the field, as the exact scope of each patent family has yet to be definitively determined. However, the involved parties reached an agreement outside the courts as recently as March 2011, in which among other terms, UMass Medical School gained the right to sublicense rights to the use of 3' overhangs to Merck.

The most recent legal action also involves Alnylam Pharmaceuticals, who is being sued by one time partner Tekmira over inventorship of certain delivery technologies. Tekmira, which owns technology relating to delivering siRNA compounds known as SNALPs resulting from its acquisition of Protiva Biotherapeutics, is a leading developer of lipid nanoparticle delivery technologies. Both companies started working together in 2006 and struck a first licensing deal in 2007 followed by a manufacturing supply deal in 2009. According to Tekmira's spokesman the delivery technology used for Alnylam's compound ALN-VSP for the treatment of liver cancer is the rightful property of Tekmira, and Alnylam has abused its collaborator status by disclosing the technology and methods to a third party and incorporating these features into its own patent filings. At present this process is yet at an early stage and the outcome difficult to predict.

As compounds near marketing approval and the economic stakes become higher, it is likely that the Courts will become busier with patent litigations from this field, and the patent landscape will probably become clearer for all involved.

As the race to join the field gained traction, big pharma, including Novartis, Roche, AstraZeneca or Merck, joined the playground with big deals signed from the end of 2006 to early 2008, the most notorious being the acquisition of Sirna Therapeutics by Merck for US$1.1 billion in October 2006.

With the deepening in knowledge of RNAi, so did the awareness of its limitations and hence the R&D to overcome these issues evolved. The main obstacles to widespread use of RNAi products are its vulnerability to degradation by RNAses ubiquitous in biological fluids, and the difficulty in targeting the compounds to the desired tissue or organ within the body. Consequently, there has been extensive patent filing on different chemical modifications useful to enhance resistance to degradation without losing silencing efficacy. Most companies in the field developed specific combinations of modifications. The main players in this area being Alnylam, through its access to the IP developed by Isis Pharmaceuticals for the use of antisense oligonucleotides, but also Silence Therapeutics, Quark Pharmaceuticals, and Santaris Pharma. The filing in this area has been so aggressive that it is probably difficult to develop a novel siRNA containing modifications which doesn't require a license to at least one, if not more, of the different patents.

Although the resistance to degradation has been overcome by technology, a huge hurdle remains in the delivery of compounds to the target tissue. This issue still remains as the main obstacle for this drug class to really flourish, and many of the more recent deals in industry have been made between purely chemical development RNAi firms and those who had developed delivery solutions. For example, Silence with Intradigm.

Bearing in mind the above described landscape, when starting on a new project from a patent perspective, one must analyse which of all these patent rights will be infringed by the commercialization of the novel compound. This includes analysis of the fundamental patents, and also those relating to nucleotide modifications and routes and vehicles for their delivery. But this is not all, for there have also been a great number of filings on specific gene targets. In fact, if one studies the dynamics of patent publications citing siRNA, the first publications appeared in 2003 and rapidly peaked to a maximum of over 400 publications in 2005, with well over 300 patent applications citing siRNA being published every year since then. Therefore every new project requires extensive analysis of the landscape to determine the best option to obtain freedom to operate in an increasingly competitive and complex area.

Regarding delivery, many approaches have been developed but none has been fully satisfying, and possibly as a consequence, the field's pace has slowed down considerably. Also, the two most advanced compounds in the clinic, both for the treatment of wet AMD were halted during 2009 before reaching or finalising phase III, their sponsors explaining the compounds weren't meeting the end-point of improved efficacy over Lucentis, the standard of treatment for this disease and a monoclonal antibody targeting the same molecule: VEGF. The exact reason remaining unknown, but all these factors probably having played their part together with the general economic crisis, in the last year or so big pharma have pulled out of many of the RNAi deals, and/or closed down their nucleotide operations. Examples of this include Pfizer having closed down its Oligo Therapeutics Unit early this year; Roche announced in November 2010 it was terminating its efforts in RNA interference, and Novartis refusing a further option to extend its collaboration with Alnylam. Consequently, at present there are a handful of companies dedicated exclusively to RNAi with programs in early clinical stages. For an overview of these companies and their programs in the clinic see Table 2.

Although the pace in the RNAi field has slowed down considerably, it remains to be seen how this area will develop and if the full potential of this technology can be harnessed. In such a case, we may witness the full revolution of drug development.

Company	Drug	Disease	Target	Clinical Stage
Opko Health	Bevasiranib	Wet AMD	VEGF	Phase III
Allergan	AGN-211745	Wet AMD	VEGF-R	Phase II
Quark Pharm.	RTP801	Wet AMD	RTP-801i	Phase II
	QPI-1002	Acute Kidney Injury / Delayed Graft Function	p53	Phase II
	QPI-1007	NAION	Caspase-2	Phase I
Sylentis	SYL040012	Glaucoma	b2-adrenergic receptor	Phase Ib
Alnylam Pharmaceuticals	ALN-RSV01	RSV	RSV nucleocapsid	Phase II
	ALN-VSP	Liver cancer	KSP + VEGF	Phase I
	ALN-TTR	TTR amyloidosis	TTR	Phase I
TransDerm Inc.	TD-101	Pachyonychia congenita	Keratin 6A N171K mutant	Phase I
Benitec / City of Hope	pHIV-shl-TAR-CCR5Z	AIDS	HIV *tat rev*	Phase I
Silence Therapeutics	Atu-027	Solid tumours	PKN3	Phase I
Calando Pharm.	CALAA-01	Solid tumours	RRM2	Phase I
Marina Biotech	CEQ508	FAP	β-catenin	Phase I
Gradalis Inc.	FANG	Solid tumours	Furin	Phase II
Duke University	NCT00672542	Metastatic melanoma	LMP2, LMP7, MECL1	Phase I
Tekmira Pharm.	TKM-ApoB	Hypercholesterolaemia	ApoB	Phase I
Zabecor	Excellair	Asthma	Syk	Phase II
Santaris Pharma	SPC3649	Hepatitis C virus	miR-122	Phase II

Table 2. RNAi-based compounds in clinical trials.

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12

P2X Receptors as New Therapeutic Targets

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1. Introduction

P2X receptors are membrane non-selective cation channels that gated in the presence of extracellular adenosine triphosphate (ATP) and related di- and tri-phosphate nucleotides, that more commonly known for providing cells with energy. Binding of ATP to the excellular pocket of P2X triggers the opening of transmembrane pore, which allows sodium, magnesium, potassium, calcium and other organic ions to flow down their electrochemical gradients. Because seven P2X receptor subtypes ($P2X_{1-7}$) are widely distributed in excitable and nonexcitable cells of vertebrates, P2X receptors mediate many physiology processes including synaptic transmission and thrombocyte aggregation. These ion channels are also involved in the pathology of several disease states, playing key roles in inter alia afferent signaling (including neuropathic pain), regulation of renal blood flow, vascular endothelium, and chronic inflammation, and thus are potential targets for drug development. The recent discovery of potent and highly selective antagonists for P2X receptors, through the use of high-throughput screening, have helped to further understand the P2X receptors pharmacology and provided new evidence that P2X receptors play specific roles, such as in chronic pain states. In this review, we place previous work of P2X in the context of three-dimensional (3D) crystal structure of Zebrafish $P2X_{4.1}$ ($\Delta zfP2X_{4.1}$), discuss how the P2X family of ion channels have distinguished themselves as potential new drug targets, and try to differentiate between drugs which are useful research tools, helpful in understanding the physiological roles of these receptors. We also summarize the key questions and challenges, which await researchers' further work as we move forward to the new drug development era of P2X receptors. We are optimistic that safe and effective candidate drugs will be suitable for progression into clinical development.

2. The P2X receptor protein family

2.1 The gene of P2X receptors

Seven P2X receptor subtypes in mammals are different in genes. Their chromosomal locations, amino acid length and mass are summarized (Table 1). $P2X_2$, $P2X_3$ and $P2X_6$ receptor genes are all on different chromosomes. $P2X_1$ and $P2X_5$ subunit genes are located close on the short arm of chromosome 13 (Table 1). $P2X_4$ and $P2X_7$ subunit genes are also both located on the long arm of chromosome 12 (North 2002). From the alignment of amino acid sequences, $P2X_4$ and $P2X_7$ are the most related pairs (North 2002). The full-length of seven P2X receptors have 11-13 exons, and they all share a common structure, with well-

conserved amino acids in the outer loop and transmembrane regions. These seven genes are considerable different in size.

Subunit	Chromosome	Length (AA)	Mass (Da)	Accession Nos.	Reference Nos.
$P2X_1$	17p13.2	399	44,980	P51575	(Longhurst PA 1996, Soledad Valera 1994)
$P2X_2$		471	51,754	Q9UBL9	(Kevin J. Lynch 1999, Lynch et al. 1999)
$P2X_3$	11q12	397	44,289	P56373	(Garcia-Guzman et al. 1997b)
$P2X_4$	12q24.31	388	43,369	Q99571	(Garcia-Guzman et al. 1997a)
$P2X_5$	17p13.3	422	47,205	Q93086	(Le et al. 1997)
$P2X_6$	22q11	441	48,829	O15547	(Urano et al. 1997)
$P2X_7$	12q24.31	595	68,586	Q99572	(Franc¸ois Rassendren 1997)

Table 1. Properties of human P2X receptors

Chromosomal localizations, amino acid length, and Mass are from Protein Knowledge base of UniProtKB (http://www.uniprot.org/). The accession numbers and references are indication for the original submission of cDNA sequences.

2.2 Amino acid sequence and structure

In the mid-1990s, the first cDNA cloning of P2X receptor led to the deduction of P2X receptors' structure and physiology. In 2009, Kawate and colleagues reported the crystal structure of a truncated mutation of the Zebrafish $P2X_{4.1}$ receptor ($\Delta zfP2X_{4.1}$) (Toshimitsu Kawate 2009), which is the first 3D crystal structure of P2X receptors. It represents a step change in our understanding of these membrane ion channels, where previously only low-resolution structure data and inferences from indirect structure-function studies were available. Due to these indirect and direct biochemical and pharmacological data, approximate structure and topology of seven distinct $P2X_1$–$P2X_7$ subtypes can be elucidated. Three individual subunits assemble to form functional homomeric (except $P2X_6$) or heteromeric receptors (except $P2X_7$). These subunit peptides are difference in size ranging from 388 ($hP2X_4$) to 595 ($hP2X_7$) amino acids long. The protein of these seven subtypes are pairwise identical (from 40% to 55%) (North 2002). All functional P2X receptors have the following topology: intracellular N-terminus/first transmembrane segment (TM1)/extracellular segment/second transmembrane segment (TM2)/intracellular C-terminus.

2.3 TM domains and the pore region

Every P2X protein has two hydrophobic transmembrane regions, which are sufficient long to cross the plasma membrane (Brake et al. 1994, North 1996, Valera et al. 1994). The presences of only two transmembrane segments distinguish P2X receptors from other ligand-gated cation channels. The first transmembrane segment (TM1) extends from residue 30 to 53, and the second transmembrane segment (TM2) from 328 to 353 (in the rat $P2X_2$ receptor) (Zhiyuan Li 2004). These two regions are both α-helix, form the central ion conduction pore and participate in the conformational changes during receptor activation. Three pairs of TM1 and TM2 domains are highly tilted to the cell membrane giving the pore

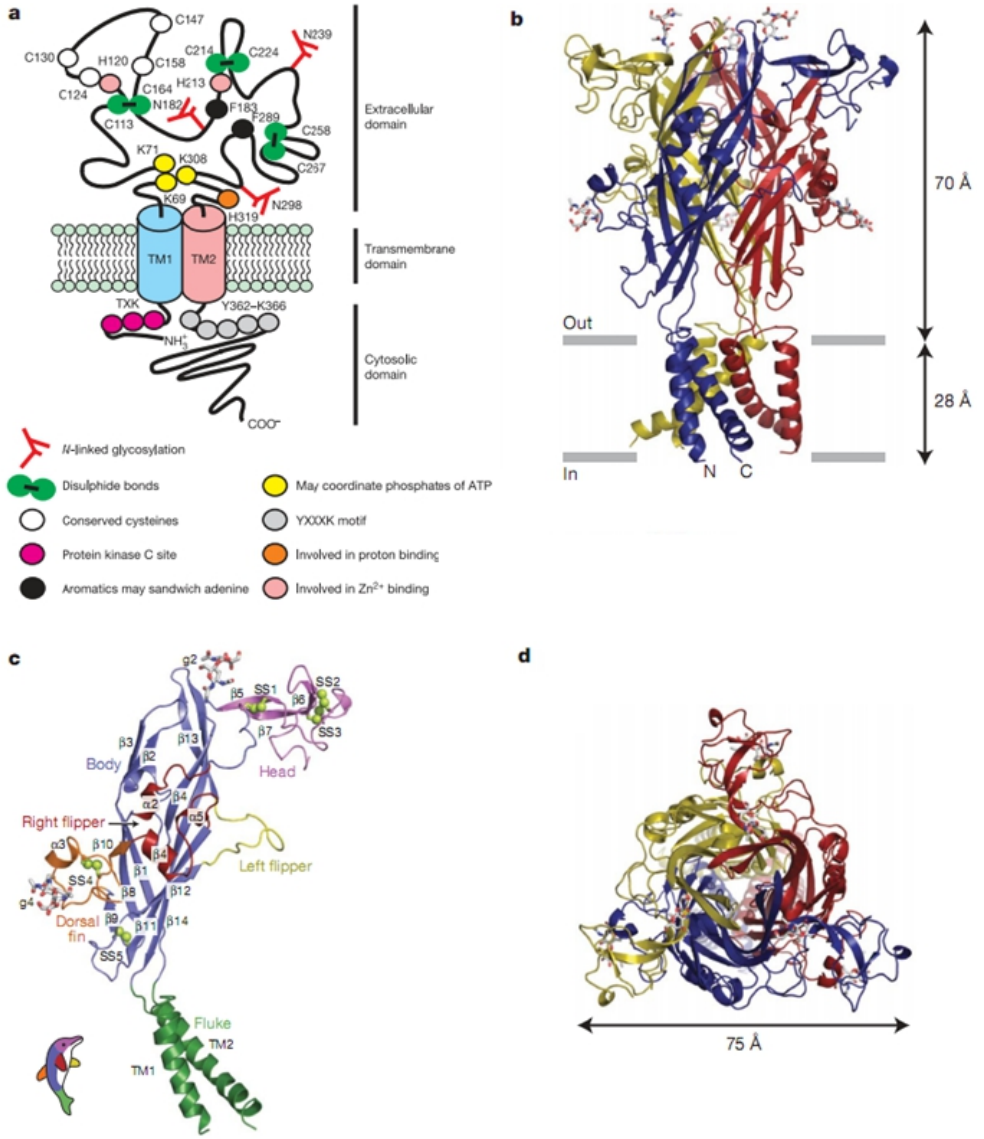

Fig. 2. Structure of P2X receptors. (a) Topology features of P2X receptors which based on the structure-function studies on $P2X_1$ and $P2X_2$ receptors. The numbers refers to rat $P2X_2$ receptors. (b) Stereoview of the homotrimeric $\Delta zfP2X_4$ structure. Three subunits are depicted in red, yellow and blue, respectively. NAG and glycosylated asparagines residues are represented in stick. This is a closed resting conformation. (c) Top view of the homotrimeric $\Delta zfP2X_4$ structure from extracellular side of the membrane. Fig. a is reproduced from reference (North 2006). Fig. b-d are reproduced from reference (Toshimitsu Kawate 2009).

an hourglass appearance (Toshimitsu Kawate 2009). The previous mutagenesis, functional work and 3D crystal structure give strong evidence that the TM2 domain plays major role in ion transduction and pore opening. TM2 domain is directly involved in the assembly of subunits into oligomeric complexes and is partly responsible for determining the rate and degree of desensitization (Evans 2010), interacts with the permeating ions and regulates specific properties of ion flow including conductance (Nakazawa et al. 1998), permeability (Migita et al. 2001), and Ca^{2+} flux (Egan et al. 2004) among P2X receptors. The diameter of the narrowest part of the open pore is thought to be about 8-20 Å (Toshimitsu Kawate 2009), which is from Thr 336 to Phe 346 (the number refers to rat $P2X_2$) (Mufeng Li 2008). Although the present data suggest that the TM1 domain involved in transducing agonist binding into channel gating, an explicit role for the TM1 has not been identified. The COOH- and NH_2-terminal regions are both in the cytoplasm.

2.4 Terminal regions

The N-terminus region is relatively short (about 25 amino acids) and contains a consensus site for protein kinase C-mediated phosphorylation; removal of this site leads to an accelerated fade in the amplitude of the ATP-gated current of the $P2X_2$ receptor. The C-terminus is longer (about 25–250 amino acids) and diverges in sequence considerably between seven subtypes and contains multiple sites that mediate subtype-specific effects. A YXXXK motif at the end of the TM2 is highly conserved among all P2X receptors, the Tyr and Lys of which have been proved to stabilize the P2X subunit in the plasma membrane, which was reported by Rassendren and his colleagues (Chaumont et al. 2004).

2.5 Extracellular domains

The extracellular loop also contains consensus sequences for the N-linked glycosylation involved in targeting the receptor to the cell surface membrane, for agonist and antagonist binding sites, and for intersubunit binding sites responsible for allosteric modulation of the ATP response by hydrogen ions and zinc.

The two transmembrane segments are connected by a long string of amino acids that form the extracellular of the receptor. This string contains ten conserved cysteines, which participate in disulfide bonds that help define the tertiary structure of the protein. From the N-terminus to the C-terminus, the cysteine pairs are 1-6 (Cys119 – Cys168), 2-4 (Cys129 – Cys152), 3-5 (Cys135 – Cys162), 7-8 (Cys220 – Cys230) and 9-10 (Cys264 – Cys273). These ten cysteine residues are very conserved in all mammalian P2X receptors (Clyne et al. 2002, Ennion et al. 2002). Two pairs of disulfide bonds which are pair 2-4 and 3-5, may exchange with each other during and after biosynthesis, because of the very close space.

Based upon mutagenesis data and binding studies, eight highly conserved residues have been proved to be involved in ATP binding. In rat $P2X_2$ these residues are Lys74, Lys76, Phe188, Thr189, Asn293, Phe294, Arg295, and Lys313 (Jiang et al. 2000, Marquez-Klaka et al. 2007, Roberts et al. 2004, 2006). In the 3D crystal structure of $\Delta zfP2X_{4.1}$, these residues are lining a cavity which is surrounded by the head domain, and left flipper of one subunit, and dorsal fin of another (Toshimitsu Kawate 2009). The exact nature of these residues is not very clear so far. How do they interact with each other is unknown. Jiang and Roberts reported that the negatively charged triphosphate moiety may interact with positively charged residues Lys74, Lys76 and Lys313, whereas Phe188, Thr189, Asn293, and Phe294 might be interacted with the adenine ring and ribose moiety (Roberts et al. 2006).

All seven P2X receptor subtypes have consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr) some of which is essential for protein to traffic to the cell surface. In rat $P2X_2$, Asp182, Asp239 and Asp298 all can be glycosylated in oocytes and HEK293 cell (Newbolt et al. 1998). If these glycosylation sites are removed or prevented by tunicamycin or mutagenesis, the full function of P2X receptors will be disturbed. Receptors give no response to ATP, if only one site is glycosylated. But if two of three sites are glycosylated, the cell can express full function receptors (North 2002). All P2X receptors have N-linked glycosylation, which are well conserved among species but different between receptor subtypes (North 2009., North 2002).

3. Physiological role of P2X receptor

Seven P2X receptor subunits ($P2X_{1-7}$) are widely expressed in tissues throughout body; for example, they are found in excitable and nonexcitable cells, such as neurons, myocytes, leukocytes and epithelial cells. And thus they play key roles in inter alia afferent signaling (including pain), regulation of renal blood flow, vascular endothelium, and inflammatory responses. Most P2X receptors are nonselective cation channels that discriminate poorly among monovalent cations, while exhibiting a stronger preference for calcium ions. The exception is $P2X_5$ receptor, which in some species such as human and chicken exhibits a modest permeability for chloride ions. The resulting rise in intracellular calcium evokes transmitter release from central and peripheral neurons and glia, promotes hormone release from endocrine glands, triggers contraction of muscle, regulates airway ciliary motility, and activates downstream signaling cascades in various of cells.

3.1 Central nervous system

ATP is a fast neurotransmitter, it can be released from the excitable and nonexcitable cells in normal physiological and pathophysiological conditions. As one member of the purinergic receptor family, P2X receptors are widely expressed on central nervous system at different mRNA and protein levels, for example, $P2X_2$, $P2X_4$ and $P2X_6$ are most abundant in neurons (North 2006). They play an important role in the regulation of neuronal and glial functions, which is participating in the synaptic process and mediating communications among them. In the central synapses, the significant function of P2X may be related to depolarizing neurons and considerable calcium permeability plays the key role (Pankratov et al. 2002), no matter it is at resting membrane potential or not.

3.1.1 Postsynaptic and presynaptic

Using post embedding immunocytochemistry, Rubio and his colleagues for the first time qualitatively and quantitatively described the precise location of $P2X_2$, $P2X_4$ and $P2X_6$ subunits in postsynaptic on the hippocampal CA1 pyramidal and cerebellar Purkinje cells (Rubio et al. 2001). Bouts of action potential firing are activated the synaptic P2X receptors. The function and densities of P2X receptors in the postsynaptic might be related to some specific proteins. Using the glutathione S-transferase pull-down experiment, it is proved that β-amyloid precursor protein-binding proteins Fe65 colocalizes and interacts with $P2X_2$ receptor in postsynaptic of excitatory synapses (Masin et al. 2006). Direct postsynaptic effects on neurons have been reported.

Khakh and Gu have reported that P2X receptors mediate presynaptic responses. Calcium ions play a key role in this responses, which are triggered and enter through the P2X

receptors or through Ca^{2+} channels (North 2006). Presynaptic P2X receptors may be active physiologically in some synaptics (Gu et al. 1997). Presynaptic actions form one component of ATP effects in hippocampus. A feed forward circuit forms among the CA3 pyramidal neurons, GABAnergic interneurons and output CA1 pyramidal neurons. The increased release of glutamate from presynaptic $P2X_2$ receptors works on the interneurons, which are depolarized by $P2Y_1$ receptors. The depolarization of interneurons is the concomitant reduction and activation of potassium and nonselective cationic conductance, respectively (Bowser et al. 2004, North 2006). ATP plays a special role in this feed-forward circuit, it might act as a physiological brake to runaway excitation.

3.1.2 Disorder

Because the ATP plays key roles in neurotransmission and neuromodulation, purine and pyrimidine receptor subfamilies have been involved in various pathological conditions. This pathophysiology of CNS disorders include brain trauma, ischaemia, neurodegenerative diseases and neuropsychiatric diseases. When injury happens, large amounts of ATP release into extracellular environment which are important for triggering cellular responses to trauma. The expression level of $P2X_4$ and $P2X_7$ has changed, which might stimulate the migration and chemotaxis of resting microglia to the site of damage (Ohsawa et al. 2007, Xiang et al. 2006), and $P2X_7$ have an important role in controlling microglia proliferation and death (Bianco et al. 2006, Franke et al. 2006). Cerebella lesions result in up-regulation of $P2X_1$ and $P2X_2$ receptors in precerebellar nuclei, and stab wound injury in the nucleus accumbens leads to increased expression of several subtypes of P2X and P2Y receptors.

Cerebral ischaemia can produce and exacerbate problems to the CNS, which include stroke and paralysis. This disease can increase the release of purines into cerebral cortical perdusates (Braun et al. 1998). In vitro studies of organotypic cultures from hippocampus, F. Cavaliere and their colleague found out that $P2X_2$ and $P2X_4$ were up-regulated by glucose/oxygen deprivation (Cavaliere et al. 2003), and this can be prevented by P2 receptor antagonists. On the other side, $P2X_4$ and $P2X_7$ receptors, which are on microglia, and may be involved in cortical damage, also produced by glucose/oxygen deprivation (Cavaliere et al. 2005).

Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS) are all belong to neurodegenerative diseases. $P2X_7$ receptor is related to Parkinson's disease and Alzheimer's disease, but the function of $P2X_7$ in the pathogenesis of the Parkinson's disease is not very clear. However, the animal model and patients with Alzheimer's disease showed that $P2X_7$ receptor is up regulated in the brain (McLarnon et al. 2006, Parvathenani et al. 2003). $P2X_7$ in microphages and microglia might enhance the degenerative (Rampe et al. 2004). Changes in P2X receptor-mediated neurotransmission in cortico-striatal projections have been found in two different transgenic models of Huntington's disease (Burnstock 2008, Burnstock et al. 2011). The density of $P2X_3$, $P2X_6$, and $P2X_7$ might be related to Diabetic neuropathy. On the diabetic rats, it is found out that they decreased in hippocampal nerve terminals.

Epilepsy seizures have devastating behavior. The pathogenesis is still unclear. P2X receptors play important roles in this disease, especially for $P2X_7$. The decrease of presynaptic P2X receptors in the hippocampus of rats might be related to the development of seizures and neurodegeneration during epilepsy (Burnstock 2008). Abnormal expression of $P2X_7$ might be associated with immunoreactivity in hippocampus and microglia (Rappold et al. 2006).

3.2 Genitourinary system

The vitro and vivo experiments showed that ATP and its analogues alter renal vascular resistance and renal blood flow. Seven P2X receptors are expressed in renal vascular, glomerular, mesangial and tubular epithelial cells. For example, $P2X_1$ expressed in preglomerular arteries and afferent arteriole (Turner et al. 2003, Zhao et al. 2005), whereas $P2X_2$ are expressed in larger intrarenal arteries (Turner et al. 2003). $P2X_4$ and $P2X_7$ are all detected in medium arteries (Lewis et al. 2001).

The recent hypothesis is $P2X_1$ receptors play an important role in renal autoregulation via tubuloglomerular feedback. Using the immunohistological studies, it has been detected that $P2X_1$ expressed in afferent arterioles, if the glomerular filtration rate or the rate of reabsorption in the proximal tubule changes; the glomerular filtration rate will change correspondingly. Changes in glomerular filtration rate are sensed by macula dens cells which will release ATP to stimulate $P2X_1$ receptors on afferent arteriolar smooth muscle (North 2009). This process triggers an increase of afferent arteriolar resistance. This reduces pressure in the glomerular capillaries and decreases glomerular filtration (Guan et al. 2007).

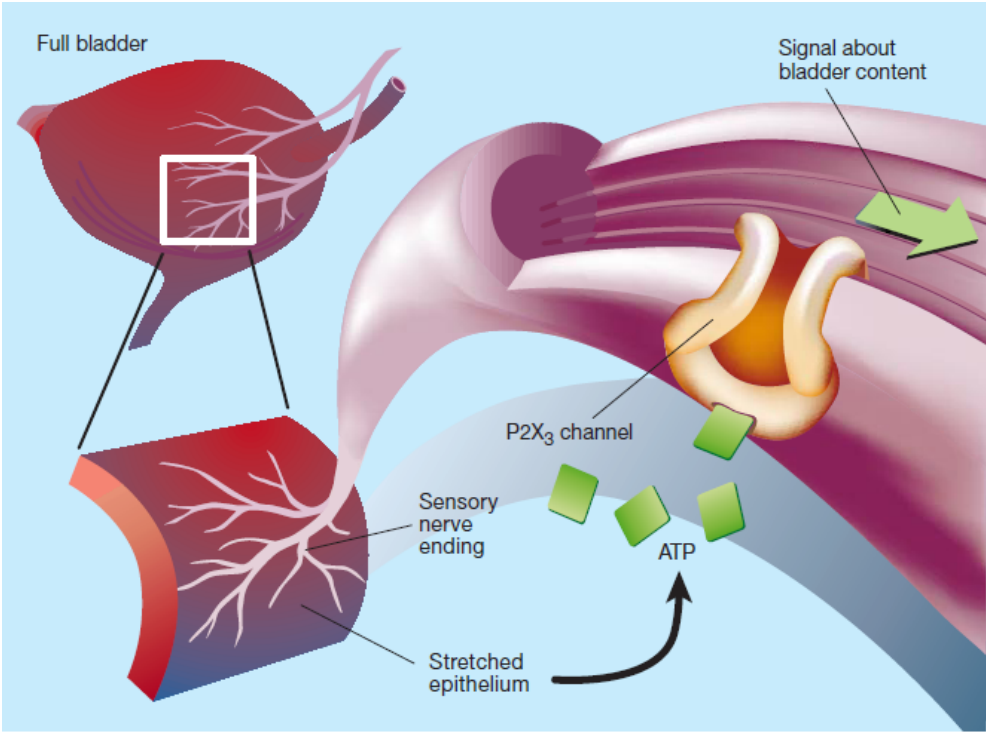

Fig. 3. Bladder urging, general view of bladder emptying. Stretching releases ATP from the inside of bladder epithelia cells, and channels made of $P2X_3$ receptors could detect ATP and trigger the neuronal pathway which responses for the bladder emptying. Fig.3 was reproduced from reference (McCleskey 2000)

Why we feel urge? The $P2X_3$ receptor knockout mice proved that $P2X_3$ receptors which are expressed only on sensor neurons, detect extracellular ATP, that is proposed to be released from the urothelium as a sensory mediator for the degree of the urinary bladder distention (Ferguson et al. 1997). If $P2X_3$ receptors are knocked out, the mice performed greatly decreased responses to the filling and stretching of urinary bladders, this means that they emptied their bladder less frequently than wild type. This urine storage disorders might be treated by antagonists of $P2X_3$ receptors.

Purinergic nerve-mediated contraction of the human bladder is increased to 40% in pathophysiological conditions such as interstitial cystitis (IC), outflow obstruction, idiopathic detrusor instability and probably also neurogenic bladder (Burnstock et al. 2011). Using bladder tissue sample of patients undergoing cystectomy or prostatectomy, Tempest et al found out that $P2X_2$ and $P2X_3$ receptors are all expressed in human bladder urothelium. Their protein expressing level did not correlate with the gene expression, which might be related to the pain with IC (Tempest et al. 2004).

3.3 Taste

A single taste bud contains three of four types of taste cells, including type I supporting cells, type II receptor cells, and III presynaptic cells, type IV basal cells. Type I cells on the outside of the type II and III. P2Y and P2X receptors are expressed on the type II cells, which are for the five tastes (umami, sour, salty, sweet and bitter), but type II cells do not synapse with afferent nerves within the taste buds. In taste system, ATP is a key neurotransmitter, which bridge between the taste bud and the nervous system. RT-PCR and immunohistochemical studies confirmed that $P2X_2$, $P2X_3$, $P2X_4$, $P2X_7$ receptor expressed on the taste bud cell. $P2X_2$ localized on the afferent nerve fibers and presynaptic cells, $P2X_3$ only localized afferent fibers, but the immunohistochemical studies showed no $P2X_4$ protein in taste bud cells and nerve fibers (Hayato et al. 2007, Kataoka et al. 2006). Homomeric and/or heteromeric $P2X_2$ and $P2X_3$ receptors play an important role in taste transduction. Finger and his colleagues found out that $P2X_2$ and $P2X_3$ knockout mice decreased the responses to sweet, glutamate and bitter substances (Finger et al. 2005). But single knockout mice recover some neural and behavioral responses to tastants (Finger et al. 2005). The exact role of $P2X_7$ is still not very clear, but it might be related to taste bud cells apoptosis (Hayato et al. 2007).

3.4 Hearing

Studies on the animal (rat, mouse) showed that $P2X_2$ and $P2X_3$ receptors might be in the inner and outer hear cells. This is only electrophysiological and immunolocalization data. There is no knockout animal model data to confirm the exact function of these P2X receptors.

3.5 Cardiovascular diseases

It has been reported that P2X receptors expressed throughout the cardiovascular system. In recent years, two tissues (the platelet and the endothelium) are studied in blood pressure and blood coagulation have advanced rapidly.

Although it has a long history that $P2Y_1$ and $P2Y_{12}$ play a main role in thrombosis, the $P2X_1$ receptors might play an important role in thrombus formation under the condition of stenosis, in which shear stress is high. It has been proved by the $P2X_1$ knockout animal model which is platelet-dependent thrombotic occlusion of small arteries. In this model

blood flow is characterized by a high shear rate. In the model of systemic thromboembolism death rate of $P2X_1$ deficient mice is reduced and the size of mural thrombi was decreased compared to the wild type, mural thrombi was made by laser induced vessel wall injury (Hechler et al. 2003). It is also contribute to transient Ca^{2+} influx and platelet shape change in response to ATP or Alpha, beta-MeATP and responsible for the platelet activation induced by low concentrations of collagen (Gachet 2008, Rolf et al. 2001, Rolf et al. 2002). This makes the $P2X_1$ receptors represent the ideal target for an antithrombotic drug.

As we all know, ATP is a cotransmitter in the heart, which is released from ischemic and gypoxicmycytes. Using quantitative PCR and situ hybridization, Musa and colleagues measured expression of mRNA of the P2X receptors in rat left ventricle, right atrium, sinoatrial node (SAN), and human right atrium. It is found out that $P2X_5$ was expressed abundantly in all three regions of rat heart. Although in human right atrium and SAN mRNA of $P2X_{4\text{-}7}$ was expressed, the expression of $P2X_4$ and $P2X_7$ mRNA was highest in these two regions. $P2X_1$ mRNA was only detected in human SAN (Musa et al. 2009). The increased expression of $P2X_1$ in the atria might be contributed to suffer from dilated cardiomyopathy. That $P2X_4$ mRNA was up-regulated contribute to ligation-induced heart failure (Burnstock et al. 2011, Musa et al. 2009).

Endothelial $P2X_4$ receptors play a crucial role in flow-sensitive mechanisms, which regulate blood pressure and vascular remodeling. It has been proved by Yamamoto et.al on the $P2X_4$ deficient mice model. $P2X_4$ knockout mice didn't have normal endothelial cell responses to flow, for example, Ca^{2+} influx and production of the potent vasodilator nitric oxide (NO) which might lead to higher blood pressure (North 2009), and it is also found out that $P2X_4$ deficient mice excrete smaller amounts of NO in urine than wild type (Yamamoto et al. 2006).

3.6 Respiratory system

ATP is playing a crucial role in the central respiratory control, which might mediate changes in the activity of medullary respiratory neurons in hypercapnia. Gourine and colleagues found out the evidence that ATP acting on $P2X_2$ receptors, which are expressed in ventrolateral medulla that contains respiratory neurons responsible for generating and shaping the respiratory rhythm. Modulation of the function of $P2X_2$ receptors in ventrolateral medulla might be related to change in activities of ventrolateral medulla respiratory neurones (Gourine et al. 2003).

It has also been discovered that $P2X_4$ receptors expressed on lung epithelial cells can control ciliary beat frequency, which make the clearance of mucus from the airways (Zsembery et al. 2003). Cystic fibrosis might be benefit from manipulation of it. Some agonists, such as β2-adrenergic ($P2Y_2$ agonist) and ATP, can increase ciliary beating in trachea and airway epithelia via activation of P2Y and P2X receptors, which provide an key therapy in respiratory diseases (Leipziger 2003). Zhao et al found out that erythromycin can block P2X mediated Ca^{2+} influx, this action of which might contribute to the treatment of airway inflammation.

3.7 Exocrine glands

Exocrine glands can secrete hormones and other chemical messengers into ducts that lead directly into the external environment. ATP work on the purine receptors that are expressed on secretory epithelia with the consequent secretion of hormonal peptides, bicarbonate,

potassium and so on (Leipziger 2003). Pochet and colleagues found out that $P2X_7$ receptors are expressed in mice submandibularductal cells, and $P2X_4$ receptors are also involved. Using the wild type and $P2X_7$ knockout mice models, they found out strong evidence that $P2X_7$ salivary secretion through the modulation of phospholipid signaling processes, especially for phospholipase A2 and phospholipase D. In the knockout mice, ATP no longer active any phospholipid signaling and saliva showed a decreased potassium content (Garcia-Marcos et al. 2006). Using the RT-PCR and immunohistochemical staining, it is found out that $P2X_4$ receptors are in choangiocytes, which is likely the primary isoform involved, representing a functionally component which modulating biliary secretion (Doctor et al. 2005).

3.8 Intestinal motility

There is some evidence show that P2X receptors are expressed in the enteric nervous system, which underlies coordinated movements of the intestine. $P2X_2$, $P2X_3$ and $P2X_7$ receptors are all involved in the intestine system in health and gut disease. It has been detected that $P2X_2$, $P2X_3$ receptors are expressed on AH cells which is the principal intrinsic afferent neuron of the enteric nervous system (Furness et al. 2004, North 2009). In the $P2X_3$ knockout mice model, the gut peristalsis was inhibited. It has been proved that $P2X_3$ receptors might contribute to detection of distention or intraluminal pressure increases and initiation of reflex contractions (Bian et al. 2003). On the other side, $P2X_3$ receptors are involved in gut disorders. Irritable bowel syndrome (IBS) is characterized by chronic abdominal pain, bloating and discomfort. In the rat model of IBS-like visceral hyperalgesia, $P2X_3$ protein expression was significantly enhanced in colon-specific DRGs 8 weeks (Xu et al. 2008), which suggesting a potential role in dysmotility and pain (Yiangou et al. 2001). Wynn et al described that $P2X_3$ receptors signaling enhancement in colitis (Wynn et al. 2004). It is suggesting that $P2X_3$ receptors are potential targets for drug development of IBS.
$P2X_3$ receptors are expressed in intrinsic sensory neurones in the submucous plexus of gut and extrinsic sensory nerves (Xiang et al. 2004). It has been found out that in the substantial distension, ATP activates moderate distension higher threshold extrinsic sensory fibers and lower threshold intrinsic enteric sensory fibers that transmit the message to the CNS, via $P2X_3$ receptors from mucosal epithelia, respectively (Bian et al. 2003, Wynn et al. 2004, Wynn et al. 2003).

3.9 Immune system and inflammation

It is found out that $P2X_1$, $P2X_4$ and $P2X_7$ receptors are coexpressed in most immune cells in different level. $P2X_7$ receptors play a crucial role in inflammation and immunomodulation (Burnstock et al. 2011).
Inflammation is initiated by some pathogen constituents, injured or dying cells which can release intrinsic host molecular (Di Virgilio 2007). As the host endogenous pro-inflammatory Nucleotides especially ATP play a key role in inflammation in which human IL-1 family proteins are key mediators of the acute immune response to injury and infection. The $P2X_7$ receptor is the key player in interleukin (IL)-1β and IL- 18 maturation and release. In inflammation process, $P2X_7$ receptors induce the rapid activation of caspase-1 with subsequent release of the pro-inflammatory cytokine IL-1β from activated macrophage and microglia. That inhibition the $P2X_7$ receptor is directly decreased the levels of IL-1β in plasma or in the area of injury.

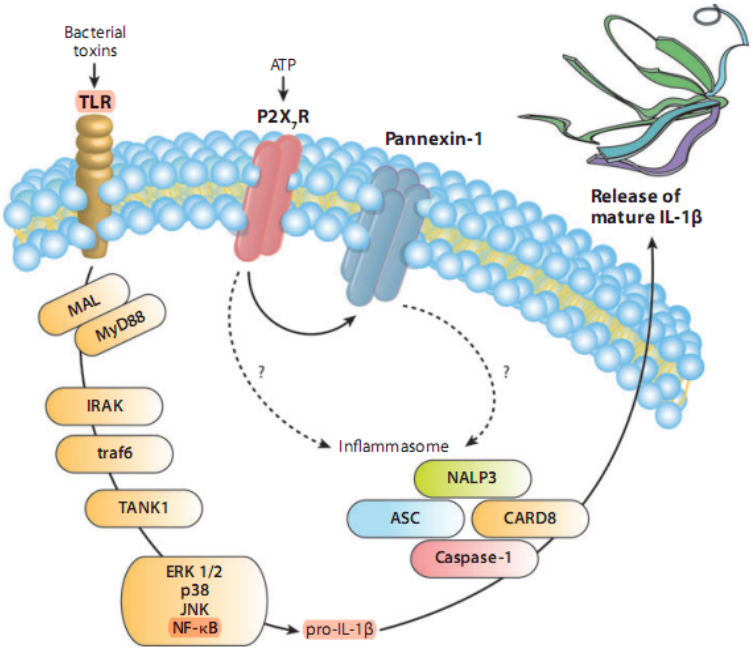

Fig. 3. $P2X_7$ receptor and the NALP3 inflammasome. The $P2X_7$ receptor is the key player in interleukin (IL)-1β and IL-18 maturation and release. High concentrations of extracellular ATP, present at sites of inflammation, activate a P2X7R/pannexin-1 protein complex which induces the rapid activation of caspase-1 with subsequent release of the pro-inflammatory cytokine IL-1β from activated macrophage and microglia. This figure is reproduced from reference (North 2009).

Mycobacterium tuberculosis (MTB) is a monocyte/macrophage (M/M) parasite. Lammas and colleagues proved that $P2X_7$ receptors associated with ATP induced apoptosis in macrophages that result in killing of the mycobacteria within them (Lammas et al. 1997). Roberta et al also proved that mycobacterial infection leads to an increased expression of $P2X_7$, and at the same time infected macrophages induced the extracellular release of ATP. Cell death can't be induced by MTB after using the oxidized ATP (oATP), which is the $P2X_7$ receptor antagonist (Placido et al. 2006). Taken together, the death of intracellular bacilli directly related to $P2X_7$ mediated macrophage apoptosis, but the mechanisms underlying the IL-1 cytokines (IL-1α, IL-1β and IL-18) cellular release are still unclear. Pelegrin and colleagues investigated the release patterns in LPS-primed peritoneal macrophage, RAW264.7 macrophage, and J774A.1 macrophage. They found out that IL-1β is the direct cause of macrophage apoptosis, and two release pathways are proved (Pelegrin et al. 2008). One is the caspase-1 mediating release of processed IL-1β that is selectively blocked by inhibition of caspase-1 or panx1, the other is a calcium independent, caspase-1/ panx1-independent release of pro-IL-1β that is selectively blocked by glycine (Pelegrin et al. 2008). Although it is proved that $P2X_7$ receptors are highly expressed in these macrophage, apoptosis of which is related to its prolonged activation, and concomitant killing of intracellular pathogens (North 2009, Pelegrin et al. 2008), it is unclear that whether the macrophage apoptosis is necessary for killing of intracellular mycobacteria.

Receptors	Distribution	Antagonist	Diseases and potential therapeutic strategies
$P2X_1$	Sympathetic neurons, Sensory neurons, Smooth muscle, Cardiac muscle, Endothelial cells, Inner ear, Osteoblasts, Platelets, Cochlea Oligodendrocytes, Exocrine secretary cells, Kupffer cells	Ip5I, MRS 2220, NF023	
$P2X_2$	Sympathetic neurons, Sensory neurons, Eye, Tongue, Parasympathetic neurons, Enteric neurons, Central nervous system, Retinal neurons, Endothelial cells, Osteoblasts, Keratinocytes, Sperm, Erythrocytes, macrophages, Endocrine secretory cells, Cholangiocytes, Inner ear, Olfactory organ, Cochlea		
$P2X_3$	Sympathetic neurons，Tongue, Parasympathetic neurons, Keratinocytes, Müller cells, Sensory neurons, Enteric neurons, Retinal neurons, Smooth muscle, Cardiac muscle, Endothelial cells, Inner ear, Cholangiocytes	A-317491, TNP-ATP,	neuropathic and inflammatory animal pain (Jarvis et al. 2002) thermahyperalgesia and mechanical allodynia, ATP-induced allodynia (Nakagawa et al. 2007)
$P2X_4$	Sympathetic neurons, Parasympathetic neurons, Enteric neurons, Central nervous system, Retinal neurons, Cardiac muscle, Osteoclasts, Epithelial cells, Microglia, Müller cells, Endothelial cells, Erythrocytes, Immune cells, Exocrine/Endocrine secretory cells,	Suramin, PPADS, Reactive Blue 2, CORMs	
$P2X_5$	Sympathetic neurons, Parasympathetic neurons, Sensory neurons, Retinal neurons, Smooth muscle, Cardiac muscle, Osteoblasts, Keratinocytes, Epithelial cells, Müller cells		
$P2X_6$	Sympathetic neurons, Central nervous system, Smooth muscle, Cardiac muscle, Epithelial cells, Cholangiocytes		
$P2X_7$	Sympathetic neurons, Sensory neurons, Enteric neurons, Central nervous system, Retinal neurons, Smooth muscle, Eye, Osteoblasts, Keratinocytes, Fibroblasts, Epithelial cells, Microglia, Cochlea, Müller cells, Enteric glial cells, Sperm, Erythrocytes, Immune cells(predominantly), Inner ear, Kupffer cells, Exocrine/Endocrine secretory cells	AZD9056 (clinic trial Ⅱ), KN62, Brilliant Blue, oATP, A-438079, PPADS, Suramim, Decavanadate	rheumatoid arthritis (Elsby et al. 2011) Parkinson disease (Marcellino et al. 2010) antinociception (McGaraughty et al. 2007)

Table 2. Characteristics of P2X receptors

Osteoclasts function in concert with osteoblasts, fibroblast lineage cells which deposit new bone. Normal bone remodeling and the maintenance of skeletal integrity need osteoblasts and osteoclasts coordinate their activity. Using extracellular monoclonal antibody, Gartland et al. performed an experiment in vivo and in vitro, which demonstrated that $P2X_7$ receptors are expressed in various stages of osteoclast differentiation (Gartland et al. 2003). Osteoclastresorptive activity was inhibited by its antibody. Comparing the $P2X_7$ knockout mice model with wild type, it is indicating that $P2X_7$ does not regulate longitudinal bone growth, but $P2X_7$ knockout mice significantly reduced in total and cortical bone content and periosteal circumference in femurs (Ke et al. 2003). Thus, the $P2X_7$ receptor represents a novel therapeutic target for skeletal disorders.

4. Novel therapeutic drugs

Since seven P2X receptors ($P2X_{1-7}$) are widely distributed in excitable and non-excitable cells of vertebrates, and play a crucial role in the pathology of several disease states, such as neuropathic pain, chronic inflammation, rheumatoid arthritis and so on. Thus P2X receptors are the potential targets for drug development. Considerable efforts have been made to synthesize therapeutic antagonists. Besides being important pharmacological tools for characterization of the pathophysiological roles of P2X receptors in native systems, such ligands may represent new therapeutic entities of potential interests in various human diseases. The most prospective areas for drug discovery at present are for the treatment of visceral pain with $P2X_3$/$P2X_{2/3}$ receptor antagonists and neuropathic and inflammatory pain with $P2X_7$ antagonists up to present.

4.1 $P2X_7$ receptors

$P2X_7$ receptors is a unique family have been extensively studied in immune cells where they are related to rapid release of pro-inflammatory cytokines and the initiation of the inflammatory cascade. Until now there are some therapeutic antagonists have been synthesized. They are evaluated in different levels from animal model experiment to clinical trial. Some of them are still evaluated in cell test as a tool for pathophysiological study. The $P2X_7$ receptors antagonists are nonselective or selective, and they show different affinity and IC_{50}.

The most promising $P2X_7$ receptor antagonist is AZD9056, which was developed for oral treatment of rheumatoid arthritis. AZD9056, a weakly basic secondary amine (pKa 9.77) with a hydrophobic adamantine moiety that is highly protein bound (97%), is in clinic trial Ⅱ (Elsby et al. 2011). Efficacy and drug-drug interaction (DDI) studies performed as part of the clinical development program for a new candidate drug. This study is prior to patient studies. Since patients with rheumatoid arthritis receive multiple co-medications, that might be taken a risk of the co-morbid conditions, it is necessary to evaluate the potential of RA drug candidates to perpetrate a drug-drug interaction (DDI) with methotrexate which is a most commonly drug treating RA (van Roon et al. 2009). Methotrexate is a substrate of the human transporters OAT1, OAT3, MRP2 and BCRP which all-mediate active renal elimination. AZD9056 can't inhibit OAT1 and OAT3 transporting methotrexate, but have a weakly inhibition effect on BCRP mediated transporting (IC_{50}=92 μM) (Elsby et al. 2011). Recently, Keystone and colleagues have reported their work on phase Ⅱ studies, which assess the effects of orally administered AZD9056 on the signs or symptoms of rheumatoid arthritis (RA). They used randomized, double blind, placebo-controlled, and parallel-group

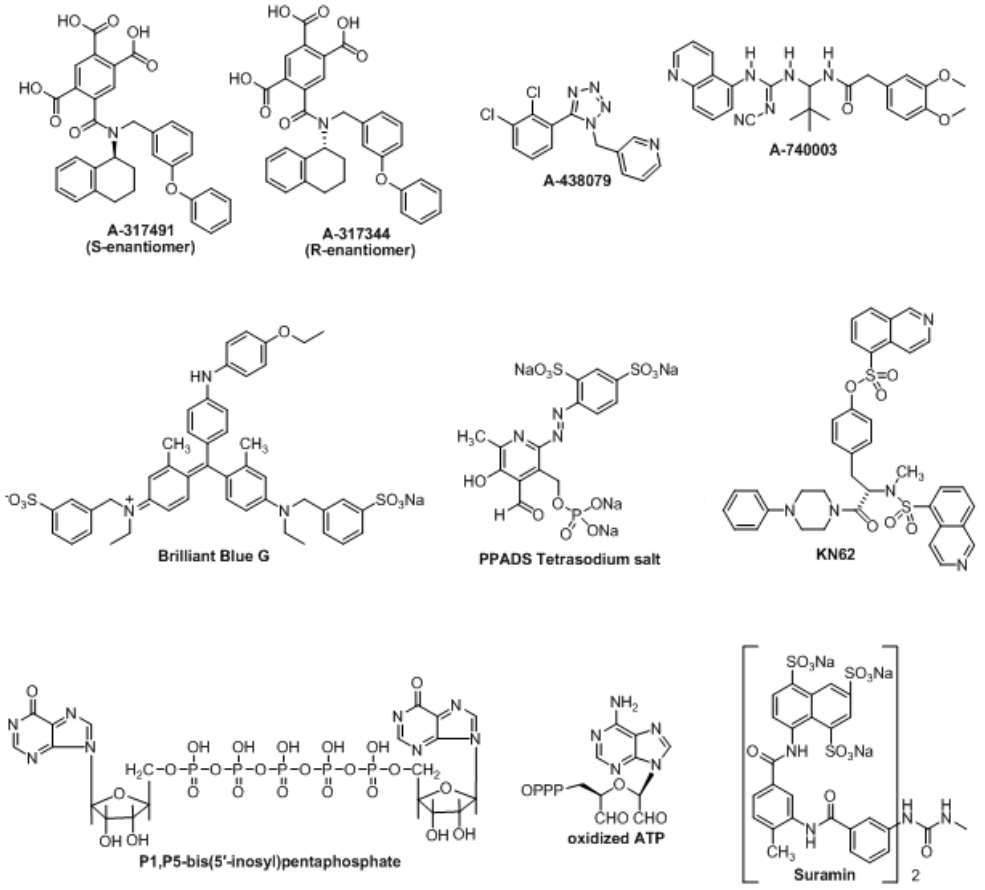

Fig. 4. Structures of P2X receptors antagonists. A-317491 which is the first non-nucleotide antagonist, has high selectivity (IC_{50} > 10 μM) to $P2X_3$ and $P2X_{2/3}$ receptors; the R-enantiomer of A-317491, A-317344, was ineffective (ED_{50} > 100 μmol/kg s.c.) in neuropathic and inflammatory animal pain models; 3-((5-(2,3-dichlorophenyl)-1H-tetrazole-1-yl)methyl pyridine (A-438079) effectively inhibit Bz-ATP stimulation for $P2X_7$ receptors; A-740003 ((N-(1-{[(cyanoimino) (5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2- (3,4-dimetho- xyphenyl)acetamide) shows highly specific and potent for $rP2X_7$ and $hP2X_7$ of which potency is 18-40 nM (Honore et al. 2006); Brilliant Blue G is a more potent and selective antagonist, IC_{50} for rat and human $P2X_7$ receptor is 10 nM and 200 nM, respectively (North 2002); KN62 (1-[N,O-bis(5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) which is isoquinoline derivative (Humphreys et al. 1998), inhibit ATP – stimulated Ca^{2+} influx and selectively inhibit calcium/calmodulin-dependent protein kinase II. TNP-ATP, PPADS and suramin is non-selective antagonist at P2X receptors; ATP 2′,3′-dialdehyde (oxidized-ATP, oATP) is a irreversible antagonist at $P2X_7$, $P2X_1$ and $P2X_2$ receptor (Di Virgilio 2003); Ip5I is a potent and selectivity antagonist for recombinant rat $P2X_1$ receptors at nanomolar concentration (pIC_{50} = 5.6) (King et al. 1999).

in the phase Ⅱa and phase Ⅱb studies. Through months studies, they found out that 65% of patients who received 400 mg/day which is a tolerated dose responded at the ACR 20 level that is American College of Rheumtology 20% response criteria, comparing with 27% of placebo-treated recipients. In Ⅱb studies 383 randomized patients who received AZD9056 had no clinically or statistically significant effects on RA compared with placebo group. But in both studies, AZD9056 was used in tolerated dose (400 mg/day) so that the proportion of patients meet the ACR20 criteria. The results showed that AZD9056 does not have significant efficacy for RA (Keystone et al. 2011).

In 2006, Abbott Laboratories disclosed two series $P2X_7$ antagonists: disubsituted tetrazaled and cyanoguanidines, which show enhanced potency and selectivity to $rP2X_7$ and $hP2X_7$ (Honore et al. 2006, Nelson et al. 2006). 1-Benzyl-5-aryltetrazoles were discovered to be novel $P2X_7$ antagonists by Nelson et al (Nelson et al. 2006). 3-((5-(2, 3-dichlorophenyl)-1H-tetrazole-1-yl) methyl pyridine (A-438079) effectively inhibits Bz-ATP stimulation. The compound can inhibit calcium flux in human and rat $P2X_7$ cell lines. It's IC_{50} for human and rat $P2X_7$ receptors are 100 and 300 nM respectively. It has devoid activity to other P2 receptors (IC_{60}>> 10 μM) and its analogues can also have the ability to inhibit IL-1β release and $P2X_7$ mediated pore formation in human THP-1 cells (Nelson et al. 2006). Daniel Marcellino et al using $P2X_7$ receptor antagonist A-438079 as a tool found out that blockade of $P2X_7$ might be a novel protection strategy for striatal DA terminals in Parkinson's disease (Marcellino et al. 2010). Mcgaraughty et al utilized A-438079 investigate $P2X_7$ related antinociception mechanism in vivo and vitro. Three different rat models of neuropathic pain showed attenuated formalin-induced nocifensive behaviors after injecting 10-300 μmol/kg (i.p.) of A-438079 (McGaraughty et al. 2007). A-438079 dose dependently (0.3-3μM) decreased the quantity of cytokine and IL-1β (McGaraughty et al. 2007). Comparing other P2 receptors, A-740003 ((N-(1-{[(cyanoimino) (5-quinolinylamino) methyl] amino}-2, 2-dimethylpropyl)-2-(3, 4-dimetho- xyphenyl) acetamide) shows highly specific and potent effects on $rP2X_7$ and $hP2X_7$ of which potency is 18-40 nM (Honore et al. 2006). It also changes intracellular calcium concentration in a competitive way. Both compounds' sufficient bioavailability in intraperitoneal administration allows for in vivo investigation on disease models of $P2X_7$ receptors (Honore et al. 2006, Nelson et al. 2006).

Suramin (IC_{50}> 300 μM for rat $P2X_7$) and PPADS (IC_{50}～50 μM) (pyridoxalphosphate- 6-azopheny2', 4'-disulfonate) are both prototypic nonselective P2X receptor antagonists (North 2002). They show noncompetitive antagonism and low affinity (Ki>10 μM). KN62 (1-[N,O-bis(5-isoquinoline-sulfonyl) -N-methyl-L-tyrosyl]-4-phe-nylpiperazine) which is isoquinoline derivative (Humphreys et al. 1998), and being a most potent compound with 13.4 nM IC_{50}, can inhibit ATP–stimulated Ca^{2+} influx and selectively inhibit calcium/calmodulin-dependent protein kinase II. But it shows significantly species differences. It is more potent to human $P2X_7$ versus rat $P2X_7$ (Humphreys et al. 1998). Brilliant Blue Gis a more potent and selective antagonist, IC_{50} for rat and human $P2X_7$ receptor is 10 nM and 200 nM, respectively (North 2002).

Michel et al reported the decavanadate, which is a polymeric form of vanadate, is a reversible and competitive $P2X_7$ receptor antagonist. But it also displays non-selectivity because it not only inhibits the $P2X_2$ and $P2X_4$ mediated response, but also interacts with IP3 binding and inhibition of ribonuclease A (Michel et al. 2006).

ATP 2', 3'-dialdehyde (oxidized-ATP, oATP), ATP with the 2'- and 3'-hydroxyl moieties oxidized to aldehydes by periodate treatment, is a irreversible antagonist required 1 or 2 h incubation to inhibit $P2X_7$ receptor (Di Virgilio 2003). oATP is used in mouse macrophage to

lock $P2X_7$ initiated responses. But except that, oATP can also inhibit $P2X_1$ and $P2X_2$ mediated responses (Evans et al. 1995). It also inhibits nuclear factor-κB (NF-κB) and cytokine release. So these limit its role as a pharmacological tool like decavanadate.

4.2 $P2X_3$ and $P2X_{2/3}$ receptors

$P2X_3$ and $P2X_{2/3}$ receptors localize on peripheral and central processes of sensory afferent nerves (Jarvis et al. 2002). Experiment with antisense oligonucleotides and $P2X_3$ knockout mice proved that $P2X_3$ receptors play a crucial role in the signaling of chronic inflammatory pain and some features of neuropathic pain.

Virginio et al. identified 2'3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) can block $P2X_1$, $P2X_3$ and $P2X_{2/3}$ receptors, but it is ineffective in blocking currents produced by activated $P2X_2$, $P2X_4$ and $P2X_7$ receptors. It can completely block fast component of ATP mediated and αβmeATP mediated sustained current in neurones of dorsal root and nodose ganglia (Bradbury et al. 1998). It is a competitive antagonist for $P2X_3$ and $P2X_{2/3}$ receptors with pA2 values of -8.7 and -8.2 (Burgard et al. 2000). The IC_{50} values for $hP2X_{2/3}$ and $rP2X_{2/3}$ are 3 to 6 nM. It displays a rapid inhibition in onset, reversible, and use independence. The $P2X_{2/3}$ receptors can fully recover from TNP-ATP after removal it for more than 5 s (Burgard et al. 2000).

A-317491 has high selectivity (IC_{50}> 10 μM) to $P2X_3$ and $P2X_{2/3}$ receptors, which can potently block human and rat $P2X_3$ and $P2X_{2/3}$ mediated calcium flux (Ki=22 – 92 nM) (Jarvis et al. 2002). Using neuropathic and inflammatory animal pain models, A-317491 can reduce nociception. They use s.c. administration found out that A-317491 dose-dependently (ED_{50} = 30 μmol/kg s.c.) reduced complete Freund's adjuvant-induced thermal hyperalgesia in the rat pain models, but ineffective in acute pain, postoperative and visceral pain (Jarvis et al. 2002). However, it is most effective (ED_{50} = 10 - 15μmol/kg) in attenuating both thermahyperalgesia and mechanical allodynia after chronic nerbe constriction injury. On the contrary, the R-enantiomer of A-317491, A-317344, was ineffective (ED_{50} > 100 μmol/kg s.c.)in neuropathic and inflammatory animal pain models. Nakagawa et al use the pain animal models found out that i.t. co-administration of ATP and A-317491 (30 nM) significantly prevented the ATP-induced allodynia (Nakagawa et al. 2007). In other drug treatment group, co-administration of suramin and PPADS significantly prevented the induction of longlasting allodynia (Nakagawa et al. 2007).

AF-353, which is synthesized by Roche Palo Alto are novel, selective and highly potent $P2X_3$ and $P2X_{2/3}$ receptor antagonist. It has been proved that AF-353 is orally bioavailable (%F = 32.9) and stable in vivo for the treatment of pain which is in clinical trial (Gever et al. 2010). The antagonistic potencies (pIC(50)) for rat and human $P2X_3$ and human $P2X_{2/3}$ receptors ranged from 7.3 to 8.5 which had little or no effect on other P2X receptors (Gever et al. 2010). Comparing with A-317491 and TNP-ATP, AF-353 inhibits activation by ATP in non-competitive fashion. It is observed that A-317491 has reasonable half-life (t(1/2) = 1.63 h) and plasma-free fraction (98.2% protein bound) (Gever et al. 2010). This compound is good for studying $P2X_3$ and $P2X_{2/3}$ receptors in animal models and optimize compound for clinic trial.

4.3 $P2X_4$ receptors

Finding antagonists for the $P2X_4$ receptor has been more problematic. Suramin, PPADS and reactive Blue 2 are conventional non-selective antagonists for $P2X_4$ receptors up to present. PPADS is a reversible antagonist required 20 to 30 min washing can partially reverse (North

2002). The $hP2X_4$ receptor is more sensitive to PPADS than $rP2X_4$. Jones et al reported that IC_{50} for mouse $P2X_4$ receptor is 10 mM (Jarvis et al. 2002, Jones et al. 2000). Recently Wilkinson and Kemp proved that CORMs which is co-releasing molecules is an effective antagonist at human $P2X_4$ receptors (Jarvis et al. 2002).

4.4 $P2X_1$ receptors

Diinosinepentaphosphate (IpnI, n is the number of phosphates), comprise two ribosylatedinosine molecules bridged by a phosphate chain. These dinucleotides are synthesized by deaminating diadenosine polyphosphates with non-specific AMP-deaminase of Aspergillus sp. (Pintor et al. 1997). P1,P5-bis(5′-inosyl) pentaphosphate (Ip5I), the member of this family, has been showing a potent antagonist at a specific dinucleotide receptor in rat brain synaptosomes (IC_{50} = 4 nM) and P2X receptor (IC_{50} = 30 μM) (Pintor et al. 1997). King et al. reported that Ip5I is a potent and selectivity antagonist for recombinant rat $P2X_1$ receptors at nanomolar concentration (pIC_{50} = 5.6). Non-linear of Schild plot proved that Ip5I is a noncompetitive antagonist (King et al. 1999). It represents a different manner in low and high concentrations. At low (≤ 100 nM) concentrations Ip5I represents a high affinity antagonist for $P2X_1$ receptors, however, in higher (> 100 nM) concentrations it represent a more complex actions.

Jacobson et al. synthesize two antagonists which are analogues of PPADS. MRS 2220 is selective antagonist. Comparing with PPADS (IC_{50} = 98.5 +/- 5.5 nM), MRS 2220 represents a lower IC_{50} (IC_{50} = 10.2 +/- 2.6 mM) at recombinant $P2X_1$ receptor, But unlike PPADS its effect was reversible with washout and surmountable (Jacobson et al. 1998).

NF023 (8, 8′-(carbonylbis (imino-3, 1-phenylene carbonylimino) bis (1, 3, 5-naphthalenetrisulf onic acid)) is suramin analogue, which represent a competitive fashion at P2X receptor-mediated responses in certain vascular and visceral smooth muscles. $P2X_1$ receptors represent a most sensitive manners with IC_{50} values of 0.24 and 0.21 μM for rat and human homologues, respectively (Soto et al. 1999). $P2X_2$ (IC_{50}> 50 μM), $P2X_3$ (IC_{50} = 8.5 and 28.9 μM for rat and human), $P2X_4$ (up to 100 μM) shown different sensitivity (Soto et al. 1999). TNP-ATP is potent antagonist at $P2X_1$ and $P2X_3$ receptors at nanomolar concentrations.

4.5 Other P2X receptors

So far, there is no selective and potency antagonists that is orally bioavailable and stable in vivo for $P2X_2$, $P2X_5$ and $P2X_6$. Suramin, PPADS and TNP-ATP are non-selective antagonists at P2X receptors. Seven P2X receptors represent different sensitivity.

5. Conclusion and future issues

P2X receptor, which is a novel family of ligand-gated cation channels, is non-selective cation channel that gated in the presence of ATP. Seven P2X receptor subunits ($P2X_{1-7}$) are widely distributed in excitable and non-excitable cells of vertebrates and play a crucial role in inter alia afferent signaling (including neuropathic pain), regulation of renal blood low, vascular endothelium, and chronic inflammation. Thus P2X receptors are potential targets for drug development. The field is still limited by the availability of agonist, antagonist and modulator which could accelerate our understanding of the physiological roles of P2X receptors. However, there are some notable antagonists of $P2X_1$, $P2X_3$ and $P2X_7$ receptors

with highly potency, selectivity and nanomolar affinity, such as AF-353, A-317491 and AZD9056. There are some important issues for the antagonist application. The first is actions at receptors other than P2X receptors. If the antagonist is developed to be a potential drug, it has to overcome the actions on other receptors or other subtype of P2X receptors. Selectivity is important for drug development. For example, TNP-ATP, PPADS and suramin are non-selective antagonists of P2X receptors. Seven subtypes of P2X receptors represent different sensitivity. But it would be welcomed to study the physiological and functional role for developing such compounds. The second is the species specificity. Human and rat P2X receptors represent different sensitivity to a compound which means that it is difficult to do animal model experiments before the clinic trial. So far, there has been promising development of clinical $P2X_7$ antagonists, notably Abbott compounds – A-438079 and A-740003. Both compounds represent highly specificity and potency for $rP2X_7$ and $hP2X_7$. Sufficient bioavailability is likely to allow for in vivo investigation on disease models of $P2X_7$ receptors. Another promising $P2X_7$ receptor antagonist is AZD9056, which was developed for oral treatment of rheumatoid arthritis. But recent clinical trail II experiments showed that AZD9056 did not have significant efficacy for RA. They concluded that $P2X_7$ receptors do not appear to be a therapeutically useful target in RA. However, it is proved that AZD9056 is a selective and potent antagonist of $P2X_7$ receptors before clinical trial II. So it might be efficacious for other diseases which are related to $P2X_7$ receptors. AF-353 is most promising compound that may reach the market introduction. The good orally bio-availability and stability of AF-353 in vivo may bring the biggest advantage for patients in pain treatment. Antagonists for some of the other P2X receptors remain to be developed. P2X receptors are likely to have widespread therapeutic usage in the future.

6. References

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Pregnane X Receptor in Drug Development

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1. Introduction

Benzoate X receptor (BXR) was identified in *Xenopus* and named BXR for its recognition of benzoates as substrates (Blumberg et al. 1998a). Subsequently, a related mouse gene was identified based on its sequence homology to other known nuclear receptors (NRs) and named pregnane X receptor (PXR) for its activation by pregnane steroids (Kliewer et al. 1998). Upon its identification, the human PXR gene was first given the name SXR for steroid and xenobiotics receptor but was subsequently coined human PXR (hPXR) (Bertilsson et al. 1998; Blumberg et al. 1998b; Lehmann et al. 1998). PXR is a broad-spectrum xenobiotic sensor and master transcriptional regulator of xenobiotic detoxification and metabolism genes, capable of being activated by structurally diverse ligands, including many commercially marketed chemotherapeutics. Upon ligand engagement, PXR binds to the promoter regions of its target genes as a heterodimer with another NR, retinoic X receptor (RXR) to initiate gene transcription (Bertilsson et al. 1998; Kliewer et al. 1998). Target genes of PXR include genes for phase I and phase II drug metabolizing enzymes (DMEs) and phase III ATP binding cassette (ABC) drug transporters. The two most important target genes of PXR are cytochrome P450 3A4 (*CYP3A4*) and multidrug resistance 1 (*MDR1*). CYP3A4 is most abundantly expressed in the liver and is the primary contributor to metabolizing most of the currently marketed therapeutic agents (Ingelman-Sundberg 2004). MDR1 is involved in drug resistance. Induction of CYP3A4 and MDR1 contributes to clinical drug-drug interactions and drug resistance. In addition, PXR plays roles in many other important physiologic and pathologic processes, such as those in bone disorders, liver diseases, inflammation, and cancers. Topics in this chapter include structural-functional analysis of PXR, regulation of PXR and its target genes, physiologic and pathologic functions of PXR, and relevant drug discovery techniques for PXR. Importantly, this chapter highlights PXR as an appealing target for both the development of novel drugs and the improvement of current drug therapies.

2. Structure of Pregnane X Receptor

PXR shares common structural features that are characteristic of NRs (Ingraham & Redinbo 2005). A DNA-binding domain (DBD) residing at the amino terminus allows for the NR to

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bind to hormone response elements (HREs), facilitated by the presence of two zinc-finger motifs. The ligand-binding domain (LBD) is the most prominent feature in PXR. Crystal structures of the LBD shed light on the promiscuity of PXR, which depict a flexible and substantially large cavity, with a volume > 1600 Å (Fig. 1) (Watkins et al. 2001). According to the 3-D structure, the LBD comprises three sets of α-helices: α1/α3, α4/α5/α8, and α7/α10. In addition, a layer of five stranded anti-parallel β-sheets includes two novel β-strands not observed in other NRs: β1 and β1′. In contrast to other NRs of known structures, PXR contains an insert of approximately 60 amino acids between helices α1 and α3, which contribute to the formation of the novel helix α2, β1, and β1′. In hPXR, a flexible loop encompassing residues 309-321 replaces helix α6 (Orans et al. 2005).

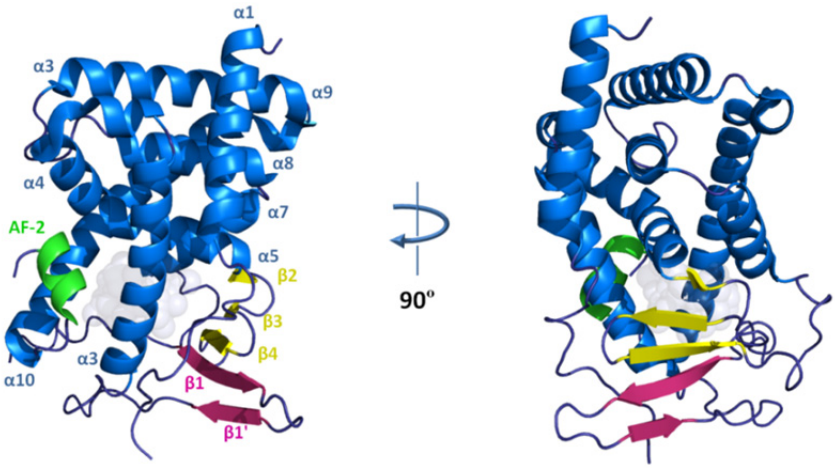

Fig. 1. Crystal structure of the hPXR LBD (PDB code 1ILH). The α-helices are rendered in blue, β-sheets present in other NRs are shown in yellow, β-sheets unique to PXR are represented in magenta, and the AF-2 helix is depicted in green. The three orientations of the agonist SR12813 are shown as transparent spheres, indicating the ligand-binding cavity.

PXR was shown to homodimerize in solution (Noble et al. 2006). Crystal structures indicate the involvement of the terminal β1′ strands from each monomer to form the dimer interface, supported by six intermolecular hydrogen bonds (Fig. 2). In addition, Trp223 and Tyr225 from each monomer interlock to form a tryptophan zipper, the first to be observed in a native protein. The Trp223Ala, Tyr225Ala double mutant prevents homodimerization without affecting protein folding. The mutant retained ligand and DNA binding capabilities but exhibited a much reduced CYP3A4 induction in response to PXR agonists SR12813 and rifampicin. This impairment was believed to be due to a disruption of coactivator recruitment.

PXR interacts with p160/SRC coactivators such as the steroid receptor coactivator 1 (SRC-1) through the active conformation of the ligand-dependent activating function 2 (AF-2) helix within the LBD (Watkins et al. 2003a). These coactivators contain three LXXLL motifs (L=Leu, X=any other amino acid), which also adopt α-helical conformations and interact with the NR via a "charge clamp". A 25-mer SRC-1 peptide containing the second LXXLL motif was co-crystallized with hPXR (Fig. 2). The LXXLL region of the peptide is buried in a

groove on the surface of the PXR LBD composed of AF-2, α3, and α4. Binding of the SRC-1 peptide stabilized the LBD. Not surprisingly, coactivator peptides were reported to be required for stable expression and purification of the protein in bacterial systems.

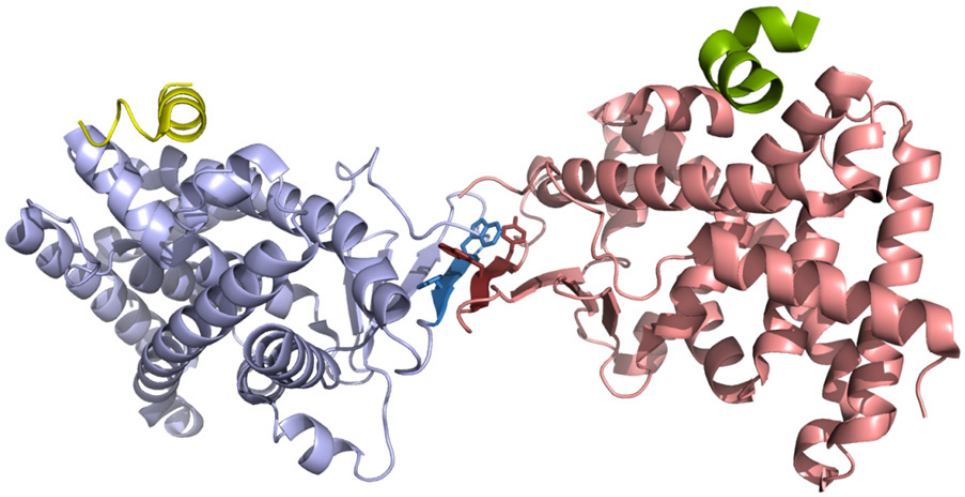

Fig. 2. Structure of hPXR illustrating the homodimerization of the receptor (PDB code 1NRL). Each monomer is represented as either marine blue or salmon red, with the respective β1′ rendered as blue or red, respectively. Trp223 and Tyr225 from each monomer are represented as sticks. The SRC-1 peptides are shown as either yellow or green.

The ligand-binding pocket is formed in large part by non-polar residues, creating a mostly hydrophobic and uncharged cavity (Fig. 3A). Of the four charged amino acid side chains present in the pocket (Glu321, His327, His407, and Arg410), a salt bridge was observed between Glu321 and Arg410. Another salt bridge occurs between Asp205 and Arg413 surrounding the ligand-binding pocket. The amino acid residues participating in these electrostatic interactions were shown to be important in the basal activity of PXR based on mutagenesis and cell-based reporter assays (Watkins et al. 2001). Some of these residues along four polar residues (Ser208, Ser247, Cys284, and Gln285) can form critical interactions with the ligand. The structural models also provide insights into the marked differences in the activation of PXR across species. For instance, SR12813 selectively activates hPXR over the mouse PXR (mPXR). When residues that are unique and are involved in SR12813 binding to hPXR were incorporated in mPXR, the mouse-human hybrid PXR responded efficiently to SR12813.

Several x-ray structures of the PXR LBD in complex with agonists have been reported. The first complex showed the cholesterol-lowering drug SR12813 in three distinct positions within the cavity (Watkins et al. 2001). A more recent crystallographically determined structure involving PXR-SR12813 in complex with an SRC-1 coactivator peptide revealed a single agonist binding mode (Fig. 3B) (Watkins et al. 2003a). Thus, Redinbo and coworkers argued that the PXR ligand-binding pocket can accommodate ligands in multiple positions, and upon coactivator binding, the ligand is stabilized into a single active orientation. In this "active" state, SR12813 interacts with Ser247 and His407 through hydrogen bonding involving 11 hydrophobic amino acid side chains. The other PXR agonists that were

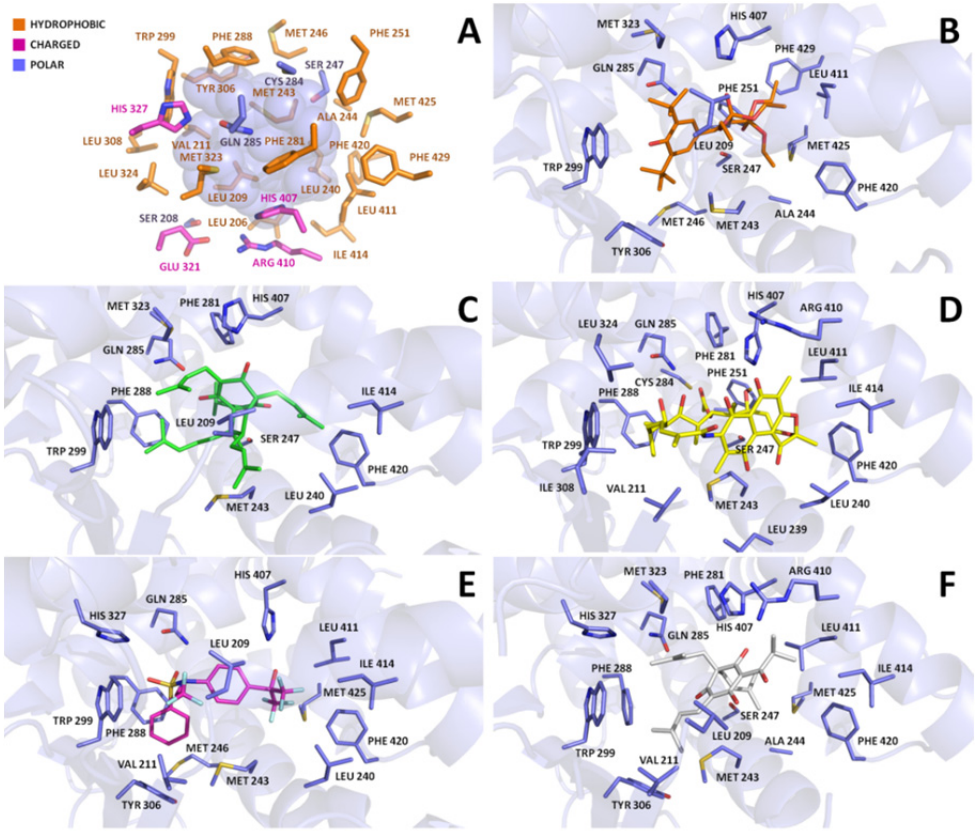

Fig. 3. A) Stick representation of the amino acids forming the ligand-binding pocket of hPXR (PDB code 1ILH). The carbon atoms of the hydrophobic residues are shown in orange, charged residues in magenta and polar residues in marine blue. The areas occupied by the three binding modes of the agonist SR12813 are shown as transparent spheres. B) hPXR LBD in complex with SR12813 in a single orientation (PDB code 1NRL). Carbon atoms of the ligand are shown in orange. C) hPXR LBD in complex with hyperforin (PDB code 1M13). Carbon atoms of the ligand are shown in green. D) hPXR LBD in complex with rifampicin (PDB code 1SKX). Carbon atoms of the ligand are shown in yellow. E) hPXR LBD in complex with TO901317 (PDB code 2O9I). Carbon atoms of the ligand are shown in magenta. F) hPXR LBD in complex with colupulone (PDB code 2QNV). Carbon atoms of the ligand are shown in gray. For panels B-F, the structures are viewed from the same orientation. The carbon atoms of the LBD residues are illustrated in marine blue. For panels A-F, oxygen, nitrogen, and sulfur atoms are depicted in red, blue, and yellow, respectively.

examined by crystallography in complex with hPXR also reveal the importance of hydrogen bond interactions of the ligand and the residues forming the cavity, in addition to the extended hydrophobic contacts. The active component of the herbal antidepressant St. John's wort, hyperforin, forms hydrogen bonds with Ser247, Gln285, and His407 (Fig. 3C) (Watkins et al. 2003b). The antibiotic rifampicin, one of the largest known PXR ligands, also

forms hydrogen bonds with Ser247, Gln285, and His407 (Fig. 3D) (Chrencik et al. 2005). The liver X receptor (LXR) agonist T0901317 was shown to interact with the hPXR LBD through polar interactions with Gln285 and His407 (Fig. 3E) (Xue et al. 2007). A third hydrogen bond involved His327, which has not been observed to interact with ligands in previous structures. The hops constituent colupulone forms hydrogen bonds with His407 and bonds to Gln285 through a water molecule (Fig. 3F) (Teotico et al. 2008).

The PXR-ligand interaction appears to be a dynamic process, leading to structural changes that quite possibly alter the interaction between PXR and its coactivator or corepressor. In contrast to the apo-PXR, binding of hyperforin changes the pocket shape and increases its volume from 1294 to 1544 Å. In addition, a novel α-helix (α6) is observed in place of the unordered loop in the 317-321 region. Interestingly, binding of rifampicin increases the disorder of three flexible loops neighboring the ligand cavity. Binding of colupulone or rifampicin increases the thermal motion in various regions of PXR, including the AF2 region and LBD sections formed by β1 and β1′.

The growing number of crystal structures will prove to be invaluable in uncovering the complex relationship among ligand, receptor, coregulators, and target DNA. Structural information from all the complexes obtained to date reveals a large and expandable ligand-binding pocket that can harbor ligands of varying sizes, with different chemical and structural properties, thus explaining the promiscuity of PXR in contrast to other NRs.

3. Regulation of Pregnane X Receptor and its target genes

The expression profile of PXR was initially thought to be limited to the liver, colon, and small intestines (Bertilsson et al. 1998; Blumberg et al. 1998b; Lehmann et al. 1998) but has since been found to have a much wider range, including the brain, bone marrow, peripheral blood mononuclear cells (PBMCs) (Albermann et al. 2005; Bauer et al. 2004; Lamba et al. 2004), ovaries (Masuyama et al. 2001), and T lymphocytes (Dubrac et al. 2010). Regulation of PXR is perhaps best studied in the liver. The adult liver is the primary organ of xenobiotic metabolism and elimination. In contrast, fetal liver is mainly involved in hematopoiesis. Toward the late stages of fetal development, the liver ceases to be the main organ for hematopoiesis and begins to express genes associated with xenobiotic detoxification, such as cytochrome P450 (CYP). During this stage, expression of PXR is transcriptionally regulated by another NR, hepatocyte nuclear factor 4α (HNF-4α) (Kamiya et al. 2003; Li et al. 2000). A closer examination of the promoter region of PXR revealed binding sites for HNF-4α. Indeed, disruption of the HNF-4α gene led to reduced expression of PXR and Cyp3a11 (Cyp3a11 is the mouse ortholog of human CYP3A4) in fetal mouse livers (Kamiya et al. 2003). Interestingly, expression of genes transcriptionally regulated by HNF-4α in fetal livers, including PXR, remained unperturbed in adult livers derived from HNF-4α–null mice, suggesting that the regulation of these genes by HNF-4α is developmentally restricted (Hayhurst et al. 2001; Kamiya et al. 2003). Although PXR is no longer subject to HNF-4α regulation in adult livers, it is still tightly regulated at many levels, as discussed below.

3.1 Transcriptional regulation of Pregnane X Receptor

3.1.1 Histone methylation

Hypermethylated CpG-rich promoter regions of key tumor suppressor genes that result in transcriptional silencing are often detected in many human cancers (Baylin et al. 1998).

Silencing of PXR through the hypermethylation of its CpG-rich promoter regions is associated with aggressive neuroblastoma (Misawa et al. 2005), while a decrease in methylation of PXR promoter is correlated with colorectal cancer (Habano et al. 2011). The restoration of PXR expression in neuroblastoma cells results in growth suppression of the cells, suggesting a tumor suppressive property of PXR (Misawa et al. 2005). However, overexpression of PXR in colorectal cancer through promoter hypomethylation suggests a tumor promoting property of PXR. Indeed, chemoresistance to irinotecan (CPT-11), a topoisomerase I inhibitor currently used for the treatment of metastatic colorectal cancer, results from increased PXR expression (Raynal et al. 2010). The role of increased expression of PXR during carcinogenesis could be explained, in part, by its antiapoptotic property independent of xenobiotic enzyme regulation (Zhou et al. 2008b). Conversely, PXR has also been shown to suppress proliferation and tumorigenicity of colon cancer cells (Ouyang et al. 2010). The role of PXR in carcinogenesis remains to be fully elucidated.

3.2 Post-transcriptional regulation of Pregnane X Receptor

3.2.1 Regulation by miRNA

Post-transcriptional regulation of PXR involves the role of microRNA (miRNA). A growing list of proteins, including NRs and CYPs, exhibit similar patterns of regulation by miRNA (Nakajima & Yokoi 2011). miRNAs are a family of non-coding RNA of ~22 nucleotides in length that regulate protein expression either through the attenuation of protein translation or mRNA degradation (Bartel 2004). An online search using the miRBase Target database revealed potential recognition sites for 16 miRNAs in the 3′ -untranslated region (UTR) of the hPXR, including miR-148a, miR-560, and miR-192 (Takagi et al. 2008). miR-148a, a highly abundant miRNA selectively expressed in the liver, was shown to modulate the expression of PXR. Overexpression of miR-148a results in a decrease in PXR levels and a concomitant decrease in *CYP3A4* mRNA induction (Takagi et al. 2008). Regulation of PXR through miRNA presents an exciting avenue that warrants further investigation.

3.3 Post-translational regulation of Pregnane X Receptor

3.3.1 Phosphorylation

Phosphorylation of PXR is often inhibitory (Pondugula et al. 2009b). Treatment of primary rat and human hepatocytes with protein kinase A (PKA) activator leads to the attenuation of *Cyp3A1* (Cyp3A1 is the rat ortholog of human CYP3A4) and *CYP3A4* mRNA levels, respectively. This can be attributed, in part, to the phosphorylation of PXR by PKA (Ding & Staudinger 2005a; Lichti-Kaiser et al. 2009b). However, a similar treatment of mouse hepatocytes resulted in an increase in *Cyp3a11* mRNA levels, suggesting species specificity within PXR (Lichti-Kaiser et al. 2009b).

Work from our laboratory demonstrated that the treatment of HepG2 cells with flavonoids leads to an increase in CYP expression through modulating the activity of cyclin-dependent kinase 5 (Cdk5). We further showed PXR to be a substrate for Cdk5 in *in vitro* kinase assays, suggesting that Cdk5 may modulate the activity of PXR through inhibitory phosphorylation (Dong et al. 2010). Cdk2 was also shown to attenuate PXR activity, in part, through inhibitory phosphorylation of PXR (Lin et al. 2008). The negative regulation of PXR by the cell-cycle–regulated Cdk2 suggests that PXR is subject to cell cycle regulation.

PXR was also reported to be a substrate in *in vitro* kinase assays for a panel of kinases; protein kinase C (PKC) (Ding and Staudinger 2005b), 70 kDa ribosomal S6 kinase (p70S6K),

glycogen synthase kinase 3 (GSK3), and casein kinase II (CK2) (Lichti-Kaiser et al. 2009b), further suggesting that PXR may be modulated by a wide range of protein kinases. However, to this end, *in vivo* phosphorylation of PXR remains undetectable. It is possible that the level of phosphorylation of PXR is below current detection limits.

A systematic approach to mutating serine/threonine (S/T) residues to aspartic acid (D) revealed that Ser8Asp, Thr57Asp, Ser208Asp, and Thr408Asp resulted in a decrease in PXR transactivation (Lichti-Kaiser et al. 2009a). Notably, we determined the phosphomimetic mutant of PXR, Thr57Asp, also exhibited an altered pattern of subcellular localization (Pondugula et al. 2009a). Collectively, phosphorylation of PXR confers a negative regulatory effect, possibly through altering its pattern of subcellular localization or affecting its interaction with corepressors or coactivators.

3.3.2 Ubiquitination, SUMOylation, and acetylation

PXR degradation plays a pivotal role in its regulation, although little is known about the mechanism behind the regulation of its stability. A semi-quantitative approach determined that the half-life of unliganded PXR is less than 4 hours. Binding of PXR to a subset of its ligand increases its half-life, in part due to the disruption of its interaction with suppressor for gal1 (SUG1), a component of the proteasome (Masuyama et al. 2002; Masuyama et al. 2005). Recent work from the Staudinger group further demonstrated an increase in ubiquitinated PXR following inhibition of the 26S proteasome with MG132 (Staudinger et al. 2011). Preliminary work from our laboratory demonstrated that PXR mainly undergoes a lysine-48 (Lys-48) polyubiquitin linkage, which signals its degradation. Proteasomal inhibition also resulted in the inhibition of PXR transactivation, suggesting interplay between PXR and the ubiquitin pathway (Staudinger et al. 2011). However, it is noteworthy that many coregulators of PXR are also subject to regulation through the proteasomal pathway (Lonard & O'Malley 2009). More work will need to be done to dissect the role of ubiquitination on PXR.

It was first observed that patients undergoing long-term treatment with rifampicin exhibit suppression of the inflammatory response in the liver through repression of nuclear factor kappa B (NF-κB) activation (Gu et al. 2006; Paunescu 1970). Activation of the inflammatory response in the liver significantly attenuates the SUMOylation of ligand-bound PXR. SUMOylation of PXR was shown to occur mainly through the SUMO2/3 chains. Although preliminary, the culmination of these studies will shed light on the role of SUMOylation of PXR and its crosstalk with the inflammatory response pathway.

Lysine acetylation was first identified and studied as histone modification and, as such, these lysine modifying enzymes were coined histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Yang & Seto 2008). Since then, lysine acetylation has been shown to extend beyond histones. NRs such as androgen receptor and estrogen receptor undergo acetylation, which modulates the NRs' ligand sensitivity (Fu et al. 2004). Little is known about PXR acetylation, but a recent report showed the presence of an acetylated species of PXR (Biswas et al. 2011). Acetylation of PXR was shown to modulate its activity, and ligand-induced deacetylation of PXR is modulated by SIRT1 (Biswas et al. 2011).

3.4 Pregnane X Receptor coregulators

Coregulators were first identified and described merely as adaptors to stabilize gene transcription. Their known functions have since expanded to include histone remodeling,

transcription initiation, RNA elongation, and protein degradation (McKenna & O'Malley 2002). Coregulators are broadly divided into coactivators and corepressors, molecules that lead to enhanced and attenuated gene transcription, respectively (McKenna & O'Malley 2002). Unliganded PXR interacts with corepressors, small heterodimer partner (SHP), silencing mediator for retinoid and thyroid receptors (SMRT), and nuclear receptor corepressor (NCoR) to inhibit gene transcription (Ourlin et al. 2003; Takeshita et al. 2002). Ligand binding to PXR in turn results in the dissociation of corepressors and the association of coactivators, such as SRC-1/NCOA1. Structure-based analysis has shown that coactivator binding further promotes the interaction between PXR and its ligand (Watkins et al. 2003a). Other coactivators of PXR include SRC-2 (GRIP1), nuclear receptor interacting protein 1 (NRIP1), peroxisome proliferator-activated receptor-gamma (PPARγ) coactivator (PGC-1), and forkhead transcription receptor (FHKR/FOXO1) (Moore et al. 2006).

3.5 Pregnane X Receptor regulation of target genes

PXR is a ligand-activated transcription factor, which upon ligand engagement becomes activated and binds to DNA in the nucleus to regulate gene transcription. Although its LBD exhibits flexibility and variability, the DBD is highly conserved across species. Several studies identified PXR response elements to be AG(G/T)TCA-like everted repeats (ERs) separated by 6 or 8 bp (ER-6 and ER-8) and direct repeats (DRs) separated by 3 or 4 bp (DR-3 and DR-4) (Kliewer et al. 2002). Next-generation sequencing techniques on the mouse cistrome using chromatin immunoprecipitation (ChIP-on-chip) and ChIP sequencing (ChIP-Seq) further revealed an *in vivo* preferred binding site to include a novel DR-(5n+4) pattern (Cui et al. 2010). ChIP studies using cryopreserved primary human hepatocytes treated with rifampicin revealed the upregulation of genes involved in drug metabolism and clearance, which is in agreement with studies performed in cell lines (Hariparsad et al. 2009). Importantly, results from a recent study suggest that while the presence of low concentrations of two different agonists, pregnenolone-16α-carbonitrile (PCN) and lithocholic acid, leads to a similar transcriptome response in primary rat hepatocytes, a divergence in the transcriptome response was observed with higher concentrations of the agonists. This observation suggests that although PXR can be activated by a wide range of agonists, downstream response may be more selective for these agonists. A negative feedback loop was also shown to be in effect for PXR, whereby ligand-induced activation of PXR inhibits the transcription of the PXR gene, limiting the cellular concentration of PXR in the presence of a high concentration of agonist (Bailey et al. 2011).

4. Physiologic functions of Pregnane X Receptor

In recent years, numerous studies have revealed the mechanisms of PXR-mediated induction of DMEs and drug transporters by which xenobiotics is detoxified. In addition, PXR also regulates endobiotic metabolism, which is important for maintaining homeostasis of cholesterol, bile acids, lipids, steroid hormone, and glucose in the human body.

4.1 Pregnane X Receptor in xenobiotic metabolism

DMEs and drug transporters play crucial roles in xenobiotic detoxification and elimination. Phase I CYPs belong to the monooxygenase superfamily and are highly expressed in the liver and intestine. They catalyze the first step of detoxification of aliphatic or lipophilic

compounds through hydroxylation or oxidation reactions and convert these compounds into more soluble derivatives (Guengerich 2001; Nebert & Gonzalez 1987; Willson & Kliewer 2002). Phase II conjugation reactions are catalyzed by a large group of transferases, such as sulfotransferase (SULT), glutathione-*S*-transferase (GST), and UDP-glucuronosyltransferase (UGT) (McCarver & Hines 2002). Polar functional groups are conjugated onto xenobiotics and endobiotics to generate water-soluble, inactive metabolites (McCarver & Hines 2002), which can then be excreted from the cell in a process regulated by ABC and solute carrier (SLC) family transporters (Ayrton & Morgan 2001; El-Sheikh et al. 2008). These transporters, together with phase I and II DMEs, orchestrate the xenobiotic metabolism process.

PXR regulates the metabolism and elimination of xenobiotics by regulating the expression of DMEs and drug transporters. Phase I DME genes regulated by hPXR include *CYP3A4*, *CYP2C8*, *CYP2C9*, and *CYP2B6* (Ferguson et al. 2005; Gerbal-Chaloin et al. 2001; Goodwin et al. 2001; Kliewer et al. 1998; Lehmann et al. 1998). Phase II DME genes regulated by PXR include SULTs (Alnouti & Klaassen 2008), GSTs (Higgins & Hayes 2011) and UGTs (Chen et al. 2003). PXR also regulates the expression of ABC transporters and organic anion-transporting polypeptides (OATPs/SLC) responsible for the influx/efflux of xenobiotics across the cell membrane in the liver and intestine, including multidrug resistance 1 (MDR1/P-gp) (Geick et al. 2001), multidrug resistance associated proteins (MRPs) (Kast et al. 2002; Teng et al. 2003) and multiple OATPs (Meyer zu Schwabedissen et al. 2008).

4.2 Pregnane X Receptor in endobiotic metabolism

Cholesterol is an essential component of the cell membrane and is important in producing bile acids, steroid hormones, and vitamin D. However, oxidized cholesterol contributes to the development of atherosclerosis (Ross 1999). Hence, cholesterol metabolism and transportation are important in the control of cholesterol homeostasis and protection against atherosclerosis (Repa et al. 2000). The biotransformation and transportation of cholesterol in most tissues are catalyzed mainly by mitochondrial sterol 27-hydroxylase, CYP27A. A recent study revealed that upon ligand activation, PXR can induce CYP27A expression and further increase the expression of cholesterol efflux transporters ABCA1 and ABCG1 in intestinal cells but not in hepatocytes (Li et al. 2007). Another study showed that PXR activation can induce hypercholesterolemia in wild-type mice associated with an elevated level of proteins in the liver related to cholesterol transportation and metabolism, such as CD36, ApoA-IV, and CYP39A1 (Zhou et al. 2009). In addition, previous clinical studies have shown that long-term treatment of patients with PXR agonists led to an elevation in cholesterol levels and was associated with an increased risk of cardiovascular disease (Carr et al. 1998; Eiris et al. 1995; Khogali et al. 1974; Lutjohann et al. 2004). These data strongly implicate PXR in mediating cholesterol homeostasis.

Bile acids play major roles in cholesterol metabolism and excretion. They are produced in the liver by CYPs-mediated oxidation of cholesterol (Ihunnah et al. 2011). Accumulation of bile acid is mainly responsible for cholestatic liver injury (Allen et al. 2011). Therefore, bile acid levels need to be tightly controlled to avoid cellular toxicity. It has been reported that PXR plays a central role in bile acid synthesis, metabolism, and transportation. PXR affects the biosynthesis of bile acids by negatively regulating the expression of Cyp7a1 (Staudinger et al. 2001), a rate-limiting enzyme in bile acid biosynthesis (Saini et al. 2004). PXR regulates bile acid metabolism by regulating the expression of CYP3A (Xie et al. 2001) and SULT2A

(Sonoda et al. 2002). In addition to bile acid synthesis and metabolism, PXR also regulates the expression of bile acid transporters (Staudinger et al. 2001; Teng & Piquette-Miller 2007; Wagner et al. 2005).

Hepatic lipid homeostasis relies on the balance of lipid uptake and synthesis (lipogenesis), lipid catabolism (β-oxidation), and secretion. Recent studies showed that activation of PXR regulates lipogenesis independent of the activation of lipogenic transcriptional factor sterol regulatory element-binding protein 1c (SREBP-1c) and is associated with the induction of the free fatty acid uptake transporter CD36, PPARγ, and stearoyl-CoA desaturase-1 (SCD-1) (Zhou et al. 2008a; Zhou et al. 2006). Moreover, PXR also regulates the expression of other hepatic genes related to lipid homeostasis, including ApoA-IV, oxysterol 7α-hydroxylase (CYP39A1), and 7-dehydrocholesterol reductase (DHCR7) (Zhou et al. 2009). In addition, PXR affects lipid homeostasis by regulating lipid catabolism. Treatment with PCN increased levels of hepatic triglycerides in PXR+/+ mice but not in PXR−/− mice (Nakamura et al. 2007). Further investigation revealed that direct interaction of PXR with FoxA2 appeared to be the underlying mechanism by which activation of PXR repressed the expression of carnitine palmitoyltransferase 1A (CPT1A) and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), two key enzymes involved in beta-oxidation and ketogenesis, respectively (Nakamura et al. 2007).

Glucocorticoids, a class of steroid hormones, perform numerous physiologic functions in growth, development, and metabolic events. Both genetic and pharmacologic activation of PXR increased the plasma levels of corticosterone and aldosterone, the mouse equivalence of primary glucocorticoid and mineralocorticoid, respectively (Zhai et al. 2007). This increase was accompanied by the activation of adrenal steroidogenic enzymes, such as CYP11 and 3β-hydroxysteroid dehydrogenase (Zhai et al. 2007). Transgenic mice carrying an activated PXR (VP-PXR) exhibited normal ACTH secretion in the pituitary and intact suppression of dexamethasone by corticosterone, indicating a functional hypothalamus–pituitary–adrenal axis despite severely disrupted adrenal steroid homeostasis (Zhai et al. 2007). Clinical observations in patients undergoing rifampicin treatment revealed ACTH-independent hypercortisolism, which may result in a misdiagnosis of Cushing's syndrome, suggesting that PXR may have broad implications in steroid homeostasis (Zhai et al. 2007).

Blood glucose levels in the body are tightly controlled through multiple processes, including gluconeogenesis, glycogenolysis, and glycogenesis. Importantly, PXR has been shown to modulate these processes. Firstly, the expression of PEPCK and G6Pase, two important rate-limiting enzymes in gluconeogenesis and glycogenolysis, are reduced in VP-hPXR transgenic mice following sustained activation of PXR in the liver (Zhou et al. 2006). Second, the PXR agonist PCN downregulates G6Pase gene expression in wild-type but not PXR−/− mice (Kodama et al. 2007). Third, cross-talk between PXR, CREB, and FOXO1 can also affect gluconeogenesis (Konno et al. 2008). It has been reported that ligand-activated PXR can bind to phosphorylated CREB and FOXO1 and further suppress their transcriptional activity, leading to the suppression of G6Pase and PEPCK1 gene expression and decreased gluconeogenesis (Kodama et al. 2004; Konno et al. 2008). These results suggest that PXR can regulate glucose levels by controlling gluconeogenesis.

5. Pregnane X Receptor as a novel target for drug development

5.1 Pregnane X Receptor in hepatic steatosis

Hepatic steatosis, also known as fatty liver, is a reversible process manifested as abnormal accumulation of lipids in the liver. Hepatic steatosis is associated with a multitude of

diseases, such as cardiovascular disease, obesity, diabetes, cancer, and liver diseases (Diehl 2010). Recent studies suggested that activation of PXR could contribute to the process of hepatic steatosis (Zhou et al. 2006). As discussed in section 4.2., PXR-humanized mice treated with rifampicin exhibited hepatic lipid accumulation. The PXR-mediated triglyceride accumulation was independent of SREBP-1c, but was linked to elevated levels of CD36 and several accessory lipogenic enzymes, such as SCD-1 and long chain free fatty acid elongase (Zhou et al. 2006). PXR directly regulates the transcription of CD36 by binding to its promoter (Zhou et al. 2008a; Zhou et al. 2006). Recently, S14, which plays an important role in the induction of lipogenic enzymes, was identified as a novel transcriptional target of PXR (Moreau et al. 2009). PXR mediates lipogenesis through the induction of S14 expression. Collectively, these studies suggested that abnormal activation of PXR may greatly contribute to the pathogenesis of hepatic steatosis.

5.2 Pregnane X Receptor in bone disorders

Vitamin D plays important roles in bone homeostasis since it regulates the absorption and excretion of calcium, a major component in bone development and maintenance. The physiologically active form of vitamin D, 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$), binds to the vitamin D receptor (VDR) to exert its function (Jurutka et al. 2007). CYP24 and CYP3A4 can catabolize $1,25(OH)_2D_3$ into its inactive form in the liver and intestine (Pascussi et al. 2005; Xu et al. 2006). Recently, *in vivo* and *in vitro* studies identified CYP24 as a PXR target gene, suggesting that PXR activation can alter the homeostasis of $1,25(OH)_2D_3$ and affect VDR activation through modulating CYP24 and CYP3A4 gene expression (Pascussi et al. 2005; Xu et al. 2006). In addition, it has long been known that prolonged treatment with antiepileptic drugs, many of which are PXR agonists, may lead to vitamin D deficiency (Andress et al. 2002). These results suggest that the activation of PXR may contribute to osteomalacia, the softening of the bones caused by defective bone mineralization.

However, the role of PXR in bone disorders is still unclear. PXR knockout mice displayed marked osteopenia with enhanced bone resorption and reduced bone formation in the trabecular bones and decreased thickness in the cortical bones (Azuma et al. 2010). Additionally, vitamin K was recently identified as a PXR ligand and exerts PXR-dependent biological functions in the bone. It has been reported that vitamin K stimulated several PXR target genes expressed in bone, such as tsukushi, matrilin-2, CD14, and Msx2 which are involved in osteoblast differentiation (Ichikawa et al. 2006; Igarashi et al. 2007). Furthermore, vitamin K supplementation increases bone density *in vivo* and is currently in clinical use to manage osteoporosis (Fang et al. 2011). These opposing data suggest PXR also plays roles in bone disorders.

5.3 Pregnane X Receptor in inflammatory bowel disease

Inflammatory bowel disease (IBD) refers to an inflammation of the intestinal tract. In IBD patients, the expression of PXR and its target genes were significantly reduced in the intestine, which led to the deregulation of PXR activity and xenobiotic metabolism, contributing to the pathogenesis of this disease (Dring et al. 2006; Langmann et al. 2004). Two recent studies have demonstrated the efficacy of rifaximin, an activator of hPXR, in the treatment of IBD (Mencarelli et al. 2010; Shah et al. 2007). In both a human colon cell line and IBD mouse models, induced by either dextran sulfate sodium or trinitrobenzene sulfonic acid, rifaximin showed protective and therapeutic activity associated with the

induction of PXR target genes related to intestinal detoxification (Mencarelli et al. 2010; Shah et al. 2007). This therapeutic effect of rifaximin was not observed in PXR knockdown colon epithelial cells or in PXR-null IBD mice (Shah et al. 2007). Other studies showed that rifaximin-activated PXR also inhibited the NF-κB signaling cascade, resulting in the suppression of the NF-κB-mediated proinflammatory response (Gu et al. 2006). These data suggest that PXR is a potential therapeutic target in the prevention and treatment of human IBD.

5.4 Pregnane X Receptor in cancer and chemotherapy

It has been well documented that PXR is expressed in many human cancers, including breast (Dotzlaw et al. 1999; Miki et al. 2006), prostate (Chen et al. 2007), colon (Ouyang et al. 2010; Raynal et al. 2010; Zhou et al. 2008b), osteosarcoma (Mensah-Osman et al. 2007), ovarian (Gupta et al. 2008), and endometrial cancers (Masuyama et al. 2003; Masuyama et al. 2007). Due to its ligand promiscuity, PXR can be activated by many anticancer drugs, including cyclophosphamide, tamoxifen, taxol, vincristine, and vinblastine (Koyano et al. 2002; Poso & Honkakoski 2006; Smith et al. 2010; Synold et al. 2001). Moreover, cancer patients are usually treated with combination therapy in addition to anticancer drugs, which also increases the possibility of drug-mediated PXR activation. Accordingly, recent studies support the idea that activation of PXR may compromise the effectiveness of anticancer drugs and contribute to acquired multi-drug resistance during anticancer chemotherapy (Chen, 2010). In PXR-expressing cancer cells such as prostate, colon, and endometrial cancer, PXR agonists can lead to increased resistance of cancer cells to chemotherapeutic agents, while the cancer cells can be sensitized to these anticancer agents by knockdown of PXR (Chen et al. 2007; Masuyama et al. 2007; Ouyang et al. 2010). On the other hand, PXR-mediated chemoresistance originating from inducible activity of PXR can also be blocked by pharmacologic intervention, leading to enhanced efficacy of chemotherapy. A recent study demonstrated that the reduced chemosensitivity of colorectal cancer cells to irinotecan was reversed by the PXR antagonist sulforaphane, while the activation of PXR decreased the effectiveness of this drug (Raynal et al. 2010). Thus the concept has been proposed to tackle resistance to anticancer drug by pharmacologically antagonizing regulating PXR (Chen 2010). The discovery and development of nontoxic, specific, and potent PXR antagonists will provide an effective way to improve the efficacy of anticancer drugs for the treatment of PXR-positive cancers.

6. Pregnane X Receptor as a target for improvement on current drug therapies

Adverse drug reactions (ADRs) induced by drug-drug interactions are major clinical problems, significantly contributing to mortality and morbidity (Wilke et al. 2007). PXR activation plays a crucial role in drug-drug interactions by inducing the expression of DMEs and drug transporters. Many prescription drugs have been found to bind to PXR, induce PXR target gene expression, and affect the metabolism and pharmacokinetics of the drugs. These drugs include calcium channel blockers felodipine, isradipine, lacidipine, nicardipine, and nifedipine (Xiao et al. 2011), HIV protease inhibitors (Dussault et al. 2001), anti-inflammatory agent dexamethasone (Pascussi et al. 2000), and others. Common herbal medicines can also activate PXR, such as licorice (Mu et al. 2006), gugulipid (Brobst et al.

2004), Ginkgo biloba (Yeung et al. 2008), and St. John's wort (Moore et al. 2000). Since activated PXR induces the expression of CYPs, it is conceivable that the activation of PXR can lead to undesirable drug–drug interactions in a large number of pharmaceutical drugs.
PXR contributes to ADRs by decreasing therapeutic efficacy and by increasing drug toxicity (Ma et al. 2008b). When co-administrated with rifampicin, the antihypertensive drug verapamil and anti-HIV protease inhibitors showed dramatically decreased efficacy because of increased drug metabolism and clearance caused by PXR-induced CYP3A4 expression (Fuhr 2000; Niemi et al. 2003). Another example comes from St. John's wort, an herbal medicine containing PXR agonist hyperforin. Long-term consumption of St. John's wort induces PXR-mediated CYP3A expression, resulting in increased metabolism and reduced efficacy of many therapeutic drugs in combination therapy, such as amitriptyline, cyclosporine, digoxin, indinavir, irinotecan, warfarin, phenprocoumon, alprazolam, dextromethorphan, simvastatin, and oral contraceptives (Mai et al. 2004; Moore et al. 2000). Besides decreasing drug efficacy, PXR also plays an important role in drug-induced toxicity. A recent study showed that pretreatment with PCN significantly enhanced acetaminophen-induced hepatotoxicity in mice, probably by inducing CYP3A and hence converting acetaminophen to its toxic intermediate metabolite, N-acetyl-p-benzoquinone imine (Cheng et al. 2009; Guo et al. 2004).
Efforts have been made to overcome PXR-induced drug-drug interactions in drug development. One approach is to chemically modify the lead compound to minimize its PXR activating function without compromising its pharmacologic activity against the therapeutic target. This approach was illustrated in a recent study on the first generation of IGF-1R inhibitors (Velaparthi et al. 2008; Zimmermann et al. 2010). However, due to PXR ligand promiscuity, tremendous chemistry efforts will be needed to remove the PXR activating function of many lead compounds. In addition, many commercially marketed drugs with PXR agonistic activity are still in the clinical use. In light of these considerations, another approach is to discover and develop new compounds that can antagonize PXR activity as co-therapeutics (Chen 2008; Chen 2010; Venkatesh et al. 2011). The feasibility of this approach was supported by recent studies that showed that the effectiveness of drugs can be enhanced by antagonizing the inducible activity of PXR with several pharmacologic interventions, such as ecteinascidin-743 (Synold et al. 2001), ketoconazole (Huang et al. 2007), sulforaphane (Zhou et al. 2007), A-792611 (Healan-Greenberg et al. 2008), and coumestrol (Wang et al. 2008a). These studies shed light on the development of PXR antagonists as an effective way to minimize PXR-induced ADRs.

7. Drug discovery techniques for Pregnane X Receptor

7.1 Structure-based modeling for predicting Pregnane X Receptor ligands

Computational studies have become useful tools geared toward understanding and predicting PXR-ligand interactions, a major focus being the determination of features within the ligand that allow for biomolecular recognition. This information can be used to predict potential drug clearance or resistance or to develop chemical PXR modulators. The promiscuity of PXR was investigated using computational solvent mapping to determine hot spots on the protein surface (Ngan et al. 2009). These studies identified five well-defined hot spots on all sides of the binding pocket, with the most important one being formed by the residues Phe288, Trp299, and Tyr306. *In silico* pharmacophore and docking analysis yielded PXR antagonists, some of which may bind to the AF-2 domain of PXR (Ekins et al.

2008). Quantitative structural activity relationships (QSARs) have been applied using data from various sources to identify ligand structural features that contribute to biological activity (Jacobs 2004). To encompass a broader spectrum of compounds, molecular descriptors for PXR agonistic effects were predicted using machine learning approaches (Ung et al. 2007). To obtain a more consistent set of biological data, Xiao *et al.* cross-evaluated compounds with varying degrees of PXR activation using an *in silico* modeling analysis with several biological assays. They concluded that potent ligands interact with residues in the PXR ligand-binding cavity through critical hydrogen bonds and π-π contacts. Molecules with low molecular weight or mismatched shapes appeared to be weak binders (Xiao et al. 2011).

7.2 Biochemical assays

Several biochemical assays have been developed to study the direct interaction of ligands with PXR. Stable and highly purified protein preparations are required to obtain reliable results. However, as with many other NRs, PXR proves to be a difficult protein to purify. Full-length recombinant hPXR expressed in *E. coli* cells was first purified as inclusion bodies, followed by resolubilization, to be used for the generation of polyclonal antibodies (Saradhi et al. 2005). Aside from this single report, only the LBD of PXR is commonly expressed and purified from bacterial systems to be used in biochemical assays and crystallographic experiments. To increase protein stability, an SRC-1 coactivator peptide is co-expressed and co-purified with PXR (Watkins et al. 2003a), or the peptide is tethered to the LBD (Wang et al. 2008b). Due to inconsistencies in the co-expression of PXR and SRC-1 peptide at an equal ratio, it is believed that the tethered protein offers the better alternative.

The scintillation proximity assay (SPA) has become a powerful technology to study PXR-ligand interactions. Microspheres or beads incorporating a scintillant and designed to bind the target protein is incubated with radiolabeled ligands. Commercially available beads come in different core materials, such as polyvinyltoluene (PVT) or yttrium silicate (YSi), coated with coupling molecules to capture the target protein. Jones *et al*. described a novel SPA assay that utilizes the tritium-labeled PXR agonist SR12813 (Jones et al. 2000). In this protocol, biotinylated hPXR was bound to streptavidin-coated PVT beads. Various PXR activators were tested for their ability to compete with the [^{3}H]SR12813.

The affinity selection-mass spectrometry system (ALIS) utilizes liquid chromatography in tandem with mass spectrometric detection. PXR homogenates are pre-incubated with the desired compound, and the complex is separated rapidly (e.g., <20 s) from the unbound compound by a size-exclusion column (Xiao et al. 2011). The protein-ligand complex elutes at the void volume and is subsequently trapped in a collection loop, with the separation being monitored by UV absorbance. The complex is injected into a reversed-phase column at high temperature (e.g., 60 °C) with the acidic mobile phase containing 0.1% formic acid. Under these conditions, the ligand dissociates from the protein and is detected and quantified by the mass analyzer within the desired mass range. Elution from the reversed-phase column follows a standard gradient procedure with increasing non-polar solvent. Using this system, binding affinities can be obtained by performing dilutions of the ligand at constant protein concentrations. The advantage of this method is that the direct binding of the ligand to the protein can be evaluated.

In the temperature-dependent circular dichroism (TdCD) method, PXR-ligand solutions are allowed to equilibrate at room temperature (Xiao et al. 2011). The ellipticity is then monitored by circular dichroism spectroscopy at 220 nm and at increasing temperature (e.g.,

2°C/min). In the reported experiment, a single unfolding transition phase was observed for the apo PXR (PXR was tethered to an SRC-1 peptide) with a melting temperature (T_m) of 41.5°C. In the presence of rifampicin, the T_m value increased to 49°C. Hypothetical K_d values can be extracted based on T_m shifts. A relatively good correlation (r^2=0.72) was shown between the data obtained by this assay and that of a reporter gene cell-based assay.

Fluorescence polarization is a widely used technique to assess protein-peptide interactions and was applied to measure the recruitment of the coactivator SRC-1 upon ligand binding to PXR. A relatively small fluorescently labeled SRC-1 peptide emits the absorbed polarized radiation in a different direction from that of the incident light due to the fast tumbling rate. The binding of the peptide to the PXR protein increases the effective mass, resulting in a slower tumbling rate, leading to an increase in polarized light. A fluorescein-labeled SRC-1 peptide containing the amino acid sequence ILRKLLQE was used as a probe to test for direct interactions between bile acid intermediates and mouse PXR LBD fused to GST (Goodwin et al. 2003). In the presence of agonist, an increase in fluorescence polarization indicated ligand-dependent recruitment of SRC-1.

Several other techniques have been broadly used to evaluate the binding affinity and molecular recognitions of protein-ligand systems (Jecklin et al. 2009). Initially limited to probing only large biomolecules, label-free tools have become sophisticated in the analysis of protein interactions with small molecules. Surface plasmon resonance (SPR) can provide data in real time, with concomitant determination of kinetic descriptors and binding affinities. Isothermal titration calorimetry (ITC) is considered to be a true label-free technique for investigating thermodynamic profiles and affinity measurements. Major drawbacks include lack of sensitivity, which requires higher concentrations of either protein or compound. This could lead to solubility issues and challenges in the analysis of weak binders. In the case of SPR technology, immobilization conditions could cause artifacts due to conformational changes. Another obvious limitation in these methods is the need for relatively pure and stable purified PXR in large amounts.

7.3 Cell-based assays

Cell-based assays to determine PXR transactivation and CYP induction are especially useful, eliminating the need for primary hepatocytes while still providing a physiologically relevant environment. The first reporter-based assay described the use of chloramphenicol acetyltransferase (CAT) as a readout (Quattrochi et al. 1995). This method was subsequently improved upon by substituting luciferase for CAT; with the expression of luciferase being controlled by the PXR response element (PXRE) (Raucy et al. 2002). This model system has since been widely used in many laboratory settings, including high-throughput screening for potential PXR agonists and antagonists (Dong et al. 2010; Zhu et al. 2004).

Another cell-based assay gaining a foothold in the area of transcription factor is the mammalian two-hybrid system to study protein-protein interactions in cells. This assay is especially useful in determining the interaction between PXR and its coregulators. Physical interactions between two proteins of interest are detected through a simple end-point luciferase readout, which provides a semi-quantitative method for detecting protein-protein interactions. This method is superior to the traditional immunoprecipitation method because the spatial regulation of these protein interactions is preserved.

The subcellular localization of PXR in the cells remains unclear, with reports showing both nuclear and cytosolic localization. As such, efforts to incorporate high-content imaging-

based screening for PXR remain a challenge. Our laboratory is currently developing a fusion protein of PXR containing a photoconvertible fluorophore. With this tool in hand, we will be able to investigate the kinetics of PXR's nuclear/cytoplasmic translocation.

7.4 Animal models

The mouse models for PXR offer several advantages over cell-based systems by providing *in vivo* context to reveal physiologic functions. Although cell-based *in vitro* systems, such as a reporter assay, are useful for identifying PXR ligands, the ultimate goal is to translate these *in vitro* findings into *in vivo* models with clinical relevance. The development of mouse models for PXR, such as PXR-null mice and PXR-humanized mice, provides useful tools to achieve this goal.

To better understand PXR-dependent signaling *in vivo*, two PXR-null mouse models were generated using similar approaches by disruption of PXR alleles with homologous recombination (Staudinger et al. 2001; Xie et al. 2000a). These PXR-null mice did not exhibit any apparent phenotypic changes, with normal development, growth, and reproduction. Extensive serum analysis did not reveal any significant changes in multiple serum biochemical profile, such as cholesterol, triglyceride, glucose, or liver enzyme levels (Staudinger et al. 2001; Xie et al. 2000a) suggesting that PXR is not essential for normal development or adult physiology under normal conditions. Although the loss of PXR does not alter the basal expression of PXR target genes, the PXR-null mice did not respond normally to xenobiotic treatment (Staudinger et al. 2001; Xie et al. 2000a). Hence, the PXR-null mouse is a valuable and reliable tool for dissecting PXR-dependent functions *in vivo*.

One critical problem in PXR functional studies lies in ligand selectivity between human and mouse PXR because of the marked species differences in amino acid sequences in the PXR LBD (Ekins et al. 2002). To overcome differences in PXR ligand recognition across species, humanized PXR mouse models have been generated. The BAC-hPXR mouse was generated by introducing a bacterial artificial chromosome (BAC) clone containing the complete hPXR gene and its 5'- and 3'- flanking sequences, whereas the Alb-hPXR mouse was developed by introducing the hPXR gene under the control of an albumin promoter (Alb), in PXR-null mouse (Ma et al. 2007; Xie et al. 2000a). As expected in these humanized PXR mice, no significant response was found after treatment with PCN, whereas rifampicin efficiently induced Cyp3a11 expression (Ma et al. 2007; Xie et al. 2000b). Another humanized PXR mouse model was generated that expressed a constitutively active hPXR (VP-hPXR, created by fusing hPXR to VP16, a potent viral transcriptional activator) in the livers of PXR-null mice (Xie et al. 2000a). In the VP16-hPXR mice, Cyp3a11 was constitutively induced in the liver. In addition, these mice exhibited growth retardation, hepatomegaly, and liver toxicity, suggesting that sustained activation of PXR may be deleterious (Xie et al. 2000a). Recently, a double transgenic mouse model expressing human PXR and CYP3A4, designated the TgCYP3A4/hPXR mouse, was generated (Ma et al. 2008a). Treatment of TgCYP3A4/hPXR mice with PXR ligands mimicked the human response but not the mouse response (Ma et al. 2008a). This model provides a useful tool to study hPXR-mediated human CYP3A4 expression and predict drug-drug interaction in the human body (Ma et al. 2008a). Overall, these PXR-humanized mouse models are more suitable as *in vivo* tools for studying xenobiotics metabolism mediated by hPXR.

8. Conclusion

Over the past decade, an enormous body of work has been invested toward understanding the mechanism of PXR regulation and its physiologic role in health and disease. Structure-based studies revealed its ligand promiscuity and the possibility of PXR existing as oligomers, while biochemical studies revealed the intricacies and the involvement of PXR in cross-talk across multiple signaling pathways. Genetic studies with animal models further support the *in vitro* findings, demonstrating that PXR protein expression is not limited to the liver and intestinal tract and revealing its role in maintaining cellular homeostasis. Hence, PXR serves as an attractive target for the development of pharmacologic modulators for mediating a plethora of diseases and, more importantly, MDR in chemotherapeutics.

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14

Crosstalk Between the Immune and Central Nervous Systems with Special Reference to Drug Development

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1. Introduction

Although the understanding of disease mechanisms becomes rapidly progressed in recent years, there remain a lot of unmet medical needs in a number of disease fields. Especially in the degenerative diseases of the central nervous system (CNS) such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, stroke and traumatic brain injury, currently available drugs only manage the symptoms, and there exist few disease-modifying therapies. Therefore, novel therapeutic interventions that modify and delay the disease progression are highly demanded. Drug development for the CNS diseases is particularly challenging (Kola I and Landis J, 2004). One of the hurdles to develop drugs for the CNS diseases is poor translation from animal models to human diseases (Pritchard JF, 2008).

One strategy to overcome this hurdle is to examine therapeutic potential of a target molecule in a number of experimental settings, assuming that a molecule that modulates pathophysiologic mechanisms underlying a range of neurodegenerative disease has a higher chance to work in human disease (Mehal WZ, Iredale J & Friedman SL 2011). Although this strategy requires much resource, it enables us to eliminate candidates that act only in a particular experimental setting that might have a lower chance to be effective in humans. One example for this strategy is a kynurenine 3-monooxygenase that catalyzes the conversion of kynurenine to 3-hydroxykynurenine in the kynurenine pathway of tryptophan degradation (Stone TW & Darlington LG, 2002; Schwarcz R, 2004). A small molecule inhibitor of kynurenine 3-monooxygenase increased the levels of neuroprotective kynurenic acid and decreased the levels of neurotoxic quinolinic acid/3-hydroxykynurenine in mice. Furthermore, the treatment with the kynurenine 3-monooxygenase inhibitor ameliorated neurodegeneration in two types of mouse neurodegenerative models for Alzheimer's disease and Huntington's disease (Reinhart PH & Kelly JW, 2011; Zwilling D et al., 2011). These observations suggest that the kynurenine 3-monooxygenase would be more promising therapeutic target than conventional therapeutic ones that are effective in animal models of a single disease.

Another strategy to increase the probability of success of drug development in the CNS diseases is to target a molecule that possess multiple pharmacological actions and hence

might affect different pathophysiological phases of the disease at once, because the CNS diseases often arise from multiple pathological steps and/or factors and conventional approaches to treat one disease phase has limited efficacy in CNS disorders.

There are at least two types of approaches for this strategy. First one is to develop multi-specific therapeutics, in which two or more different mechanisms of actions are given in one molecule. Several classical drugs are known to exhibit multiple therapeutic actions in a single molecular form (e.g. aspirin, thalidomide etc.), and this strategy has been developed in industries, especially in a field of biologics such as bi- or multi-specific antibodies and co-agonistic peptides (Day JW et al., 2009; Fitzgerald J & Lugovskoy A, 2011). For example, a marketed antibody Herceptin, which targets human epidermal growth factor receptor 2 (HER2), was modified to have the simultaneous interaction to HER2 and vascular endothelial growth factor (VEGF) with high affinity in order to develop more efficient antibody-based therapeutics (Bostrom et al., 2009). Actually, the modified bi-specific antibodies inhibited the growth of both human colon cancer and human breast cancer in animal models, whereas an antibody mono-specific for HER2 or VEGF are only effective for either of them, indicating that the modified bi-specific antibodies are therapeutically effective for a range of types of cancers (Bostrom et al., 2009). In addition, it is speculated that co-inhibition of HER2 and VEGF in breast cancer is more beneficial than single inhibition of either molecules, because tumor proliferation mediated by HER2 and tumor angiogenesis mediated by VEGF are expected to be concurrently inhibited (Bostrom et al., 2009). Theoretically, bi- or multi-specific therapeutics are expected to be more effective and useful than most of mono-specific therapeutics. However, it is generally more difficult to develop bi- or multi-specific therapeutics than mono-specific therapeutics, since simultaneous optimization for multiple biological actions in one molecule are required.

Second strategy is to target a single molecule that plays critical pathological roles in the multiple phases of the disease progression, which is consistent with our current hypothesis that a molecule that possesses multi-functions and hence modulates multiple pathophysiologic phases of a disease will be a more promising therapeutic target for neurodegenerative diseases than current drugs that acts only a single phase of the disease process.

In recent years, we pursue the latter strategy, especially focusing on the CNS diseases that consist of an inflammatory/immune-mediated pathological phase and a neurodegenerative phase such as multiple sclerosis and spinal cord injury. Given the recent progress of understandings of the mutual crosstalk between the immune and the central nervous systems, we speculate that a number of molecules could play physiological roles in both immune and the central nervous systems, and some of them would be involved in the inflammatory/immune-mediated pathological phase and a neurodegenerative phase.

In this chapter, we introduce our recent activities to develop novel and efficient therapeutic interventions by targeting a molecule that play multiple pathological roles in the inflammatory and degenerative phases of neurodegenerative diseases. In the following sections, first we briefly describe the background information and recent updates on the physiopathological interactions between the immune and nervous systems, and then illustrate the pathogenesis of the inflammatory/immune-mediated CNS diseases such as multiple sclerosis and spinal cord injury. Finally we introduce our efforts on a multi-functional molecule and its therapeutic potential in neurodegenerative disease.

2. The mutual interactions between the immune and the nervous systems

The CNS was previously recognized as an immune privileged site, meaning the complete absence of immunosurveillance within the CNS. This concept had been supported by the fact that the CNS is devoid of the classical lymphatic drainage and has the endothelial blood-brain barrier that is a tight barrier between the cerebrospinal fluids (CSF) surrounding the CNS parenchyma and the systemic circulation, being isolated from immune system. However, accumulating evidences have demonstrated that a mutual interaction between the immune system and central nervous system does exist both in the physiological and pathological situations (Steinman L, 2004; Schwartz M, 2009&2010).

The CNS influences and controls the immune system at least partly through the autonomic nervous system (Steinman L, 2004). Anatomically, the autonomic nervous system via the vagal nerve and sympathetic nerve innervates the sites of the immune system such as spleen, bone marrow, thymus, lymph nodes and gastrointestinal system, and directly regulates the functions of the immune system. Similar to the central neural circuit where neurons communicate with each other via many types of neurotransmitter, the parasympathetic nervous pathways communicate with the immune system via the neurotransmitter acetylcholine, and the sympathetic nerves utilize norepinephrine as the neurotransmitter for the communication with the immune system. Lymphocytes express the receptors not only for these neurotransmitters but also for other neurotransmitters such as histamine, serotonin, substance P, vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide and so on (Steinman L, 2004). Receptors for neuroendocrine mediators such as corticotrophin-releasing factor, a-melanocyte-stimulating hormone exist on lymphoid tissue (Steinman L, 2004). In addition, corticotrophin-releasing factor released by hypothalamus stimulates the secretion of ACTH from the pituitary gland, and consequently induces the release of glucocorticoids from the adrenal glands, which systemically suppress the inflammation (Sternberg, 1997). The wide variety of mediators enables the brain to properly coordinate the immune system and to keep the homeostasis of the whole body by responding to the environmental changes such as infections in an appropriate manner. For example, endotoxins such as lipopolysaccharide (LPS) produced by gram-negative bacteria activate inflammatory responses e.g. release of proinflammatory cytokines that are potentially lethal when overproduced. Acetylcholine, the principle vagal nerve transmitter, effectively suppressed LPS-induced inflammatory cytokine production. In addition, direct electrical stimulation of vagal nerve inhibited the *in vivo* shock response induced by LPS in rats possibly through the secretion of acetylcholine. These observations indicate that the brain negatively regulates systemic inflammatory responses to endotoxin via a parasympathetic vagal nerve pathway by limiting the inflammatory responses (Borovikova et al, 2000; Tracey KJ, 2002).

In some cases, the influences of immune cells on the CNS are more manifested under physiological and pathological conditions. A typical example of regulation of the brain functions by the immune systems is the fever onset and the subsequent behavioural responses such as sleep, feeding and appetite (Watkins LR & Maier SF, 1999; Steinman L, 2004). When infected with virus, bacteria and parasites, the immune system feels the danger to the body and releases pro-inflammatory cytokines such as interleukin 1 (IL-1), tumor necrosis factor (TNF) and IL-6. Regarding the fever onset, these cytokines boost the fever response through the neurons in the preoptic area of the hypothalamus, acting directly on these neurons and/or generating prostaglandin E2 (PGE2) that also regulates the fever

responses of these neurons (Morrison SF & Nakamura K, 2011). Consistent with the involvement of PGE2 in the fever response, cyclooxygenase inhibitors, which block PGE2 production, are widely used as fever reducers. Of note, fever itself dampens the production of IL-1 from immune cells in the blood by modulating the proteolytic processing of this cytokine, again indicating the existence of the negative feedback loop that suppresses the overload of the inflammatory responses to keep the homeostasis of the body (Boneberg EM & Hartun T, 2003).

In addition to the influences of the immune system on the CNS functions under pathological conditions, it has recently been hypothesized that the immune system play physiological roles in the maintenance of the fundamental CNS functions such as adult neurogenesis and spatial learning and memory (Schwartz M & Shechter R, 2010).

Although the CNS was considered to be an immune privileged site under healthy conditions as mentioned above, accumulating experimental evidences have demonstrated that continuous leukocyte trafficking into the CNS occurs even in healthy subjects, although the penetration of the leukocytes is mostly observed in CSF, the choroid plexus and the meninges, but not in the brain parenchyma (Ransohoff RM et al., 2003; Engelhardt B & Ransohoff RM, 2005; Schwartz M & Shechter R, 2010). Furthermore, recent investigations have revealed that specialized immune cells such as resident microglia, bone marrow-derived monocytic lineage cells, do exist in the brain parenchyma from embryonic developmental stage (Chan WY, 2007; Hanisch UK & Kettenmann H, 2007).

The physiological functions of the leukocytes trafficking into the CNS have remained unclear. However, it has been hypothesized that these CNS-migrating leukocytes, especially autoimmune T lymphocytes that react with self-antigens in the CNS, contribute to the physiological CNS functions such as adult neurogenesis in the dentate gyrus of the hippocampus and spatial learning and memory (Ziv Y et al., 2006).

In the hippocampus, an essential region for the formation of certain types of memories such as episodic memory and spatial memory, continuous generation of neurons is observed even in the adulthood, suggesting the importance of the adult hippocampal neurogenesis for fundamental neural functions (Deng W, 2010). With regard to the involvement of immune system in the hippocampus function, immune-deficient mice devoid of T cells (severe combined immune deficiency (SCID) mice) showed less adult neurogenesis in the dentate gyrus of the hippocampus compared to that in normal mice (Ziv Y et al., 2006). Conversely, transgenic mice engineered to produce autoimmune T cells, which react with the CNS antigen (myelin basic protein) and are therefore activated in the CNS, display more adult hippocampal neurogenesis than that in their wild-type counterparts (Ziv Y et al., 2006). Furthermore, hippocampus-dependent spatial learning and memory was impaired in immune-deficient mice devoid of T cells, and was enhanced in transgenic mice with a large number of autoimmune T cells (Kipnis et al., 2004; Ziv Y et al., 2006). Collectively, these observations suggest that autoimmune T cells play critical roles in the maintenance of fundamental function of hippocampus through the regulation of neurogenesis. In addition, other groups also reported the impacts of T cells on adult hippocampal neurogenesis (Wolf SA et al., 2009a & 2009b; Huehnchen P et al., 2011), further supporting the concept that the immune system contributes to certain types of brain functions.

On the contrary, in certain types of the degenerative CNS diseases, inflammatory and autoimmune responses by immune cells against the nervous system are considered to be harmful. Under pathological conditions such as autoimmune neurological diseases like multiple sclerosis and inflammatory brain/spinal cord traumatic injuries, a large number of

immune cells directly migrate to the CNS possibly due to the breakdown of BBB and these pathological infiltration of immune cells to the CNS mostly causes detrimental impact on the disease progression (Donnelly DJ & Popovich PG, 2008; Rezai-Zadeh K et al., 2009). Along with this concept, anti-inflammatory steroids and immunosuppressive therapeutic interventions are currently used to treat autoimmune neurological diseases and brain/spinal cord injuries. However, these therapies have limited clinical benefits while showing severe side effects (Kim Y et al., 2009; Barten LJ et al., 2010; Hilas O et al., 2010; Samantaray S et al., 2010). In contrast, several evidences have demonstrated that the immune cells rather have beneficial impacts on the pathology of the CNS diseases (Wyss-Coray T & Mucke L, 2002; Donnelly DJ & Popovich PG, 2008). For example, the implantation of activated macrophages into the injured CNS exhibits therapeutic benefits rather than harmful actions (Lazarov-Spiegler O et al., 1996; Rapalino O et al., 1998).

These observations suggest that the effects of the immune system on the CNS under pathological conditions are more complex than originally thought, depending on nature of pathologic mechanisms of respective disease. Development of a novel therapeutic intervention beyond the classical anti-inflammatory/immunosuppressive therapeutic approaches is essential for the treatment of these CNS diseases (Donnelly DJ & Popovich PG, 2008; Rezai-Zadeh K et al., 2009; Schwartz M et al., 2009).

In the following sections, we describe more detailed characteristics of multiple sclerosis and spinal cord injury, especially focusing on the pathological mechanisms and discuss on benefits and limitations of currently available drugs and desired profiles of novel therapeutic interventions.

3. The inflammatory/immune-mediated CNS diseases

3.1 Multiple sclerosis (MS)

Multiple sclerosis (MS) is a complex CNS disease and typically begins between age 20 and 50. Women are affected twice more often than men, and 50% of the patients will need help walking within 15 years after disease onset. Its clinical symptoms include sensory and visual deficits, balance and gait disturbances, limb weakness, neurogenic bladder and bowel problems (Noseworthy JH et al., 2000). Also, in some patients, mental problems such as emotional liability, depression and cognitive impairment are associated.

MS is classified into four distinct types: (1) relapsing-remitting MS; (2) secondary progressive MS; (3) primary progressive MS; and (4) progressive-relapsing MS (Noseworthy JH et al., 2000; Hilas, O et al., 2010). Approximately 80 % of the patients are categorized in relapsing-remitting MS, in which patients develop the symptoms over a period of several days, and then stabilize or even improve the symptoms within weeks. In some cases, neurological deficits persist after a relapse, and the disease progressively becomes worse between relapses (secondary progressive MS). Within 6-10 years from the disease onset, almost half of the patients with relapsing-remitting MS progress to secondary progressive MS. Twenty percent of the patients display primary progress MS that is characterized by a gradual and progressive disease course from the onset without an obvious remission stage.

In general, this type of MS shows poor prognosis (Noseworthy JH et al., 2000; Hilas, O et al., 2010). A fourth type of MS, referred to progressive-relapsing MS, is unusual and shows a progressive phenotype with obvious relapses with or without recovery. However, discrimination of primary progressive MS and progressive-relapsing MS on their clinical

characteristics is often unclear and remains controversial (Lublin FD & Reingold SC, 1996; Andersson PB et al., 1999).

The precise etiology of MS remains uncertain, but it is assumed that immune responses against self-antigens such as myelin constituents are dysregulated in genetically susceptible subjects and cause destructive inflammation and autoimmune responses to the CNS components. This autoimmune reactions lead to demyelination and subsequent neurodegeneration in the CNS. Traditionally, therapeutic interventions of MS exacerbations in an acute phase are conducted with an anti-inflammatory steroid such as methylprednisolone to suppress neuroinflammation and to shorten the duration of exacerbation (Barten LJ, 2010). In order to reduce the frequency of relapses and slow the disease progression, disease-modifying drugs are also used. Current disease-modifying therapeutics include interferon-β (IFN-β) products and glatiramer acetate, which show modest efficacy with 30-40% reduction in MS relapse rates compared with placebo (Brinkmann V et al., 2010). These agents have a number of immune-related actions including anti-inflammatory functions, but the precise mechanisms of therapeutic actions are incompletely understood (Noseworthy JH et al., 2000). Although they are established first-line therapies, they are unable to reverse existing CNS damage and have no influence on the development of permanent disability (Compston A & Coles A, 2002). Another marketed MS drug, Natalizumab is a humanized monoclonal antibody that is specific for α-4 subunit of very late antigen 4 integrin expressed on lymphocytes. Natalizumab blocks the migration of leukocytes from the blood stream into the CNS across the BBB, and decreases the relapse rates of MS by 68% at 1 year (Polman CH et al., 2006). Despite its higher clinical efficacy compared to other approved therapeutics, Natalizumab is currently used only as a second-line treatment due to its association with progressive multifocal leukoencephalopathy, which is a rare but life-threatening demyelinating neurological disorder caused by the reactivation of John Cunningham (JC) virus under immunosuppressive conditions (Barten LJ, 2010; Brinkmann V, 2010). Fingolimod (FTY720), an orally active drug with immunomodulatory actions, is recently approved as a first-line treatment for relapsing MS (Brinkmann V, 2010). Fingolimod influences the leukocyte trafficking through the modulation of sphingosine 1-phosphate receptors expressed on the leukocytes. In phase III trials, fingolimod demonstrated greater pharmacological efficacy in the reduction of the relapse rates as compared with placebo and IFN-β (Cohen JA et al., 2010; Kappos L et al., 2010). However, fingolimod treatment causes some adverse events such as infections, cardiovascular and ocular events in several cases, requring long-term follow-up clinical studies in order to provide further information on the benefit-risk profile of fingolimod as a novel oral treatment for relapsing MS (Brinlmann V, 2010).

Collectively, current therapeutic interventions for MS are mainly focusing on suppression of the immune system; inhibition of lymphocyte activation and the blockade of leukocyte trafficking into the CNS. Histopathological analyses in MS patients and MS animal models such as autoimmune encephalomyelitis (EAE), suggest that certain subsets of $CD4^+$ T lymphocytes (helper T (Th) cells) and antigen presenting cells (APCs) such as dendritic cells and macrophage/microglia (CNS tissue macrophage) play key roles in the pathogenesis of MS (Chastain EML et al., 2011). Upon encountering CNS self-antigen such as myelin antigen, APCs become maturated and migrate to lymph nodes where they activate antigen-specific $CD4^+$ T cells by presenting the CNS self-antigen (Guermonprez J et al., 2002; Bailey SL et al., 2007). During the activation process, $CD4^+$ T cells differentiate into mature effector subsets such as Th1, Th2, Th17 and Treg cells. Direction of $CD4^+$ T cell differentiation is at

least partly dependent on cytokines produced by APCs (Weaver CT et al., 2006; Zhu J & Paul WE, 2010). Accumulating evidences suggest that Th1 and Th17 cells are involved in the pathogenesis of MS (Olsson T, 1992; Segal BM, 2010). Once activated, $CD4^+$ T cells penetrate into the brain and spinal cord by crossing the BBB, and are re-activated by APCs located in the CNS, which triggers the disease induction and progression by attacking myelin structures of the CNS neurons (Chastain EML et al., 2011). Mild but significant clinical efficacy of the existing therapeutic interventions for MS via immunosuppressive actions supports the autoimmune-mediated onset of MS pathology.

However, current therapeutics such as IFN-β and glatiramer acetate are ineffective in reversing axon degeneration, another hallmark of certain types of MS such as secondary progressive MS (Compston A & Coles A, 2002; Bjartmar C et al., 2003). Thus, a novel intervention that concurrently inhibits autoimmune reactions and neuronal damages such as axon degeneration is a desirable treatment for MS patients.

3.2 Spinal cord injury

Traumatic injuries to the adult mammalian CNS often cause serious and long-lasting sensory and motor problems, because the CNS shows very poor regenerative ability. One of the reasons for poor regenerative ability of the CNS is that the injured CNS axons following brain trauma and spinal cord injury show very limited regeneration in contrast to those in the peripheral nervous system. The lack of appropriate axon regeneration in the CNS results in permanent neuronal deficits such as paralysis, and the permanent neuronal deficits have great impacts on quality-of –life of the injured subjects.

The pathology of the CNS injuries, particularly spinal cord injuries, has been studied at a molecular level in animal models, and the lack of regeneration of injured CNS axons is attributed, at least partly, to the CNS environment itself rather than to any intrinsic disability of CNS nerve fibers (Richardson et al, 1980; David and Aguayo, 1981). Among myelin proteins of the CNS, there exist axon outgrowth inhibitors such as Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp), which are suggested to inhibit the regenerative axon growth around the lesioned site after traumatic injury (Mueller et al, 2005; Yamashita et al, 2005; Kubo et al, 2007).

During our effort to identify a new member of axon outgrowth inhibitors, we discovered that the repulsive guidance molecule a (RGMa), which is expressed in neurons and oligodendrocytes in the CNS, also functions as a myelin-associated neurite outgrowth inhibitor (Hata et al, 2006; Yamashita et al, 2007). RGMa significantly inhibited neurite outgrowth in cultured neurons (Hata et al, 2006; Kubo et al, 2008). Furthermore, a neutralizing anti-RGMa antibody exhibits therapeutic effects on rat spinal cord injury by enhancing spinal axon outgrowth, suggesting that RGMa plays a key pathological role in suppressing axon regeneration and functional recovery after spinal cord injury (Hata et al, 2006; Yamashita et al, 2007).

In addition to the myelin-associated proteins that inhibit axonal regeneration, immune components play critical roles in the recovery process of the injured CNS. Traumatic injuries expose the CNS directly to the immune cells by destruction of the BBB, and are followed by the infiltration of a variety of immune cells around the damaged sites post injury (Jones TB et al., 2005; Donnelly DJ & Popovich PG, 2008). Given the immune-mediated pathology of the CNS diseases such as MS, it had been assumed that the penetrating active immune cells were harmful, and therefore should be eliminated or suppressed (Popovich PG, 2000; Dheen

St et al., 2007). Along with this concept, anti-inflammatory steroids are currently used for the treatment of spinal cord injury. However, the therapeutic efficacy of steroids such as methylprednisolone is limited, and therefore, the original concept that the CNS inflammation is simply harmful is currently re-considered.

Among the immune cells that accumulate at the lesion site, macrophages and microglia are reported to have both beneficial and detrimental impacts on the recovery from CNS injury (Jones TB et al., 2005; Donnelly DJ & Popovich PG, 2008; Popovich PG & Longbrake EE, 2008). The beneficial role of macrophages/microglia at the lesion site is the clearance of debris of the damaged axon and myelin that inhibit axon regeneration, through their phagocytotic actions and protease secretion. The concurrent secretion of neurotrophic factors that promote axon elongation/neuronal survival is potentially beneficial for the recovery of the damaged CNS (Jones TB et al., 2005; Donnelly DJ & Popovich PG, 2008; Popovich PG & Longbrake EE, 2008). Of note, it was reported that the implantation of activated macrophages promoted axon growth and functional recovery following CNS injury (Lazarov-Spiegler O et al., 1996; Rapalino O et al., 1998).

On the other hand, the excessive inflammatory responses induced by macrophages/microglia upon CNS injuries are detrimental to the recovery of the injured CNS (Jones TB et al., 2005; Donnelly DJ & Popovich PG, 2008; Popovich PG & Longbrake EE, 2008). For example, pro-inflammatory cytokines such as TNF-α and/or IL-1 expressed by activated macrophages/microglia enhance neuronal degeneration after spinal cord injury (Lee YB et al., 2000; Nesic O et al., 2001; Genovese T et al., 2006).

These lines of evidences described above suggest that the complex crosstalk between the immune system and CNS exists through a variety of molecules in the inflammatory/immune-mediated CNS disease. Interestingly, we found that RGMa is expressed not only in neurons and oligodendrocytes, but also in accumulated and activated macrophages/microglia in the injured spinal cord, suggesting RGMa might play multi-pathophysiological roles in the degenerative CNS disorders through neural and immune systems. This hypothesis prompted us to uncover the function of RGMa in the immune system and the involvement of this molecule in the immune-mediated degenerative CNS diseases.

In the next section, we describe our recent data showing the functions of RGMa in the immune system and its potential involvement in the pathogenesis of one of the immune-mediated CNS diseases, MS by using an animal model of MS, EAE.

4. Repulsive guidance molecule a (RGMa) that has pivotal roles both in the CNS and the immune system is a promising drug target

RGMa was originally discovered as an axon guidance molecule in the chick visual system (Stahl B et al., 1990). Recently, it was reported that RGMa regulated cephalic neural tube closure in mouse embryo and neuronal apoptosis (Niederkofler V et al., 2004; Matsunaga E et al., 2004; Yamashita T, 2007). In the pathological context, we demonstrated that RGMa inhibited neurite outgrowth and hindered functional recovery after spinal cord injury (Hata et al., 2006; Kubo T et al., 2008). While the functions of RGMa in the CNS have been well-established, its functions in other organs have remained uncertain. As described in the previous section, we found that RGMa was also expressed in macrophages/microglia in the injured spinal cord, implying the pathological roles of RGMa in the immune system. In order to address this possibility, we further evaluated RGMa expression in dendritic cells,

which are key components to evoke the immune reactions as APCs through the activation and differentiation of $CD4^+$ T cells (Weaver CT et al., 2006; Zhu J & Paul WE, 2010). We found that bone marrow derived dendritic cells produced RGMa upon inflammatory stimulation with lipopolysaccharide (LPS), suggesting the involvement of RGMa in the immune system, especially in the activation process of $CD4^+$ T cells triggered by dendritic cells (Muramatsu R et al., 2011). The activation process of $CD4^+$ T cells includes the enhancement of the adhesion property of $CD4^+$ T cells to intercellular adhesion molecules such as ICAM-1. Enhanced adhesion of $CD4^+$ T cells to intercellular adhesion molecules enhanced the penetration of circulating $CD4^+$ T cells into tissues including the CNS under pathological conditions such as MS. Actually, *in vitro* exposure of $CD4^+$ T cells to RGMa led to increased adhesion to ICAM-1, showing the direct activation of $CD4^+$ T cells by RGMa. These data strongly suggest that RGMa, which inhibits axon regeneration in the CNS, also plays regulatory roles in the immune system.

This encouraged us to further explore the pathological involvement of RGMa in the immune-mediated CNS disease, mouse EAE where neurodegenerative symptom was evoked by immunization with myelin antigens thereby dendritic cells and activated $CD4^+$ T cells plays pivotal roles in its pathogenesis. Immmunohistochemical analyses demonstrated that RGMa expression was upregulated in dendritic cells located in spinal cords, lymph nodes and spleen after the induction of mouse EAE. In order to evaluate the functional involvement of RGMa in the pathogenesis of EAE, we produced pharmacological effects of a neutralizing antibody against RGMa (Hata et al., 2006). The administration of the neutralizing antibody significantly suppressed the pathological changes such as demyelination of spinal axons and the paralysis of hind limbs in association with reduction of immune cell infiltration into the spinal cord and decline of inflammatory cytokine (IL-2, IFN-γ, IL-17, IL-4) production from $CD4^+$ T cells (Muramatsu R et al., 2011).In another mouse EAE models where neurological deficit was induced by adoptive transfer of activated dendritic cells that are pretreated with CNS myelin antigens *in vitro*, adoptive transfer of the activated dendritic cells lacking RGMa caused less severe neurological deficit , further suggesting the pathological role of RGMa in mouse EAE. Moreover, we found that expression levels of RGMa in dendritic cells increased in the brain and spinal cord of human subjects with MS. Moreover, the blockade of RGMa with the neutralizing antibody against RGMa inhibited the proliferation and the inflammatory cytokine (IL-2, IFN-γ, IL-17A, IL-4) production by peripheral blood mononuclear cells obtained from MS patients (Muramatsu R et al., 2011). These observations suggest that dendritic cell-derived RGMa also plays a key role in T cell activation in MS subjects. Intriguingly, RGMa polymorphisms are associated with MS and levels of pro-inflammatory cytokines (IFN-γ and TNF) in CSF of MS patients (Nohra, R et al., 2010). Collectively, these lines of evidences strongly suggest that RGMa plays critical roles in the pathogenesis of MS, especially via activating autoreactive $CD4^+$ T cells (Muramatsu et al., 2011; Flemming A, 2011).

In the current experimental settings where the neutralizing antibody to RGMa was administered via a peripheral route, the therapeutic benefits of RGMa inhibition is assumed to be mainly based on the blockade of the immune system i.e. reduction of the penetration of immune cells such as activated $CD4^+$ T cells from circulation into CNS, since the antibody did not effectively reach to the CNS (Muramatsu R et al., 2011). Nevertheless, we could not exclude the possibility that the therapeutic effect of anti-RGMa antibodies was partially mediated by the therapeutic action on the CNS because a small amount, but significant levels of the antibodies were detected in the spinal cord.

Current therapeutic interventions for MS mainly focus on anti-inflammatory effects and they are ineffective in reversing the CNS damage (Noseworthy JH et al., 2000; Compston A & Coles A, 2002; Bjartmar C et al., 2003).The approach to inhibit RGMa, an endogenous axon regrowth inhibitor, might enhance the recovery of neuronal deficits not only by suppressing excessive autoimmune responses but also by promoting regeneration of the damaged axons and restoration of the injured neural circuit in EAE. However, the pathological actions of RGMa on the axon damage in the CNS are needed to be further addressed in EAE.

Other molecules that are originally identified as regulatory factors in the CNS are also reported to have key roles in the pathogenesis of the inflammatory/immune-mediated diseases. For example, semaphorins that regulate axon guidance during neural development are reported to directly modulate the immune reactions in animal disease models including EAE (Suzuki K et al., 2008; Kumanogoh A & Kikutani H, 2010). This further supports the idea to target a molecule that acts both in the inflammatory/immune-mediated pathological phase and a neurodegenerative phase in the neurodegenerative diseases.

A therapy with a neutralizing antibody provides a highly target-selective intervention, but is also associated with critical drawbacks such as poor exposure to the CNS as mentioned and parenteral route administration. Identification of a small molecule drug that mimics antibody and interferes directly with the ligand-receptor interaction or subsequent signal transductions is an alternative strategy to overcome the drawbacks of therapeutic antibodies. However, development of a small compound that blocks direct interactions of large proteins often encounters high hurdles. In this context, we identified a key signal pathway that mediates the inhibitory action of RGMa on neurite outgrowth (Hata K et al., 2006; Kubo T et al., 2008). RGMa expresses its actions through the receptor, neogenin and its inhibitory actions are mediated by the downstream effectors, Rho, Rho kinase and myosin IIA. Therefore, small molecule that inhibits the actions of downstream molecules (Rho, Rho kinase and myosin IIA) would be alternative approaches to the therapeutic antibodies. Since inhibitors for Rho, Rho kinase and myosin IIA are currently available, it is worth examining their therapeutic potentials on EAE and spinal cord injury (Straight AF et al., 2003; Mueller BK et al., 2005; Kubo T & Yamashita T, 2007). Actually, fasudil, a Rho kinase inhibitor, exhibits therapeutic effects on experimental spinal cord injury, supporting the strategy to block downstream signals of a target molecule for the treatment of the inflammatory/immune-mediated neurodegenerative diseases (Hara M et al., 2000; Sung JK et al., 2003; Ding J et al., 2010).

5. Conclusion

In this chapter, we illustrate our recent strategy to develop a novel and effective therapeutic interventions, especially focusing on the identification of potential targets that have multiple pathophysiological functions in the neurodegenerative diseases such as MS and spinal cord injury. Our recent efforts to discover a molecule that displays multiple actions in the immune system and the CNS resulted in the identification of RGMa that plays a key role in the autoimmune-mediated pathological process in the CNS disease. Furthermore, therapeutic potential of RGMa inhibitor is demonstrated by the pharmacological benefits of the anti-RGMa neutralizing antibody in EAE animal model. Although the central effects of the neutralizing antibody has remained to be addressed, current experimental data suggest that the blockade of RGMa functions might provide improved therapeutic effects on autoimmune encephalomyelitis through the inhibition of harmful autoimmune reactions

and the promotion of axon regeneration of damaged neurons (Figure 1). In addition, the effectiveness of a small molecule inhibitor of the downstream signal pathway suggests the alternative drug discovery approach with higher probability of success.
This type of strategy to target a single molecule with multiple pathological actions will give us an opportunity to develop an efficient therapeutic intervention, which will be further elucidated in the future.

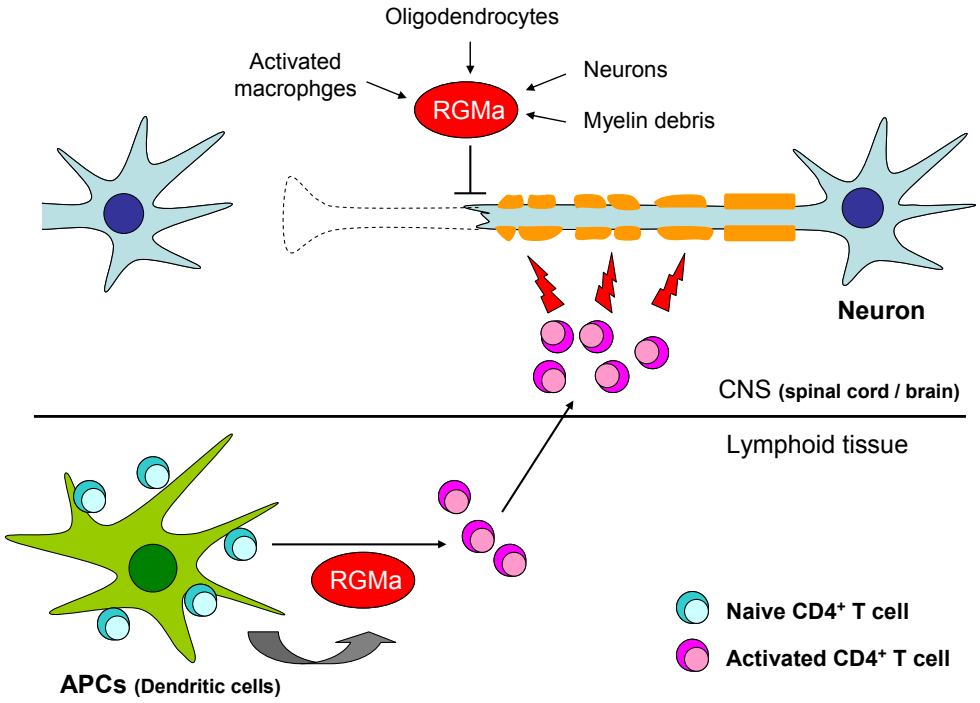

Fig. 1. RGMa plays key pathological roles both in the activation of autoreactive $CD4^+$ T cells and the inhibition of regeneration of damaged CNS axons, which renders RGMa as a promising drug target for the treatment of inflammatory/immune-mediated CNS diseases.

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Liquiritigenin Attenuates Alzheimer's-Like Neuropathology in an Amyloid Protein Precursor Transgenic Mouse Model and the Underlying Mechanisms

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1. Introduction

Estrogen plays a key regulatory role in a number of biological processes and, in addition to its classic function as a sex hormone, it has been linked to neurodegenerative diseases, including Alzheimer's disease (AD) (Wickelgren, 1997; Brann et al., 2007; Zhang et al., 2007). However, long-term compliance with estrogen therapy is often estimated to be no more than 15%–40%, due to its undesirable side-effects, such as an increased risk of developing breast and uterine cancer (Warren, 2004). Therefore, the body of recent research has focused on finding neuro-selective estrogen receptor agonists (Zhao et al., 2005) that mimic the beneficial effects of estrogen in the brain but which also exert negligible adverse effects on non-neural estrogen-responsive tissues. For instance, propylpyrazole triol and diarylpropionitrile – each of which exhibits relative specificity for the estrogen receptors ERα and ERβ respectively – have been proven to differentially regulate AD-like changes in female AD model mice (Carroll and Pike, 2008). Additionally, some phytoestrogens with fewer side-effects and potential neuroprotective effects have been developed for use in alternative treatment strategies (Zhao et al., 2002; Bang et al., 2004).

Liquiritigenin (7, 4'-dihydroxyflavanone) is a flavonoid extracted from the radix of Glycyrrhiza, an herbal that is frequently used to treat injury or swelling, or for detoxification in traditional Oriental medicine. Liquiritigenin is also one of the major active compounds of MF101 (Kupfer et al., 2008), an herbal extract currently used in clinical trials for the treatment of hot flushes and night-sweats in post-menopausal women. Our interest in liquiritigenin is based upon the following observations. First, liquiritigenin is shown to be a selective agonist of estrogen receptor-β (ERβ) (Mersereau et al., 2008). ERβ is expressed in the brain centre related to learning and memory, but it is unlikely to be related to sex (Shughrue et al., 1997; Gustafsson et al., 2003). Second, studies have already proven that liquiritigenin exerts cytoprotective effects in vitro and in vivo (Kim et al., 2004; Kim et al.,

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2006; Kim et al., 2008); in particular, we have previously observed that liquiritigenin inhibits amyloid β-peptide-induced neurotoxicity, not only in hippocampal neurons (Liu et al., 2009), but also in rats (Liu et al. 2010a). Third, we have found that liquiritigenin does not induce the proliferation of MCF-7 or T47D breast cancer cells (unpublished observations), which is consistent with the results of other studies (Gustafsson et al., 2003). Finally, pharmacokinetics data has demonstrated that liquiritigenin is absorbed well by the intestine amongst rats, with an in vitro blood-brain barrier penetration rate of (29.7±6.8)% within 90 min, similar to that of chloramphenicol (34.0±4.9)%, a known blood-brain barrier and highly penetrative drug (Kupfer et al., 2008; Lu et al., 2008; Kang et al., 2009). Taken together, these observations suggest that liquiritigenin may be a potentially effective therapy for AD.

2. Methods

2.1 Animals

Male and female 10-month-old transgenic 2576 (Tg2576) mice expressing the human 695 aa isoform of amyloid protein precursor (APP) and containing the mutation (K670N and M671L) were purchased from the Chinese Academy of Medical Sciences. Tg2576 mice and age- and strain-matched wild type (WT) mice (C57/BL6J) were housed with free access to standard food and water at a room temperature of 21±2°C, relative humidity of 45±15%, and 12 h light/dark cycles. All experimental manipulations and data analysis in this study were conducted in a blinded fashion.

All animal experiments were conducted in accordance with NIH guidelines under a protocol adhering to the guidelines of the Chinese Society of Laboratory Animal Sciences.

2.2 Experimental design

Liquiritigenin was synthesised at the Beijing Institute of Radiation Medicine (Beijing, China) and it was shown to be >95% pure by HPLC. Tg2576 mice – a widely used AD model (Spires et al., 2005; Howlett et al., 2009) – were randomly assigned to one of four liquiritigenin treatment groups (n= 5 male and 5 female/group): 30 mg/kg/d, 10 mg/kg/d, 3 mg/kg/d and 0 mg/kg/d (control vehicle treatment). Each treatment group received liquiritigenin continuously for 90 d (i.g.); the WT and vehicle-treated Tg2576 mice were treated with an identical volume (20 ml/kg/d) of vehicle (0.1% sodium carboxymethyl cellulose).

Animal behaviour was assessed from the 91st day from the beginning of treatment, with the tests carried out sequentially in accordance with the experimental schedule shown in Fig. 1.

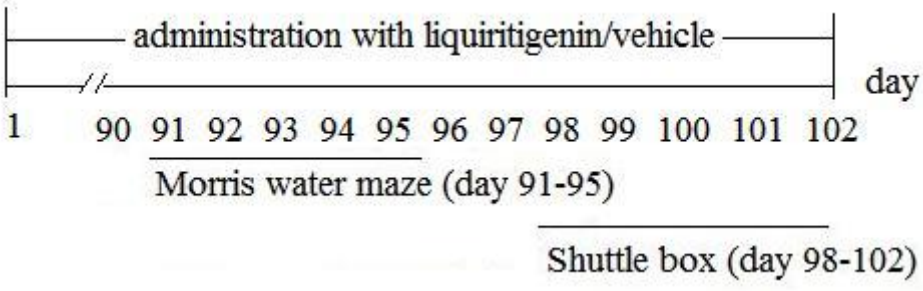

Fig. 1. Experimental schedule of behaviour tests.

2.3 Morris water maze test

A circular water tank (100-cm diameter, 40 cm tall, purchased from Chinese Academy of Medical Sciences) was divided into four equally spaced quadrants. A transparent platform was set at the east quadrant of the tank, 20 cm from the wall and 1 cm below the surface of the water.

Reference memory task: The task was conducted twice a day for 4 consecutive days. In each trial, the mouse was placed in the water at one of three starting positions (which were spaced equally around the rim of the tank), with the sequence of the positions selected at random. The mouse was allowed to swim until it found the platform or until 90 s had elapsed. In this last case, the trial was terminated and the animal was put on the platform for 30 s. The latency of escape onto the platform was recorded using Morris system software (Chinese Academy of Medical Sciences).

Probe task: On day 95, the platform was removed from the pool and the animals underwent a 90 s spatial probe trial. The time taken to reach the place where the platform had been located during training and the length of time spent in that quadrant was recorded.

2.4 Two-way shuttle avoidance task

The two-way shuttle avoidance box, placed in a dimly-lit, ventilated, sound-attenuated cupboard, is a rectangular chamber (44×28×23 cm, purchased from Chinese Academy of Medical Sciences) used to condition animals with a shock stimulus. During the conditioning stimulus (5 s of singing on a loudspeaker located on the ceiling), the animals had to move to the other side of the shuttle box in order to avoid a 2 s electrical shock of the foot (unconditioned stimulus). Between trials, the animals were able to move with impunity for 40 s. Every mouse was subjected to 10 trials per day for 4 consecutive days, and the time spent in avoidance (shuttling to the other part of the apparatus while the tone was on so as to avoid the shock), the frequency of the shock, and the length of time in shock were recorded.

2.5 Brain slice and homogenate preparation

After the above behavioural assessment was completed, all the mice were anesthetised with pentobarbital (50 mg·kg-1, i.p.), transcardially perfused with cold physiological saline, and killed by decapitation. Each cerebrum was divided into 3 parts. One part of the cerebral hemispheres was collected immediately and immersion-fixed in 4% paraformaldehyde for paraffin sections. Half of the remaining cerebral hemisphere were weighed and immediately used to make a 10% homogenate with saline by centrifugation at 3500 rpm for 10 min at 4 °C, and which were stored in -80°C until use. The remaining half of the hemispheres were lysed with 4°Ccell lysis buffer (Beyotime, P0013, China) for protein extraction – as described by the manufacturer – and stored at -80°C.

2.6 Detection of acetylcholinesterase and choline acetyltransferase activity

Acetylcholinesterase (AchE) and choline acetyltransferase (Chat) activity was detected in the 10% brain homogenate using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The absorbencies were determined with a spectrophotometer (DU-640, Beckman, USA).

2.7 Aβ/A4 immunostaining

Fixed hemibrains were blocked, sectioned (4 μm) exhaustively in the horizontal plane, and then processed for immunohistochemistry using a standard avidin-biotin peroxidase

complex protocol. Briefly, every section was immunostained using an antibody directed against Aβ/A4 (Santa Cruz, sc-28365, 1:100 dilution) at 4°C overnight, followed by signal development using an avidin-biotin peroxidase complex immunohistochemistry kit (Zhongshan Goldenbridge Bio Co., Ltd., PV-9002, China), diaminobenzidine for colour substrate reactions, and haematoxylin for counterstaining. The level of Aβ/A4 immunoreactivity in the hippocampus CA1, subiculum, amygdala, and cortex was quantified. In the hippocampus CA1, three adjacent but non-overlapping fields were captured for load analysis. In the subiculum and amygdala, we similarly captured two fields per section; in the cortex, six adjacent, non-overlapping fields were captured. The fields of immunolabelled sections were imaged and digitised using a video-capture system (a CCD camera coupled to an Olympus Optical Microscope, Tokyo, Japan) and the density of the images was quantified with Motic Images Advanced 3.2 software (on the internet at http://www.microscopeworld.com), using stereological approaches. All these assessments were performed by experimenters who were blinded to the genotypes of each brain section.

2.8 Determination of Aβ/A4 protein levels by Western blotting

Western blotting was performed to further validate the results of immunostaining. Briefly, protein concentrations were measured using a bicinchoninic acid protein determination kit (Walterson, H10020, China), and 40 μg protein per lane was analysed. SDS-PAGE and Western blotting were performed according to standard protocols (Zhang et al., 2007). The same Aβ/A4 antibody was used (1:500 dilution) and β-tubulin (Walterson, China, 1:1000 dilution) served as an internal control. A goat anti-mouse IgG-HRP secondary antibody was used (Zhongshan Goldenbridge Bio Co., Ltd., China, 1:5000 dilution). Levels of Aβ/A4 were expressed as a ratio of Aβ/A4 to β-tubulin signal, which was determined with Scion Image software (Scion Image for Windows β 4.02, on the internet at http://www.scioncorp.com).

2.9 Nissl staining for neurons

Nissl staining was directly used for routine histology with cresyl violet (C 1791, Sigma, USA). Briefly, sections were dipped into an aqueous solution of cresyl violet (0.5%) at 37°C for 20 min and then washed with distilled water followed by 70% ethanol. The density of CA1 and cortex pyramidal neurons was quantified with Motic Images Advanced 3.2 software using stereological approaches.

2.10 Detection of astrogliosis by immunostaining

Polyclonal rabbit anti-mouse glial fibrillary acidic protein (GFAP, Zhongshan, ZA-0117, China, 1:100 dilution) antibody was used for the immunostaining of astrocytes. The staining and analysis were conducted as described in Aβ/A4 immunostaining; the level of GFAP immunoreactivity was quantified in the hippocampus CA1 only.

2.11 Determination of Notch-2 expression by Western blotting

SDS-PAGE and Western blotting were performed according to standard protocols (Zhang et al., 2007) with an antibody against Notch-2 intracellular domains (Notch-2 IC, ab-52302, Abcam, UK, 1:300 dilution); 100 μg protein per lane was analysed. Otherwise, the procedure and reagents used were as described with Western blotting.

2.12 Aβ peptide oligomerisation assay in vitro

Soluble Aβ1-42 (100 μM) was dissolved in saline with 0, 0.02, 0.2 or 2 μM liquiritigenin (doses chosen based upon our preliminary tests) or 100 μM melatonin (positive control) and incubated at 37°C for 7 d so as to induce fibril formation. We then added 10 μl of this, conditioned Aβ1-42 to 990 μl 5 μM thioflavin-T (Th-T, Sigma, USA) dissolved in 50 mM glycine-NaOH (pH 8.5), and measured the fluorescent intensity of the liquid with a fluorescence spectrophotometer (excitation=435 nm, emission=485 nm).

Primary cerebrum neuron cells were obtained from newborn (<12 h postnatal) C57 mice following methods already reported (Zhao et al., 2002). These neurons were seeded into poly-D-lysine-coated 96-well plates at a density of 1.0×105 cells per well. To evaluate the toxicity of these, conditioned Aβ1-42, 7 day-old neuron cells were treated with the conditioned Aβ1-42 for 72 h. Cell viability was measured with a 3-[4,5-dimethyl-thiazolyl]-2,5- diphenyl-2- tetrazolium bromide (Sigma, USA) assay (Zhao et al., 2002), and cellular membrane penetrability was evaluated by measuring the amount of cytoplasmic lactate dehydrogenase released into the medium (Zhao et al., 2002) with a commercial kit (Biosino Bio-technology and Science Inc., Beijing, China).

2.13 Investigation of the mechanism underlying Notch-2 inhibition in vitro

We used the specific ER antagonist ICI 182 780 (Faslodex) to ascertain whether there was an inhibitory effect of liquiritigenin on Notch-2 involved ER. Primary cerebrum stem/precursor cells were obtained from newborn (<12 h postnatal) C57 mice using reported methods (Ray and Gage, 2006); these were grown for 7 days and seeded into 6-well plates at a density of 1.5×10^6 per well. The cells were treated with either vehicle ICI 182 780 (200 nM) alone or liquiritigenin alone (0.02, 0.2 or 2 μM), or else 2 μM liquiritigenin plus 200 nM ICI 182 780. ICI 182 780 was added 1 h prior to the liquiritigenin treatment, and liquiritigenin was incubated with ICI 182 780 for 5 d. Throughout the treatment, the medium was changed every other day; each change of media contained the appropriate drug concentrations.

Reverse transcriptase-polymerase chain reaction analysis and Western blotting were performed in order to determine changes of Notch-2 mRNA (GenBank accession no. NM_024358) and protein expression. Total RNA was extracted and reverse-transcribed to yield cDNA with commercial kits, as indicated by the manufacturer (Promega, A3500, USA). Polymerase chain reaction was performed using Notch-2 specific primers (forward: 5'-GCA TCC TGG TCA TCG TGGT-3', reverse: 5'- GAG CCT ATT ATC TCC TGT TCC TG-3'). The predicted size of the amplified product was 229 bp. As an internal control, specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5'-CAT GAC CAG AGT CCA TGC CAT CACT-3', reverse: 5'-TGA GGT CCA CCA CCC TGT TGC TGTA-3') were used. Amplification was carried out for 40 cycles: 30 s at 94°C, 30 s at 59°C and 30 s at 72°C in a thermal cycler (Gene Amp 2400, USA). The PCR products were visualised after 1% agarose gel electrophoresis.

For Western blotting, the procedures and reagents used were similar to those described above, with an antibody against the Notch-2 intracellular domains (Notch-2 ^{IC}, ab-52302, Abcam, UK, 1:300 dilution), and 100 μg protein per lane was analysed. Otherwise, the procedures and reagents used were as already described.

2.14 Data analysis

All data was expressed as mean ± SEM. Assays in vitro were repeated in at least three independent experiments, each of which was performed in triplicate. Behavioural results

were analysed with a two-way ANOVA followed by Dunnett's test; other raw data was compared among groups by one-way ANOVA followed by Dunnett's test. $P<0.05$ was assumed so as to indicate statistical significance.

3. Results

3.1 Liquiritigenin treatment rescued behavioural impairment in the Morris water maze test

Changes of escape latency in the reference memory task are shown in Fig. 2(A). The escape latency of mice in every group decreased gradually with increased training time, but the vehicle-treated Tg2576 mice exhibited significantly prolonged escape latency from day 2 to day 4, compared with the WT mice. Compared with the vehicle-treated Tg2576 mice, the liquiritigenin-treated mice required less time to reach the platform. A 90-s spatial probe trial was carried out after the 8th training trial. As shown in Fig. 2(B), the time required to reach the platform for the WT mice was 11.55±2.78 s. The vehicle-treated Tg2576 mice required a considerably longer time (23.65±4.84 s, $P<0.05$ vs. control), while the liquiritigenin-treated mice (10 and 30 mg/kg/d) performed better than the vehicle-treated mice (10 mg/kg/d: 17.43±3.08 s, 30 mg/kg/d: 16.76±4.02 s, $P<0.05$). The vehicle-treated Tg2576 mice (16.8±2.1 s) also showed a significant decrease in the length of time spent in the quadrant where the platform had been located during training as compared to the WT mice (23.7±2.6 s). Only the highest dose of liquiritigenin treatment increased this parameter (20.8±2.7 s), as shown in Fig. 2(C).

3.2 Liquiritigenin-treated mice perform better in the shuttle box task

The results of the shuttle box test are shown in Fig. 2(E), (F) and (G). Compared with the WT mice, the vehicle-treated Tg2576 mice were shocked more times and for longer periods, and their initiative escaping time was significantly decreased. Compared with the vehicle-treated Tg2576 mice (shocks: 6.7±1.3, shock time: 9.9±1.8 s, escaping time: 7.9±1.2 s), the liquiritigenin-treated mice (10 and 30 mg/kg/d) were shocked fewer times (10 mg/kg/d: 3.7±0.6, 30 mg/kg/d: 2.3±0.4, $P<0.05$) and spent less time being shocked (10 mg/kg/d: 4.6±0.8 s, 30 mg/kg/d: 3.0±0.5 s, $P<0.05$); their escaping time also increased (10 mg/kg/d: 16.6±1.8 s, 30 mg/kg/d: 16.9±2.2 s, $P<0.05$). The performance of mice treated with a lower dose of liquiritigenin (3 mg/kg/d) was similar to that of the vehicle-treated mice.

3.3 Changes in AchE and Chat activities upon liquiritigenin treatment

The results of AchE and Chat activity determination are shown in Table 1. The activities of AchE and Chat in the vehicle-treated Tg2576 mice were set as 100% and used as the baseline. The results in Table 1 show that the vehicle-treated Tg2576 mice exhibited nearly a 1.7-fold increase in AchE activity and a 0.6-fold decrease in Chat activity compared to WT. The AchE activity of mice treated with the middle and high doses of liquiritigenin decreased markedly to 88.0±7.9% and 67.9±5.9% respectively compared with that of the vehicle-treated Tg2576 mice. Chat activity in these mice increased to 119.0±12.7% (middle dose) and 150.9±11.2% (high dose) compared with the vehicle-treated mice.

3.4 Liquiritigenin inhibited the expression of Aβ/A4 in different brain regions

Aβ/A4 load was quantified in the hippocampus CA1, subiculum, cortex and amygdala. Fig. 3(A) shows representative images of Aβ/A4 immunostaining in the brain. Not all the

liquiritigenin-treatment groups are shown due to limitations of space. According to a report by Carroll (Carroll et al., 2007), amyloid A4 exhibits a different staining pattern to that of Aβ; amyloid A4 localises to the periphery of the cell body, while Aβ deposits localised throughout the cell body, often with a punctuate distribution. The localisation of Aβ/A4 is shown in Fig. 3(A), and some dense extracellular accumulation of Aβ could also be seen, especially in the cortex. However, because it is difficult to distinguish between different types of immunoreactive cells when doing quantitative analysis, we counted all positive staining in. For the quantitative analysis of immunostaining, the Aβ/A4 load of the vehicle-treated Tg2576 mice was set as 100%. We found that the vehicle-treated Tg2576 mice had a higher Aβ/A4 load in the hippocampus CA1, subiculum, cortex and amygdala, as compared with the WT mice. Liquiritigenin treatment attenuated the increase in the Aβ/A4 load to some extent. The Aβ/A4 load in the hippocampus was 93.3±14.6% (low dose), 65.3±7.1% (medium dose) and 55.1±9.0% (high dose) of that observed in the vehicle-treated Tg2576 mice; in the cortex, the load decreased to 91.4±13.2%, 72.1±9.1% and 67.2±7.5%, and in the amygdala, the load decreased to 87.6±8.7%, 66.9±6.1% and 53.7±5.7%, as shown in Fig. 3(B). The Aβ/A4 in the subiculum was not markedly changed by liquiritigenin treatment.

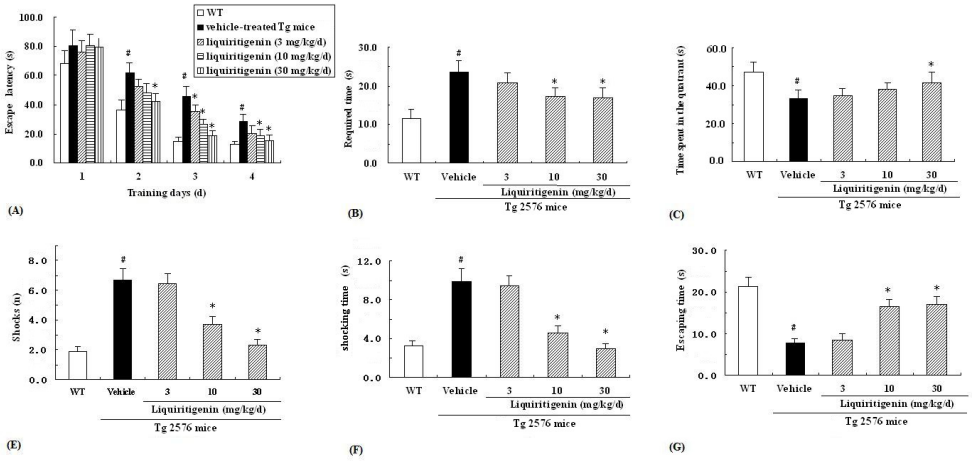

Fig. 2. Effects of liquiritigenin on the performance of Tg2576 mice in the Morris water maze task and shuttle box task. (A) Escape latency with reference to the memory task in the Morris water maze test. (B) The time required to reach the previous platform location in the Morris water maze test probe task. (C) Time spent in the platform quadrant in the Morris water maze test probe task. (D) Shocks received during training in the shuttle box task. (E) Length of shocks received in the shuttle box task. (F) Initiative escaping time in shuttle box task. Morris water maze tasks were given on days 91-95 and shuttle box tasks were given on days 98-102, following the administration of liquiritigenin. The values shown are mean±SEM for 10 animals. # $p<0.05$ vs. WT, * $p<0.05$ vs. vehicle-treated Tg2576 mice (Dunnett's test).

Group	Dose (mg/kg/d)	AchE (%)	Chat (%)
WT	-	61.4±6.2	171.4±14.2
Vehicle	-	100.0±8.5#	100.0±11.7#
liquiritigenin	3	102.0±10.2	105.8±13.1
	10	88.0±7.9*	119.0±12.7*
	30	67.9±5.9*	150.9±11.2*

Table 1. Effect of liquiritigenin on brain AchE and Chat activity in Tg2576 mice (mean±SEM, n=10/group). Acetylcholinesterase (AchE) and choline acetyltransferase (Chat) activity. The activities in the vehicle-treated Tg2576 mice were defined as 100%. # $p<0.05$ vs. WT, * $p<0.05$ vs. vehicle-treated Tg2576 mice (Dunnett's test).

3.5 Liquiritigenin treatment alters the protein levels of Aβ but not amyloid A4

Aβ/A4 levels were further detected by Western blotting. As shown in Fig. 4(A), the two bands near 100 kDa are amyloid A4 and the band near 40 kDa is oligomeric form of Aβ, according to the manufacturer. The protein level was calculated as a ratio against the loading control β-tubulin. As shown in Fig. 4(B), the vehicle-treated Tg2576 mice exhibited a higher level of both amyloid A4 (2.14-fold increase) and Aβ (2.8-fold increase) load compared with the WT mice. There was no difference in amyloid A4 load between the liquiritigenin-treated groups and the vehicle-treated Tg2576 group. However, the oligomeric form of Aβ protein levels were significantly lower in mice treated with the middle and high doses of liquiritigenin, decreasing to 0.71- and 0.57-fold of the level in the vehicle-treated Tg2576 mice, respectively.

3.6 Liquiritigenin treatment promotes an increase in neuronal cell number

Neuronal cell loss was examined by nissl staining with cresyl violet. Fig. 5(A) is a representative image of nissl staining, which shows that the vehicle-treated Tg2576 mice exhibited a loss of neurons in the hippocampus CA1 and cortex, and that liquiritigenin treatment attenuated this loss to a degree. Quantitative analysis in Fig. 5(C) shows that the number of neurons in the vehicle-treated Tg2576 mice decreased to 54.5±8.5% of WT mice in the hippocampus and 63.2±9.2% in the cortex. Liquiritigenin treatment markedly increased the neuron numbers compared with the vehicle-treated Tg2576 mice: in the 10 mg/kg/d dosing group, the number of neurons increased to 122.5±14.7% in the hippocampus and 117.5±15.0% in the cortex while, in the 30 mg/kg/d dosing group, the numbers increased to 163.8±17.7% in the hippocampus and 134.1±15.0% in the cortex.

3.7 Liquiritigenin treatment attenuates astrocytosis

In AD, deposition of Aβ in senile plaques is associated with astrocytic proliferation. Thus, we investigated whether liquiritigenin would decrease astrogliosis. Astrocytes were identified by GFAP immunoreactivity. We quantified the GFAP load specifically in the hippocampus CA1 due to the importance of this region in memory, and we have found in this study that astrocytosis was apparent in CA1. Fig. 5(B) shows representative images of GFAP immunohistochemistry in CA1. Quantitative analysis of CA1 subfields in Fig. 5(D) shows that the vehicle-treated Tg2576 mice had a 2.43-fold increase in GFAP load compared

with the WT mice, and that GFAP load was decreased significantly in mice subjected to middle and high doses of liquiritigenin compared to the vehicle-treated Tg2576 mice (10 mg/kg/d: 85.5±9.0%, 30 mg/kg/d: 73.8±7.6%).

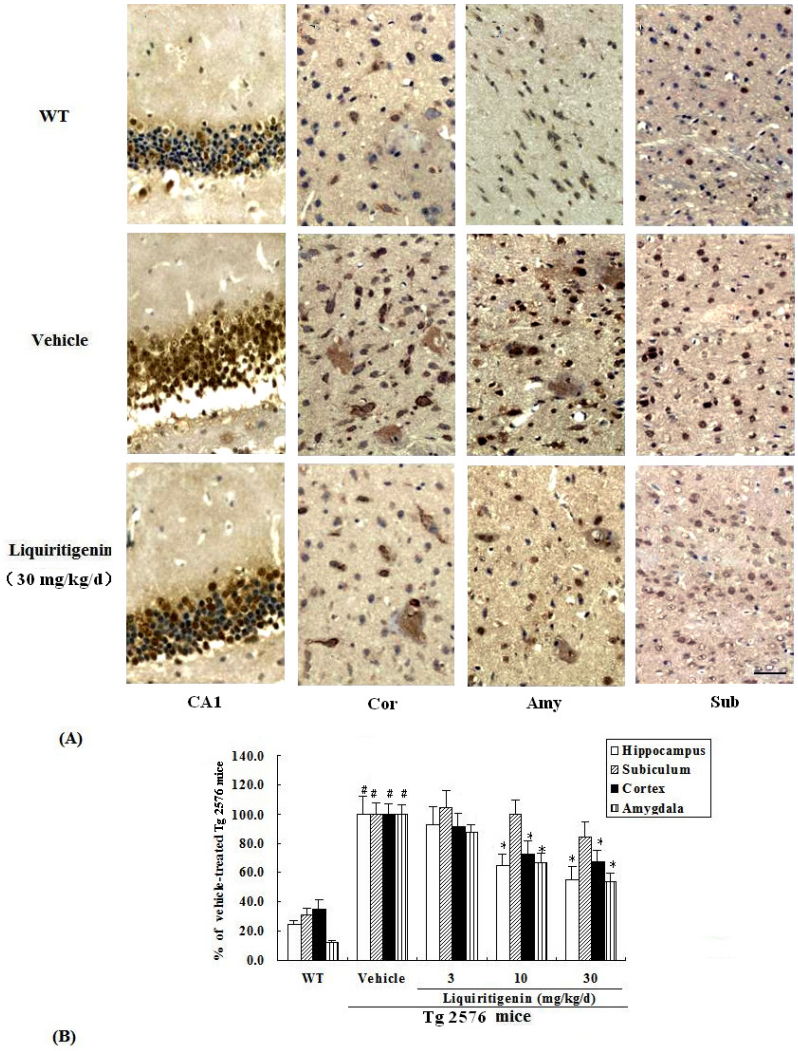

Fig. 3. Liquiritigenin attenuated Aβ/A4 accumulation in different brain regions. (A) Representative images of Aβ/A4 immunohistochemistry of different brain regions. Scare bar: 50 μm. (B) Quantification of Aβ/A4 load. The level of Aβ/A4 immunoreactivity was quantified in the hippocampus CA1, subiculum, amygdala and cortex, by Motic Images Advanced 3.2 software. The average Aβ/A4 load of the vehicle-treated Tg2576 mice was defined as 100%. The values shown are mean±SEM for 10 animals. # $p<0.05$ vs. WT, * $p<0.05$ vs. vehicle-treated Tg2576 mice (Dunnett's test).

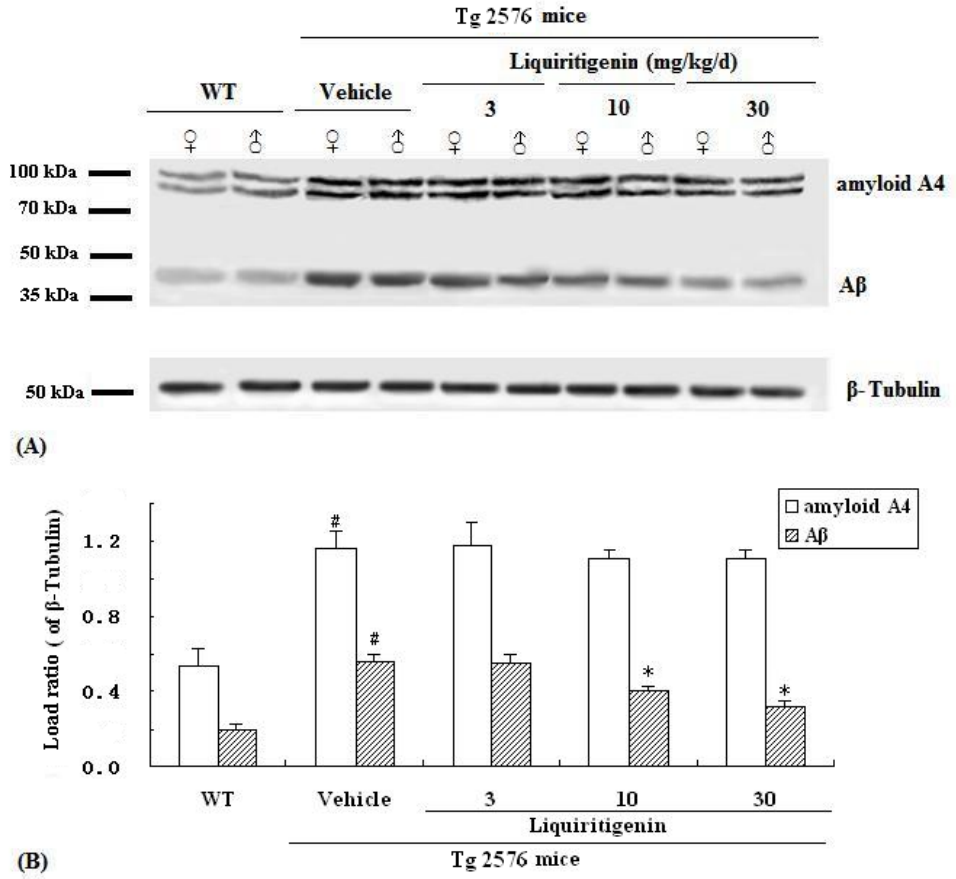

Fig. 4. The effect of liquiritigenin on Aβ/A4 accumulation, assayed by Western blotting. (A) Representative Western blots. The two bands near 100 kDa are amyloid A4 and the single band near 40 kDa is oligomeric form of Aβ. (B) Quantification of Western blotting. The optical density of Aβ bands was quantified with Scion Image software. ♀ indicates female and ♂ indicates male. The values shown are mean±SEM for 10 animals. # $p<0.05$ vs. WT, * $p<0.05$ vs. vehicle-treated Tg2576 mice (Dunnett's test).

3.8 Liquiritigenin decreased the expression of Notch-2 in vivo and in vitro

Notch signalling is involved in many critical cellular processes, such as neurogenesis, proliferation and apoptosis etc. The preliminary experiment results of a microarray assay performed in our lab suggested that liquiritigenin had a potential inhibitory activity on Notch-2 mRNA (down-regulated for 5.34 times) and protein expression (down-regulated for 3.12 times) in normal rat hippocampal neurons (unpublished data). Thus, we investigated whether liquiritigenin had a similar inhibitory effect on Notch-2 in vivo and in vitro. As shown in Fig. 6(A), Notch-2^{IC} (the active fragment) appears as a band near 100 kDa. Fig. 6(B) shows that the vehicle-treated Tg2576 mice expressed a 1.73-fold higher level of Notch-2^{IC}

than WT mice. The treatment of Tg2576 mice with liquiritigenin resulted in a mild but significant decrease (10 mg/kg/d: 18.4%; 30 mg/kg/d: 23.7%) in Notch-2^{IC} expression compared with vehicle-treated ones, while the dose of 3 mg/kg/d liquiritigenin did not seem to influence Notch-2 levels.

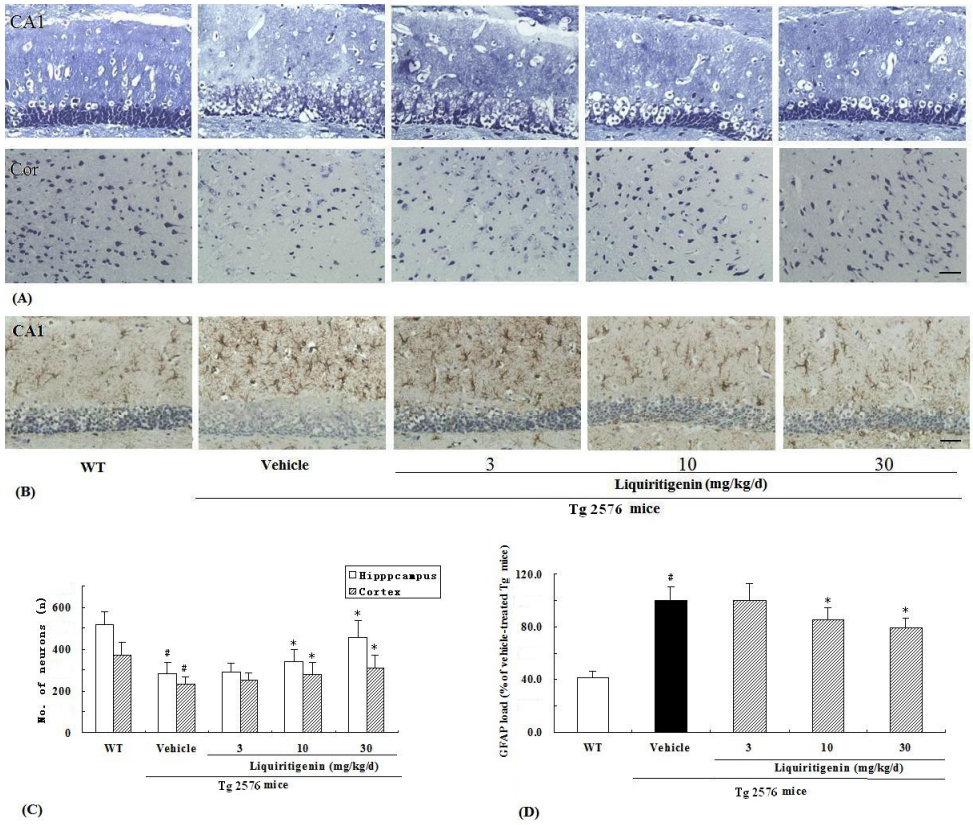

Fig. 5. Liquiritigenin attenuated neuron loss and excess proliferation of astrocytes in the brains of Tg2576 mice. (A) Representative image of nissl staining. (B) Representative image of GFAP immunostaining. (C) Quantification of nissl staining. (D) Quantification of GFAP load. Quantification was performed with Motic Images Advanced 3.2 software. The values shown are mean±SEM for 10 animals. # $p<0.05$ vs. WT, * $p<0.05$ vs. vehicle-treated Tg2576 mice (Dunnett's test).

In vitro studies showed similar results. The treatment of primary neurons obtained from newborn C57 mice with 0.2 μM or 2 μM liquiritigenin resulted in a 37.1% to 48.6% reduction in Notch-2^{IC} as assayed by Western blotting. The introduction of the ER inhibitor ICI 182 780 blocked the effects of liquiritigenin, as shown in Fig. 6(C). RT-PCR showed that the treatment of primary neurons with 0.2 or 2 μM liquiritigenin decreased levels of Notch-2 mRNA by 30%-60% compared with the control. Treatment with ICI 182 780 blocked the effect of liquiritigenin completely, but had no independent effect on Notch-2 expression, as shown in Fig. 6(D).

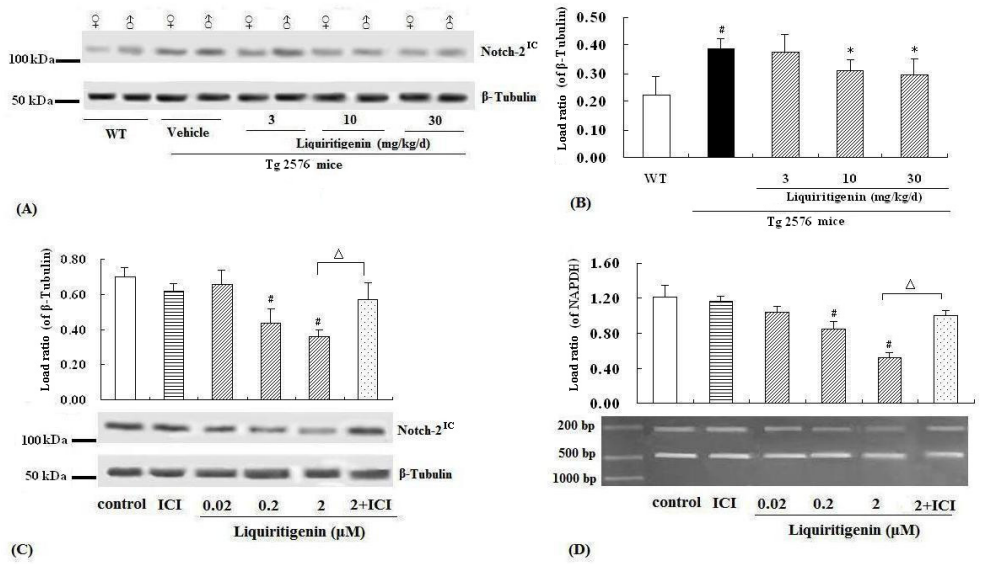

Fig. 6. Liquiritigenin inhibited Notch-2 expression both in vivo and in vitro.
(A) Representative Western blots from tissue samples. (B) Quantification of Western blots. (C) Effect of liquiritigenin on Notch-2 protein expression in vitro shown by Western blotting. (D) Effect of liquiritigenin on Notch-2 mRNA expression in vitro shown by RT-PCR. Band densities were quantified with Scion Image software. The values shown are mean±SEM. # $p<0.05$ vs. WT or control, * $p<0.05$ vs. vehicle-treated Tg2576 mice, Δ$p<0.05$ vs. liquiritigenin (0.2 μM)-treated cells (Dunnett's test).

3.9 Liquiritigenin inhibits amyloidosis in vitro

To explore the mechanisms underlying the liquiritigenin-mediated improvement in AD-type cognitive function and Aβ neuropathology, we investigated whether liquiritigenin influenced Aβ peptide oligomerisation in vitro. Aβ is a 39-43 amino acid peptide that derives from APP. $Aβ_{1-42}$ is the most easily amyloidogenic form, so it was used in the present study. Liquiritigenin was added to soluble $Aβ_{1-42}$ and these tubes were stored at 37 for 7d to allow fibril formation. The thioflavin-T assay shown in Fig. 7(A) revealed that the fluorescence of $Aβ_{1-42}$ incubated with 0.2, 2 and 20 μM liquiritigenin was decreased to 96.9±2.5%, 77.2±7.0% and 72.5±6.4% of the fluorescence observed for $Aβ_{1-42}$ incubated alone. Treatment with melatonin caused the fluorescence to decrease to 49.9%.

Primary cerebral neurons obtained from newborn C57 mice were treated with the conditioned $Aβ_{1-42}$ for 72 h. Cell viability was measured by 3-[4,5-dimethyl-thiazolyl]-2,5-diphenyl-2- tetrazolium bromid assay and LDH release. As shown in Fig. 7(B), incubation with $Aβ_{1-42}$ reduced cell viability to 69.5±3.8% ($P<0.05$ vs. control) while neurons incubated with melatonin-treated $Aβ_{1-42}$ were 85.4±2.4% viable ($P<0.05$ vs. Aβ25-35). When the $A_{β1-42}$ were treated with 0.2, 2 or 20 μM liquiritigenin, the cell viability was increased to 71.4±5.8 % ($P>0.05$ vs. $Aβ_{25-35}$), 78.0±4.7% ($P<0.05$ vs. $Aβ_{25-35}$) and 82.2±3.7% ($P<0.05$ vs. $Aβ_{25-35}$), respectively. These results showed that $Aβ_{1-42}$ alone caused a 4.3-fold increase in LDH

leakage compared to controls (P<0.05 vs. control), and that LDH leakage in 0.2 and 2 μM liquiritigenin-treated groups decreased to 80.3±4.2% and 70.1±4.8% that of $A\beta_{1-42}$ alone, respectively (P<0.05 vs. $A\beta_{25-35}$). Treatment with 0.2 μM liquiritigenin did not affect LDH leakage, as shown in Fig. 7(C).

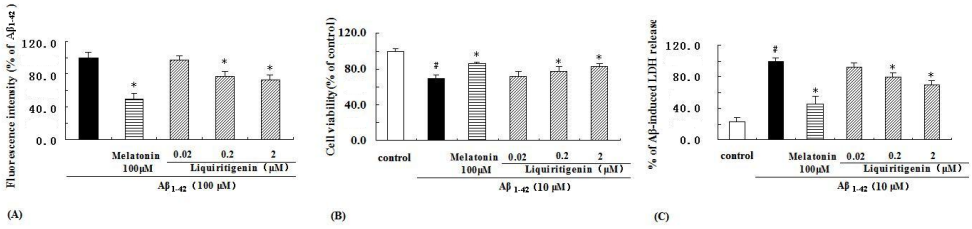

Fig. 7. Liquiritigenin inhibited $A\beta_{1-42}$ fibril formation in vitro. Soluble $A\beta_{1-42}$ (100 μM) was incubated with liquiritigenin (0, 0.02, 0.2 and 2 μM) or 100 μM melatonin (positive control) at 37°C for 7 d so as to induce fibril formation. (A) The inhibitory effects of liquiritigenin on fibril formation were measured by thioflavin-T. The fluorescence intensity indicates fibril formation. (B) Cell viability and (C) LDH release were detected in primary neurons treated with the conditioned $A\beta_{1-42}$ for 72 h. Values shown are mean±SEM. # p<0.05 vs. control, * p<0.05 vs. $A\beta_{1-42}$-treated alone (Dunnett's test).

4. Discussion

A primary clinical concern regarding hormone replacement therapy in postmenopausal women is the increased risk of breast and uterine cancer associated with prolonged treatment with estrogenic compounds. However, hormone replacement therapy is also reported to be associated with a reduced incidence and severity of AD. Therefore, the development of neuro-selective estrogen receptor agonists that exert beneficial effects on the brain but minimal effects on other estrogen-responsive tissues is of critical importance. In the present study, we investigated the efficacy of the newly found selective estrogen receptor agonist liquiritigenin in regulating neuropathology in the Tg2576 mouse model of AD. We observed that liquiritigenin treatment significantly improved behavioural performance in such mice. At the molecular level, liquiritigenin treatment reduced Aβ accumulation in some brain regions, regulated the function of the acetylcholine system, increased the average neuron number in the brain, and attenuated astrogliosis in the hippocampus. More interestingly, we have found that liquiritigenin can inhibit Notch-2 expression in an ER-dependent way. Previously, work from Carroll has shown that administration of propylpyrazole triol (an ERα agonist) but not diarylpropionitrile (an ERβ agonist) can improve behavioural performance of 3×Tg-AD model mice, and that both propylpyrazole triol and diarylpropionitrile can reduce Aβ accumulation in the brain (Carroll and Pike, 2008). Our results are not inconsistent with these observations, and it may that, although liquiritigenin has been reported as a ERβ agonist, it also has been shown to exhibit some affinity to ERα (Hillerns et al., 2005) – a report that we are agree with, based on the results of a reporter gene-based cell screening method for ER subtype ligands in our lab (unpublished data). Besides, the difference in behavioural assays used in these studies might also cause some discrepancies in results.

Since the Morris water maze and shuttle box tests investigate distinct motivations, and different skills are required for a good performance in each task, we used both of these tests

to examine the memory skills of the mice in this study. In the Morris water maze test, the mice in all three liquiritigenin dosing groups exhibited a better performance in the reference memory task. The middle and high dosing groups also needed less time to reach their destination in the probe task, but only mice receiving the 30 mg/kg/d dose of liquiritigenin treatment showed improvement in the time spent in the quadrant where the platform had been located. In the shuttle box test, the mice that received the middle and high dosages of liquiritigenin performed better than the vehicle-treated mice, indicating that a 3 mg/kg/d dose may be too low to be effective in this test. Furthermore, a dose higher than 30 mg/kg/d is likely to be unnecessary as higher doses did not cause further improvement in other AD models that we used previously (unpublished observations).

The cholinergic system plays a crucial role in cognitive functions, and most AD patients exhibit a reduction in cholinergic functioning. Estrogen treatment has been found to directly enhance the activity of the cholinergic system by some researchers (Kompoliti et al., 2004; Bora et al., 2005). In the present study, we found that the selective ERβ agonist liquiritigenin enhanced the activity of Chat and decreased AchE activity, thus enhancing cholinergic functioning. Given that liquiritigenin is a kind of phytoestrogen, our observations are consistent with other research showing that phytoestrogens, such as soy isoflavones, can reverse the increase of AchE observed in ovariectomised rats (Monteiro et al., 2007). The effects of liquiritigenin on the cholinergic system may be related to its activity on ER, and further research will be required to verify this hypothesis.

Excessive deposition of Aβ in the brain is a key characteristic of AD patients. In APP Tg mice, Aβ deposits appears in a few months after birth and they progressively accumulate (Reilly et al., 2003). We chose to use 10-month old mice in this study because serious Aβ accumulation has already occurred within this population, and we wanted to observe the therapeutic effects of treatment with liquiritigenin, rather than any preventive effects. In this study, we used both immunohistochemistry and Western blotting to measure Aβ accumulation. The antibody that we used cross-reacted with amyloid A4 and oligomeric form of Aβ (toxic fragment), both of which are given rise to from APP, and they could both be clearly and easily discriminated in Western blots, while immunohistochemistry with this antibody could effectively detect Aβ /A4 load in different regions of brain. Accordingly, the two methods were used. We report in the present study that liquiritigenin treatment attenuates Aβ /A4 load in the hippocampus, cortex and amygdala, but not in the subiculum. This conclusion is consistent with what is known about ER distribution in the brain. Studies in both humans and rodents have demonstrated a differential distribution of ERα and ERβ in brain regions affected in AD, including the hippocampus, frontal cortex and the amygdala. *In situ* hybridisation studies have demonstrated a higher density of ERβ-expressing cells compared with ERα-expressing cells in the hippocampus (Mehra et al., 2005) and layers 4-6 of the cortex (Shughrue et al., 1997 ; Mitra et al., 2003), but similarly high levels of ERβ- and ERα- expressing cells were expressed in the amygdala (Shughrue et al., 1997). A recent study showed that ERβ in the hippocampus and/or cortex is required for enhanced performance in cognitive tasks and anti-anxiety behaviour in mice (Walf et al., 2009). Our results are also consistent with the hippocampus-dependent nature of the behavioural tasks. Further complicating this issue, the mechanisms by which estradiol or selective estrogen receptor agonists propylpyrazole triol, diarylpropionitrile and liquiritigenin reduce levels of Aβ have not been clearly elucidated. Here, we have shown that liquiritigenin can inhibit amyloidosis in a cell-free system in vitro, so it is possible that

the regulation of Aβ involves non-genomic cell signalling besides classic genomic pathways (Zhang et al., 2004), potentially involving the molecular structure of liquiritigenin itself. It is worth noting that liquiritigenin doesn't have a significant effect on amyloid A4, which is another form of APP, and it may indicate that liquiritigenin can regulate the abnormal cleavage of APP, to some extent, but that it does not influence the expression level of APP. Clearly, the underlying mechanisms of liquiritigenin should be explored further.

Nissl staining was used to assess whether liquiritigenin could attenuate the neuronal cell death observed in APP transgenic mice. We hypothesise that the positive results of this assay may be due to liquiritigenin treatment triggering neurogenesis, as we have previously observed that liquiritigenin can induce the differentiation of primary stem cells into neurons through the pathway of Notch in vitro (Liu et al., 2010b). Consistent with this, liquiritigenin inhibits Notch-2 expression both in vivo and in vitro in the present study. It has been shown that Notch signal activation can inhibit neuronal differentiation, whereas a glial progenitor derived from a stem cell differentiates into an astrocyte with the help of Notch signals (Louvi et al., 2006). Therefore, it is possible that when neurons are damaged, the Notch signal pathway may be inhibited with the help of liquiritigenin, and as a result, more endogenous stem cells in the brain may develop into neurons. Our finding that Notch-2 is increased in Tg2576 mice is supported by other researchers' observations that Notch-2 is upregulated in aged and cognitively impaired rats (Bowe et al., 2007), which indicates that elevated Notch-2 signalling may correlate with memory disorders and aging.

Recent research indicates that estradiol inhibited Notch activity in ERα-positive breast cancer cells, and the ER inhibitors tamoxifen and raloxifene, can block this effect by reactivating Notch, suggesting a model in which estrogen inhibits Notch via ERα (Rizzo et al., 2008). In the present paper, we propose that liquiritigenin may exert its inhibitory effect on Notch-2 through ERβ because liquiritigenin has a much higher transcriptional activation activity on ERβ than on ERα. However, as ICI 182 780 is an ER blocker on both ERα and ERβ, it is impossible to distinguish between these two possibilities in our culture system. We have begun to test our hypothesis with ERβ knockdowns, which are ongoing at the time of writing. In any case, we consider that this triggering of neurogenesis is of great importance, and may work as a universal, endogenous protective mechanism against neuronal insults in a damaged brain, because the newly generated neurons are not subject to immune rejection and may have a good chance of integration into the original neural circuits.

As an exploration of the possibility of AD treatment with selective estrogen receptor agonists, the present report shows that the ERβ agonist liquiritigenin has a promising future. Our data confirms the potential of selective estrogen receptor agonists, especially Neuro-selective estrogen receptor agonists, in protecting against AD neuropathology, and it supports continued development and investigation in this field. Interestingly, we found that liquiritigenin has effects on both male and female mice. This may be due to the fact that ERβ is less correlated with sex than is ERα (Shughrue et al., 1997 ; Gustafsson, 2003). However, more research is needed to confirm the effects of liquiritigenin. From a mechanistic standpoint, there is a significant need for targeted research in order to better elucidate the signalling involved in both estradiol and selective estrogen receptor agonists mediated activation of genomic and non-genomic kinase signalling pathways. Relative studies has shown recently that estradiol could work through any of several ERs, including the novel G-protein coupled receptor GPR30, to enhance the learning and memory ability in ovariectomised rats (Hammond et al., 2009). As such, and subsequently, in our gonadally

intact models, animals could be simultaneous treated with an antagonist of ER and examined as to whether the effects of liquiritigenin would be antagonised. Nevertheless, the following research should move this interesting field forward, and aim at answers to many of the key questions that remain.

5. Conclusion

In summary, as a newly found selective ERβ agonist, liquiritigenin could improve the behavioural performance of Tg mice and attenuated Aβ accumulation pathologies both in vivo and in vitro. The effects of liquiritigenin may be due to its inhibition of neuron loss and astrocytosis in the brain, which could be through the Notch signalling pathway. These findings provide evidence for the beneficial activities of the selective estrogen receptor agonist liquiritigenin in a mouse model of AD and support the continued investigation of selective estrogen receptor agonists as an alternative to estrogen-based treatment in reducing the risk of AD.

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16

Novel Therapeutic Strategies for Chagas` Disease

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1. Introduction

Chagas' disease, also called American trypanosomiasis, is one of the most neglected parasitic diseases in the world. An estimated 10 million people are infected worldwide, mostly in Latin America where Chagas disease is endemic. More than 25 million people are at risk of the disease. It is estimated that in 2008 Chagas disease killed more than 10,000 people. Its infectious agent is the protozoan parasite *Trypanosoma cruzi* with symptoms progressing from mild swelling to intestinal disease and ultimately heart failure.

Currently, 2 antiparasitic drugs are recommended for the treatment of chagasic patients: nifurtimox and benznidazole. However, the effectiveness of both varies according to (i) the phase of the disease (acute and early latent infection), (ii) different parasite isolates, (iii) period of treatment and dosage and (iv) age of patient. Also, their well-known toxicity and limited effect make the search for new drugs imperative. Many trypanocidal compounds have been screened in the past few decades and some promising targets have been reported since the introduction of nifurtimox and benznidazole (1960-1970).

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2. Nitro-heterocyclic derivatives as trypanocidal agents in the treatment against Chagas` disease

The history of chemotherapy for Chagas' disease can be divided into three phases: The first phase begins with the discovery of the disease in 1909 by the researcher Carlos Chagas and ends with the publication of "Manual of Tropical and Infectious Diseases" in 1935, at which point no major advances in the discovery of trypanocidal drugs had been made. In the second phase, between 1936 and 1960, numerous substances were empirically tested for the treatment of the disease, generating controversial results. The third phase, which started in 1961, was characterized by studies that clearly demonstrated the effectiveness of some compounds, e.g. nitrofurazone (1) (Figure 1), in experimental models of infection with T. cruzi in mice. The clinical trials showed that nitrofurazone (1) might be effective in the therapy for the disease but most patients were unable to tolerate the side effects at the doses and the time required for healing. However, the discovery of nitrofurazone (1) began a new era in the therapy of Chagas' disease (Coura & de Castro, 2002).

2.1 Nifurtimox and benznidazole

In the beginning of the 1970s, the discovery of the nitroheterocyclic derivatives nifurtimox (Lampit®, Bayer) (2) and benznidazole (Rochagan®, Roche) (3) brought new perspectives for the treatment of Chagas disease, due to their efficacy in the acute phase and tolerance (Bock et al., 1969; Richle, 1973).

Recently, the patent of benznidazole (3) was transferred to LAFEPE (the Pharmaceutical Laboratory of Pernambuco State, Brazil). Since the1980s, nifurtimox (2) sales were discontinued in Brazil and then in other Latin American countries.

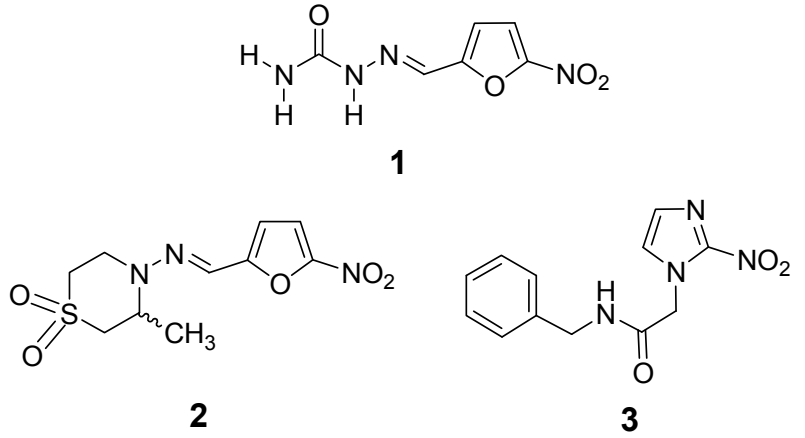


Fig. 1. Representation of chemical structures of nitrofurazone (**1**), nifurtimox (**2**) and benznidazole (**3**).

The adverse reactions associated with nifurtimox include digestive tract disturbances, such as lack of appetite, nausea, vomiting and weight loss and those induced by treatment with benznidazole (**3**) are dermo- and polyneuropathies. The main limitations of these drugs are the long-term administration and the severe side effects (Coura, 1996; Cansado, 1997).

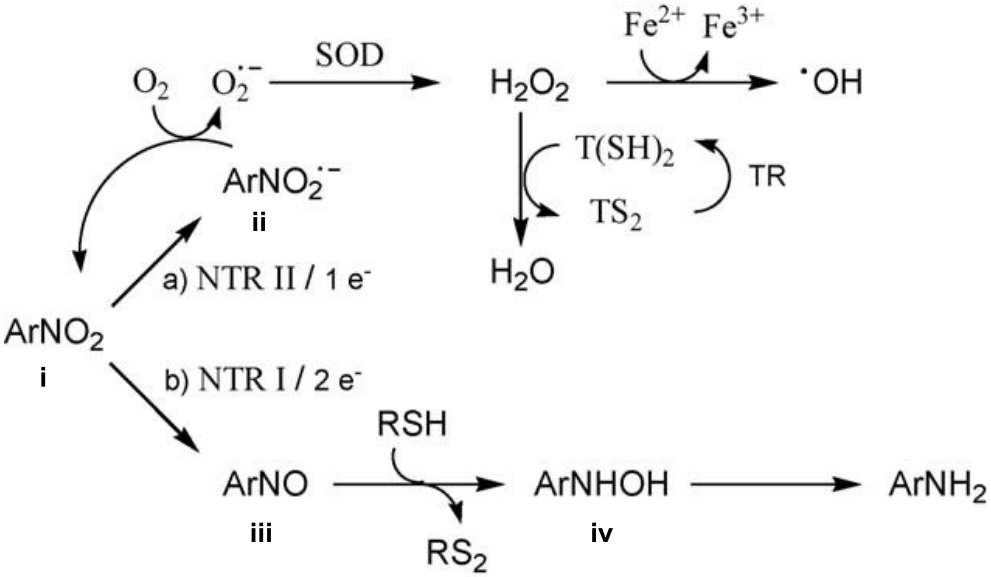
Additionally, another limitation lies in the fact that most cases of Chagas Disease are diagnosed in the chronic phase (de Castro, 1993), where these compounds have a reduced efficacy profile.

The mode of action of nifurtimox (**2**) is not yet fully elucidated despite being studied by many research groups,. The hypothesis that nifurtimox (**2**) acts through an oxidative stress has been based on the differences between the detoxification mechanisms of trypanosomatids and humans. The trypanosomatids have low activity of superoxide dismutase, absence of catalase and glutathione peroxidase (except for *Crithidia fasciculata*) (Turrens, 2004, Wilkinson & Kelly, 2003), and further explore a detoxification system based on the redox cycle of trypanothione (Fairlamb *et al.* 1985; Krauth-Siegel *et al.*, 2007), a system similar to that of glutathione. For these reasons the defence system of the parasite against reactive oxygen species (ROS) has been deficient, when compared to the host (Krauth-Siegel *et al.*, 2003; Turrens, 2004).

Scheme 1 shows the two possible routes proposed for the reductive process and bioactivation of nitro-heterocyclic derivatives with a general structure (**i**). Currently the most likely mode of action is one in which nifurtimox (**2**) exerts its biological activity through bioreduction of the nitro group (Maya *et al.*, 2007; Docampo, 1990). This process begins with the reduction of the nitro group to the nitro-anion radical (**ii**) in a reaction catalyzed by NADPH / NADH-nitroredutase (Maya *et al.*, 2007). Under aerobic conditions, the nitro-anion radical (**ii**) reacts with oxygen regenerating (**i**) and forming the superoxide anion, which is transformed into hydrogen peroxide by reacting with superoxide dismutase (SOD). The accumulation of H_2O_2 in the presence of iron can generate the hydroxyl radical via the Haber-Weiss reaction (Haber & Weiss, 1932; Koppenol, 2002). The accumulation of these ROS would lead to a possible oxidative stress in the parasite, which an impaired detoxification mechanism (Docampo & Moreno 1984; Cadenas, 1989). The hypothesis that the trypanocidal activity of nifurtimox (**2**) depends on the aerobic reductive cycle is based on various evidences, such as: (a) treatment of *T. cruzi* Tulahuen strains with **2** resulted in increased consumption of O_2, H_2O_2 production and the release of a superoxide ion (Nuñez-Vergara *et al.*, 1997; Maya *et al.*, 2007; Giulivi *et al.*, 1998). Additionally, after the use of different concentrations of **2**, it was also detected the presence of nitro-anion radical (**ii**) able to promote the anti-proliferative effect against the parasite, in the same range of serum concentrations achieved in adults after a single dose of 15 mg/kg (Docampo & Stoppani, 1979). The presence of nitro-anion radical (**ii**) was also observed by electron-spin resonance spectroscopy (ESR) (Nuñez-Vergara *et al.*, 1997) and (b), there was a reduction of essential thiols (mainly glutathione and trypanothione) in the parasite after treatment with nifurtimox (**2**) (Maya *et al.*, 1997).

Although the formation of ROS by nifurtimox (**2**) could be involved in its anti-*T. cruzi* activity, this does not seem to be the main mode of action. A study carried out by Wilkinson *et al.* (2000) provided evidence that a cell line over-expressing peroxidases was also likely to be vulnerable to oxygen metabolites generated by nifurtimox (**2**) that the typical lineage, suggesting that the trypanocidal activity of nifurtimox (**2**) is not predominantly mediated by ROS damage. One study with *C. fasciculate*, showed that organisms with low levels of the enzyme trypanosomatid catalase maintain their sensitivity to nifurtimox (**2**). These results contradict the hypothesis that the action of nifurtimox (**2**) involves the accumulation of hydrogen peroxide due to the absence of catalase (Gutteridge *et al.*, 1982). Moreover, recently, five peroxidases were identified in *T. cruzi*: two tryparedoxin peroxidases (Wilkinson *et al.*, 2000; Piacenza *et al.*, 2008; Trujillo *et al.*, 2004), two glutathione-dependent

peroxidases (Wilkinson *et al.* 2002a; Wilkinson *et al.*, 2002b), and one ascorbate-dependent heme peroxidase (Wilkinson *et al.*, 2002c) showing, in contrast to previous studies that the parasite has a complex and effective system to deal with oxidative stress.



Scheme 1. Two possible routes for the metabolic reduction of the nitro group in nitro-heteroaromatic derivatives (**i**). The aerobic route (a) and the anaerobic route (b). NTR = nitroreductase; SOD = superoxide dismutase; TSH = trypanothione; TR = trypanothione reductase. Adapted from Chauviere *et al.*, 2003.

Another likely possibility for the mode of action of nifurtimox (2) considers that the metabolic reduction of the nitro group is a process mediated by two electrons that result in the formation of the nitroso intermediate (**iii**) and then the hydroxylamine derivative (**iv**) (Kedderis & Miwa, 1988; Morello, 1988; Viodé *et al.*, 1999) (Scheme 1). Recently, Wilkinson et al. (2008) identified a nitroreductase (NTR) responsible for activation of nifurtimox (**2**) and benznidazole (**3**) in *T. cruzi* and *T. brucei* strains. This NTR of subtype I works through a sequence of two-electron reductions of species (**i**) by using NADH as a cofactor. Decreased activity of this NTR confers resistance to nitro heteroaromatic drugs, demonstrating its role in the metabolic activation of these compounds in trypanosomatids through the formation of nitroso species (**iii**) and hydroxylamine (**iv**), rather than the nitro-anion (**ii**). Additionally, it is known that nitroso compounds are electrophilic and promote the reduction of thiol levels, which can also be related to the mechanism of action of some anti-*T. cruzi* agents (Kedderis & Miwa, 1988; Viodé et al., 1999).

The oxidative stress was discarded as the main mode of anti-*T. cruzi* action of benznidazole (**3**), since at the concentrations where the drug has trypanocidal activity there was no production of superoxide ion and H_2O_2. Only at concentrations much higher than those needed to affect the parasite, *i.e.* 10 mM, was the formation of the corresponding nitro-anion radical detected (Moreno *et al.*, 1982). Some authors believe that benznidazole (**3**) is reduced

by the anaerobic route (b) and that its mode of action on *T. cruzi* may involve a direct effect on the biosynthesis of macromolecules by covalent or other interactions between its nitroreduction intermediates (Polak & Richle, 1978; Goijman *et al.*, 1985; Docampo & Moreno, 1986, Diaz de Toranzo *et al.*, 1988). Diaz de Toranzo and colleagues (1988) described the binding of benznidazole (**3**) to DNA, lipids and proteins from the epimastigote forms of *T. cruzi*. Also nifurtimox (**2**) and benznidazole (**3**) inhibited the synthesis of *T. cruzi* nucleic acids and proteins (Goijman & Stoppani, 1985; Goijman *et al.*, 1985; Gonzalez & Cazzulo, 1989).
Nifurtimox (**2**) and benznidazole (**3**) also act on the replication, transcription and translation processes in *T. cruzi* strains, inhibiting the biosynthesis of DNA, RNA and proteins respectively (Goijman & Stoppani, 1985; Goijman *et al.*, 1985).

2.2 Megazol

Few synthetic compounds have the same remarkable curative effects for the treatment of Chagas disease as megazol [2-amine-5-(1-methyl-5-nitro-2-imidazol-2-yl) 1,3,4 thiadiazole] (**4**) (Berkelhammer & Asato, 1968), a 1,3,4-thiadiazole nitroimidazole derivative that in experiments with mice infected with Colombian and ϒ *T. cruzi* strains showed higher rates of cure compared to the standard treatment using nitrofurazone (**1**), nifurtimox (**2**) and benznidazole (**3**) (Table 1) (Filardi & Brener, 1982).

T. cruzi strain	**Compounds**	**Dose (mg.kg^{-1})**	**Number of Doses**	**No. cured/ No. treated**	**% cured**
Y	Megazol (4)	25	20	9/18	50.0
		50	20	19/19	100.0
		100	20	17/17	100.0
		50$^{\blacklozenge}$	20	17/20	85.0
		500^{T}	1	8/9	88.8
	Nitrofurazone (1)	100	20	5/18	27.7
	Benznidazole (3)	100	20	5/17	29.4
	Nifurtimox (2)	25	20	1/17	5.8
		500^{T}	1	0/10	0.0
Colombiana	**Megazol (4)**	50	20	13/15	85.6
		100	20	14/14	100.0
	Nifurtimox (2)	50	20	0/15	0.0

$^{\blacklozenge}$Treatment began 5 days after inoculation, when the parasitemia was apparent. / ^{T}single dose given in the day after inoculation.

Table 1. Percentage of cured mice inoculated with *T. cruzi* strains treated with nitrofurazone (**1**), nifurtimox (**2**), benznidazole (**3**) and megazol (**4**) after oral administration.

Chauvière and co-workers (2003), who synthesized a series of megazol analogs with several structural modifications, studied the structure-activity related to their antiprotozoal profile. These new derivatives were tested against *T. cruzi*, *Trypanosoma brucei* (*T. brucei*) and *Leishmania infantum* (*L. infantum*), and their activities were compared with those exhibited by specific standard drugs, *e.g.* sodium stibogluconate (**5**) for Leishmania, suramin (**6**) for *T. brucei* and nifurtimox (**1**) for *T. cruzi* (Figure 2).

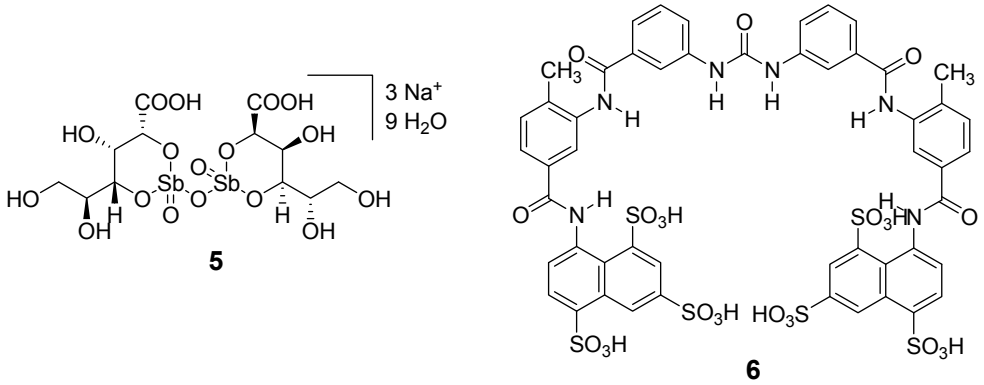


Fig. 2. Chemical structures of sodium stibogluconate (5) and suramin (6).

All derivatives tested by Chauvière and colleagues (2003) produced a decrease in trypanocidal activity; however the molecular architecture of the prototype megazol (**4**) is the one that presents the best bioactivity profile against *T. brucei*, *T. cruzi*, *L. infantum* and *L. donovani*, without cytotoxicity to macrophage cells.

Little is known about the metabolism of megazol, and despite its nitro-heterocyclic nature, some authors disagree on the metabolic reduction of the nitro group by nitroreductases as being critical for its trypanocidal activity. The biochemical studies performed by Tsuhako *et al.* (1989), with megazol (**6**) in the presence of NAD(P)H/cellular fractions of *T. cruzi* (*Y* strain) or in the presence of NAD(P)H/rat liver microsomes (Figure 3) were not able to show the presence of it corresponding nitro-anion radical (Figure 3, B).

The ineffectiveness of the rat liver microsomes in reducing megazol (**4**) was confirmed by experiments showing the slow disappearance of the band corresponding to the nitro chromophore measured by absorption spectroscopy of visible light (Figure 4). Although *T. cruzi* nitro-reductases are not well characterized (Marr and Docampo, 1986, Kuwahara *et al.*, 1984, Henderson *et al.*, 1988), the results (Figure 3) show that NADPH: cytochrome P450 reductase (Em = -0.328 mV) (McLane *et al.*, 1983) is also inefficient in promoting the reduction of the nitro group present in megazol (**4**) to the corresponding nitro-anion radical (Figure 3, B).

Under these experimental conditions, the nitro-anion radical of megazol (**4**) was not detected, while the corresponding radicals of nifurtimox (**2**) and benznidazole (**3**) (Figure 3) were clearly detected. However, in the presence of NADPH and ferredoxin: $NADP^+$ oxirredutase (E_m = -0.442 mV) (Batie & Kamin, 1981), the megazol nitro-anion radical was easily detected by electron-spin resonance spectroscopy (ESR) directly under anaerobic conditions (Figure 5A), and was well marked by computer simulation (Figure 5C) (Rao *et al.*, 1987). Corroborating these data, Tsuhako *et al.*, (1989) demonstrated that the metabolic

reduction of megazol (**4**) generates the nitro-anion radical (B), but requires enzymes with low reductive potential, suggesting that the trypanocidal activity of megazol (**4**) is not related to the metabolic bio-reductive process, differing from the mode of action of nifurtimox (**2**) and benznidazole (**3**).

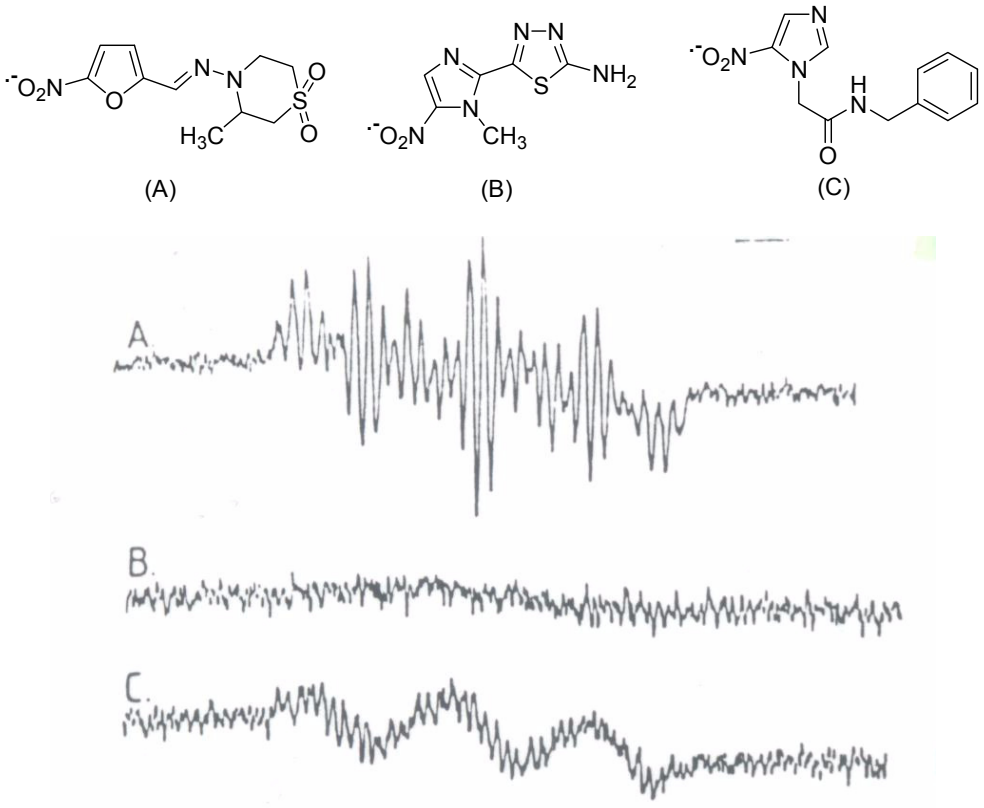


Fig. 3. ESR spectrum obtained during anaerobic incubation of rat liver microsomes with different nitro-heterocyclic compounds. The reaction mixtures in phosphate buffer (20 mM), pH 7.4, containing rat liver microsomes (2 mg protein /mL), NADPH (5 mM), DTPA (1 mM), KCl (150), DMSO (10% v/v) and: (A) nifurtimox (5 mM) (B) megazol (10 mM), and (C) benznidazole (10 mM). Adapted from Tsuhako *et al.*, 1989.

Viodé and colleagues (1999) studied the interaction of megazol (**4**) with three different enzymes with distinct redox potential, and compared the results with those of nifurtimox (**2**) and metronidazole (**7**) (Figure 6). The results indicated that the reduction of megazol (**4**) and nifurtimox (**2**) by the enzyme cytochrome b_2 (E_m = -0.01 V) occurred at similar rates, however with the enzyme cytochrome P-450 reductase (E_m = -0.328 mV), the reaction of the nitrofuran derivative was faster by one order of magnitude (Table 2). The reduction of nifurtimox (**2**) with the ADR enzyme (adrenodoxin reductase) was also faster when compared to megazol (**4**). The Km values for these enzymes (Tshako *et al.*, 1989) as well as

other reductases (Table 2) indicate that the catalytic efficiency is correlated with the reduction potential of the corresponding nitro-heterocyclic derivatives.

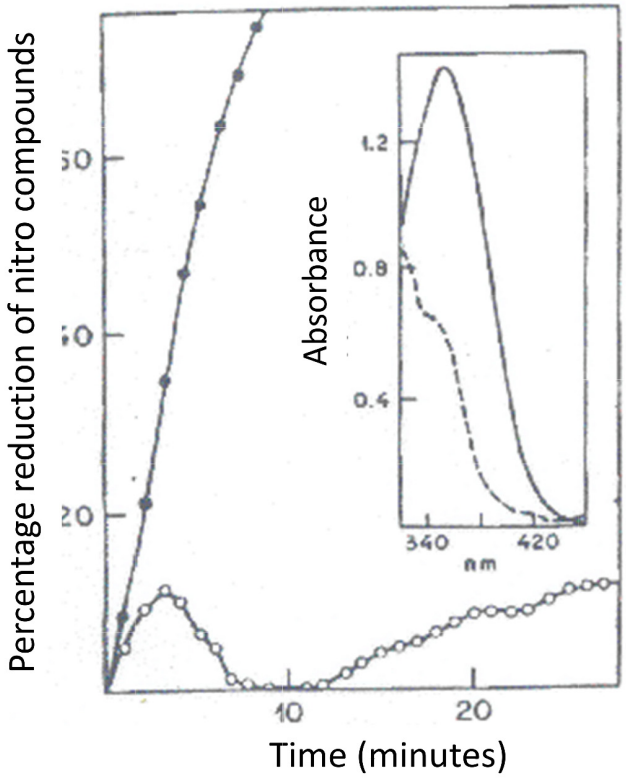


Fig. 4. Reduction of the nitro-heterocyclic compounds under anaerobic conditions by rat liver chromosomes. (•) nifurtimox (0.2 mM), and (o) megazol (0.2 mM). The graph shows the spectrum of visible light absorption of megazol (0.2 mM) in buffer (—) before and after (- - -) the addition of sodium dithionate (control).

The activities of ADR and cytochrome P-450 reductase represent the rate of NADPH oxidation at 25°C. The activity of L-lactate cytochrome *c* reductase was determined by measuring O_2 consumption. The k_{cat} values of the other enzymes were obtained from the coupled cytochrome *c* assays. ND = not determined.

The ESR experiments showed expressive enzymatic production of the nitro-anion radical of megazol (**4**), under anaerobic conditions, in comparison with nifurtimox (**2**), used as control (Figure 7).

The corresponding spectra are consistent with those published for the nitrofurane derivative (**2**) and those obtained for megazol (**4**) after reduction by ferredoxin: $NADP^+$ oxidoreductase (Tsuhako et al., 1989). The signal of ESR spectra obtained from the reduction of megazol (**4**), even at a lower intensity, under the same conditions, lasted longer than that of nifurtimox

(**2**). The half-lives determined under the same conditions were 10 and 3 minutes, respectively.

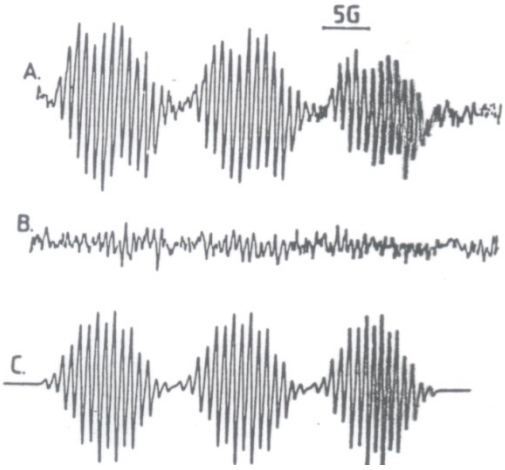


Fig. 5. ESR data obtained during the reduction of megazol. Megazol (1 mM) was incubated with ferredoxin: $NADP^+$ oxidoreductase and NADPH in phosphate buffer containing DTPA and DMSO under: (A) anaerobic conditions and (B) air ; (C) computer simulation of (A).

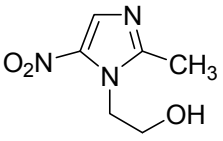


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Fig. 6. Chemical structure of the antiprotozoal agent metronidazole (**7**).

Nitro compound E^7_1 (mV) (Tshako *et al.*, 1989)	Nifurtimox - 260	Megazol - 438	Metronidazole - 485
Enzyme	K_{cat}/K_m $[M^{-1} . sec^{-1}]$		
L-lactase cit. c reductase	$2.8X10^3$	$4,7X10^3$	ND
ADR	$5.0X10^4$	$3,0X10^3$	$3,0X10^2$
Cit. P-450 redutase	$2.0X10^4$	$2,0X10^3$	ND
T. cruzi LipDH	$7.6X10^2$	$2,3X10^3$	ND
Pig heart LipDH	$3.0X10^2$	$4,3X10^3$	ND
T. cruzi TR	$1.5X10^3$	$1,8X10^3$	ND
Human GR	≤14	≤5	ND

Table 2. Enzymatic reduction of nitro-heterocyclic compounds by different reductases. Adapted from Viodé *et al.*, 1999.

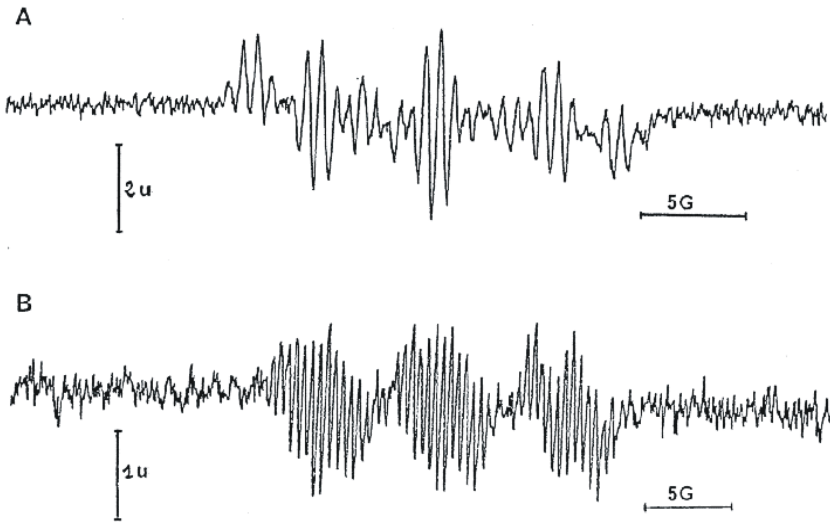


Fig. 7. The ESR spectrum of the nitro-anion radical obtained during the reduction of megazol (B) and nifurtimox (A) under anaerobic incubation at 37°C with microsomes, NADPH in phosphate buffer.

The generation of the nitro-anion radical by cytochrome P-450 reductase and rat liver microsomes also confirms that megazol (**4**) is significantly reduced, since the corresponding nitro-anion radical can be accumulated under anaerobic conditions.

The values of k_{cat}/K_m indicate that megazol (**4**) has a slower enzymatic reduction than the other two nitro-heterocyclic compounds (Table 2), whose high trypanocidal activities are not related to the first step of the reductive process.

Viodé and co-workers (1999) conclude that there is no doubt that megazol (**4**) can interfere with the oxygen metabolism of the parasite by inducing oxidative stress, but its greater potency compared to nifurtimox could be related to factors not yet known at this time.

De Castro and Meirelles (1990) studied the megazol mode of action by incorporating macromolecular precursors, *e.g.* leucine, uridine, and thymidine. Megazol (**4**) showed a potent effect on selective inhibition of protein biosynthesis, where benznidazole (**3**) and nifurtimox (**2**), even at high concentrations, caused no change in the incorporation pattern.

Maya *et al.* (2003) tested the hypothesis that nitroreduction metabolites of **4**, *e.g.* nitroso species (**iii**) (Scheme 1), act as electrophilic scavenging bionucleophilic species from the parasite. Consequently the effect of **4** on the amount of free thiols, *i.e.* $T(SH)_2$ and GSH, was investigated.

Megazol (4) caused a progressive decrease in the amount of thiol in the parasite in two hours, with a half life of 88 minutes for $T(SH)_2$ and greater than 200 minutes for GSH (Figure 8). It is an efficient scavenger of thiols, in contrast to the profile shown by nifurtimox (2).

However, the mechanism involved in the trypanocidal activity of **4** is strictly dependent on the bioformation of reactive oxygen species (Declerck *et al.*, 1986; Declerck *et al.*, 1987), produced by the reduction of its nitro group, which interacts with DNA producing mutagenicity (Ferreira & Ferreira, 1986; Nesslany *et al.*, 2004; Poli *et al.*, 2002). This undesirable profile limits the use of megazol (**4**) as a drug for Chagas' disease.

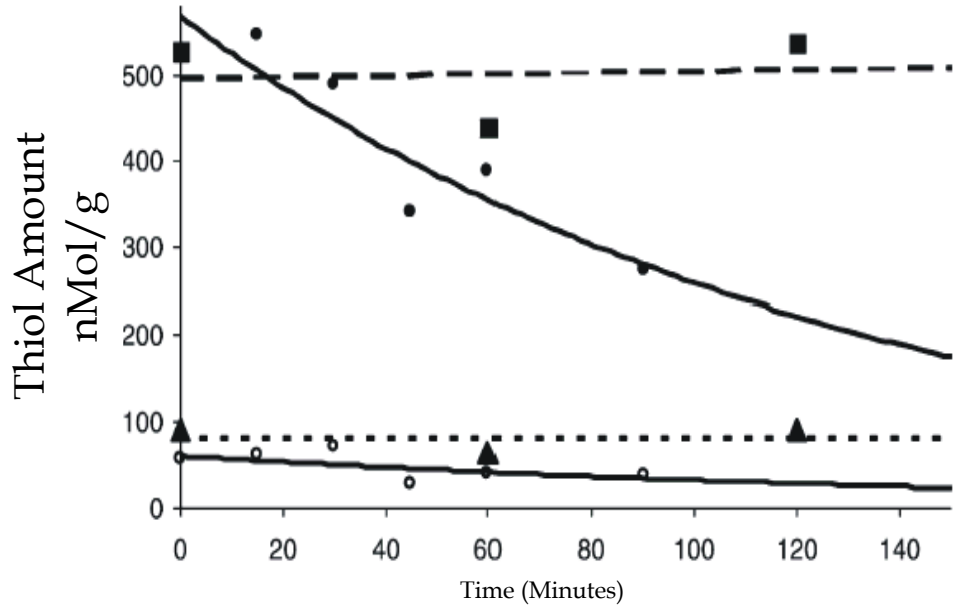


Fig. 8. Effect of megazol (**4**) on the amount of thiols in epimastigote forms of *T. cruzi*. The half-life of trypanothione is 88 min. Control -T $(SH)_2$ (■), T $(SH)_2$-megazol (•), GSH-control (▲), GSH-megazol (o).

2.3 Discovery of brazilizones A and N

Considering this background, Carvalho *et al.* (2004, 2008a) synthesized novel 1,3,4-thiadiazole derivatives structurally designed by exploiting the molecular hybridization of megazol (**4**) with the guanylhydrazone derivative (**8**), which proved to be active against trypomastigotes forms of *T. cruzi* with IC_{50}/24h = 17 μM (Messeder *et al.*, 1995). The molecular design explored the introduction of the pharmacophoric *N*-arylhydrazone subunit (A) from **8** (Figure 9) to the nitroimidazole derivative (**4**), in order to act as a radical scavenger (Mahy *et al.*, 1993; Prusis *et al.*, 2004) that would abolish the oxidative stress that induces the formation of toxic species resulting from the formation of reactive nitro-derivatives.

Among a series of substituted arylhydrazone derivatives tested as trypanocidal agents, the catechol derivative called brazilizona A (**9**) (Figure 9) showed remarkable activity (IC_{50} = 5.3 μM), being two fold more active than megazol (**4**) (IC_{50} = 9.9 μM) against blood trypomastigote forms of *T. cruzi* (Carvalho *et al.*, 2004). Parasites treated with **9** displayed an expansion of the flagellar membrane structure, dilation of the nuclear envelope, the formation of autophagosome-like structures, and cellular disorganization (Salomão *et al.*, 2010). The profile confirmed that the hydrazine framework introduced into this novel molecular pattern was successful to optimize the trypanocidal action of megazol (**4**), since the nitro imidazole group is kept. The corresponding phenyl analogue (**10**) (Figure 9) is twelve fold less active than **9** as a trypanocide agent, confirming the pharmacophoric behaviour of the nitro heterocyclic ring (Carvalho *et al.*, 2004).

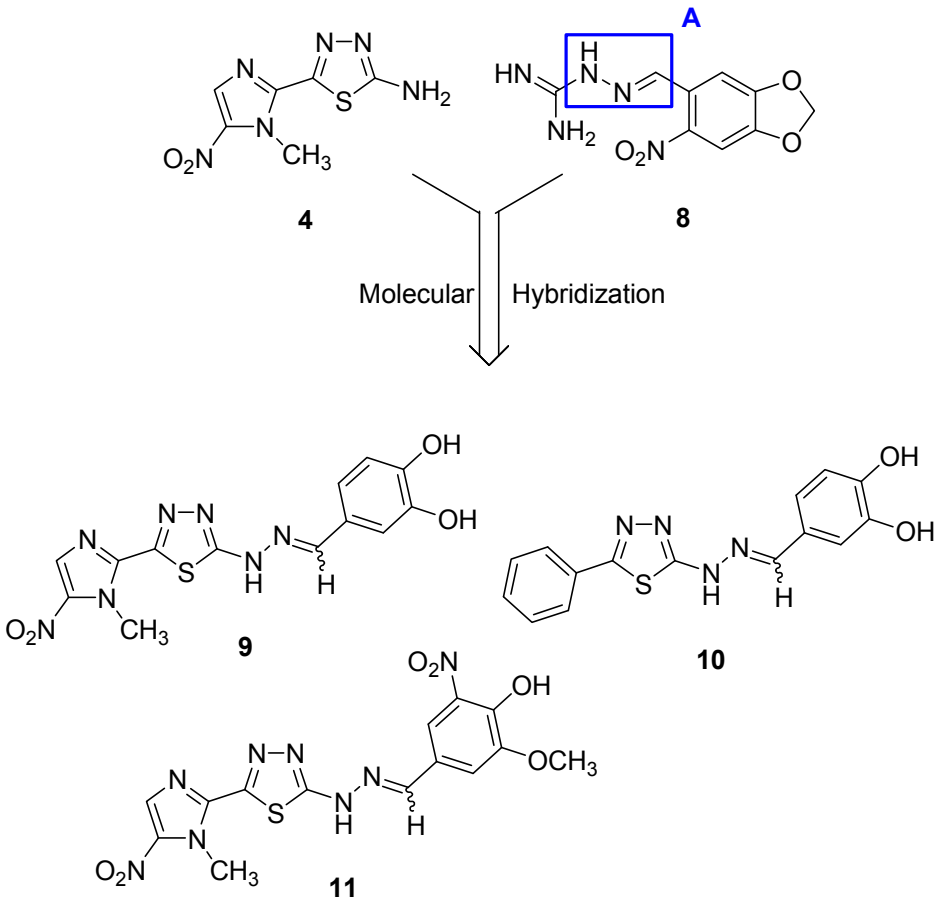


Fig. 9. Structural planning of the novel 1,3,4-thiadiazole-*N*-arylhydrazone derivatives Brazilizone A (**9**), (**10**) and Brazilizone N (**11**).

The construction of QSAR-3D models for these derivatives exploiting the CoMFA program allowed as to identify the brazilizone N (**11**), nitro vanillin analogue of **9** that also presented good trypanocide activity, *i.e.* IC_{50} = 19.0 μM) (Carvalho *et al.*, 2008).
Theoretical calculations have shown that brazilizone A (**9**) and brazilizone N (**11**) have good potential for absorption *in vivo* (Carvalho *et al.*, 2008). For an in vivo screening, compound **9** was administered to infected mice in a single dose of 200 mg/kg at the onset of parasitemia, and the body weights, levels of parasitemia, and mortality rates of the mice were monitored up to 40 days post-infection. There was no significant decrease in parasitemia or mortality in the two different regimens of treatment in vivo (Salomão *et al.*, 2010). The treatment of infected mice with **9** (200 mg/kg) did not interfere with the course of infection, with the values of body weight, parasitemia, and mortality being similar to those of the infected control group and 50% cumulative mortality occurring at 16 days post-infection (Salomão *et al.*, 2010). This behaviour is indicative of the necessity to optimize the pharmacokinetic

properties of brazilizone A (**9**) so that the use of this potent trypanocide agent as an anti-Chagasic drug can become viable.

3. Essential oils: State-of-the-art and Chagas' disease

Essential oils are very complex mixtures composed of natural volatile organic compounds characterized by a strong aroma. They are produced as secondary metabolites in plants, where the biosynthesis can be carried out in a constitutive way, independently of the action of pathogens (phytoanticipins) or induced as a defensive response of the plant against infection by bacteria, fungi and nematodes (phytoalexins). Indeed, they can be synthesized by different organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes (Bakkali et al., 2008; Jalali-Heravi & Parastar, 2011). In general, we can classify the effective components of essential oils into two main groups, volatile and nonvolatile fractions. The former group contains monoterpenes, sesquiterpenes and their oxygenated derivatives, aliphatic aldehydes, alcohols and esters. The volatile compounds are the main fraction of the oil. The non-volatile fraction is mainly composed of hydrocarbons, fatty acids, sterols, carotenoids, waxes, coumarins, psoralens and flavonoids (Jalali-Heravi and Parastar, 2011). In fact, the chemical composition of essential oils may vary according to many factors such as (i) the difference between plant species and varieties, (ii) different climates, (iii) seasonal and geographical conditions and (iv) harvest periods. In addition, different parts of the same plant can provide varying compositions of essential oils. So, in order to obtain essential oils of constant composition, they have to be extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been picked in the same season.

Plant essential oils are extracted from various aromatic plants generally localized in temperate to warm countries like Mediterranean and tropical countries. They are usually obtained by steam or hydro-distillation and analyzed by gas-chromatography mass spectrometry. In fact, there are other methods for extracting essential oils. These may include use of liquid carbon dioxide or microwaves, as well as low or high pressure distillation with boiling water or hot steam. Depending on the type of extraction the chemical profile of the essential oils could also differ not only in the number of molecules but also in the stereochemical type of molecules extracted (Masotti et al. 2003; Angioni et al., 2006).

In nature, essential oils play an important role in protecting plants against numerous organisms of bacterial, fungal and viral origin. Sometimes these oils can have a double function; on one hand they can attract some insects to ensure pollination and seed dispersal, and on the other hand they can repel predators. These valuable natural products can be used in many fields based on their properties already observed in nature, i.e. for their antimicrobial and repellent activities. The ethnopharmacological approach, which is used as a strategy to investigate essential oils, combines information acquired from users of medicinal plants (traditional communities and experts), with chemical and pharmacological studies. Brazil has a rich cultural and biological diversity and is also home to 55,000 species of higher plants as well as almost 7% of the global animal diversity (Garcia et al., 2010). Despite the large Brazilian biodiversity and the extensive work of researchers in the search for new chemotherapeutic agents against Chagas' disease, only one study is related to the

essential oil of a native species, *Croton cajucara*. Other studies are being carried out with exotic species, commonly used as spices and medicinal plants.

Today there are approximately 3,000 known essential oils, 10% of which are commercially used for the perfume, cosmetic, food and pharmaceutical industries. From a pharmaceutical point of view, many herbs and related essential oils have revealed medicinal properties including anticancer, antibacterial, antiviral, antitoxigenic and antiparasitic activities (Bakkali et al., 2008; Alviano & Alviano, 2009). Indeed, the complex composition of essential oils makes them both a promising and challenging source of drug candidates. Essential oils may contain up to 100 individual components at completely different concentrations, many of them with one or more biological activitie. Structures of natural products feature wide chemical diversity, biochemistry specificity and other molecular properties that make them favourable as lead structures in drug discovery. Therefore, besides the rational drug design and novel synthetic efforts, natural products, such as essential oils, are still undergoing investigation for novel chemical structures that may interact with known and unknown microbial targets.

With the discovery of Chagas' disease by Carlos Chagas in 1909, a large number of drugs with trypanocidal activity have been evaluated by several research groups, but all without therapeutic success. So since the late 1960s nifurtimox (4 [(5-nitrofurfurilideno) amino]-3-metiltiomorfolino-1,1-dioxide), a nitrofuran derivative, and benznidazole (Nbenzil-2-nitroimidazole-1-acetamide), a nitroimidazole derivative have been used to treat Chagas' disease. The healing potential of nifurtimox and benznidazole varies depending on the stage of the disease, treatment period and dosage, and patient age. Generally, satisfactory results are achieved when the patient is treated in the acute phase, in recent chronic infection, in congenital infection and in laboratory accidents. The main limitation of these compounds is the low efficacy in the treatment of patients in the chronic phase (Coura & Castro, 2002; Urbina, 2002). The reasons for the difference in the effectiveness of treatment between the acute and chronic stages of the disease are not known. The major disadvantage in the treatment of Chagas' disease is the development of resistance to the chemotherapeutic drugs currently being used. Due to their high toxicity, these drugs are administered at low doses, and thus resistance may appear (Coura & Castro, 2002). In addition, the high cost of treatment and the consequent abandonment of it, along with the permanence of infected people in endemic areas have contributed to parasite resistance which causes a major impact on controlling this disease.

The treatment with nifurtimox can lead to side effects such as psychological disorders, anorexia, somnolence and gastrointestinal symptoms (nausea, vomiting and diarrhea). The recommended doses for benznidazole in the treatment of Chagas' disease may cause hypersensitivity and dermatitis with skin rashes and generate neuropathologies such as paresthesia and peripheral nerve polyneuritis (Maya et al., 2007). However, more serious reactions to benznidazole include bone marrow depression, which can lead to thrombocytopenia purpurea, and agranulocytosis. While thrombocytopenia purpurea can cause hemorrhagic manifestations, agranulocytosis may lead the patient to septicaemia. Because of these characteristic side effects, nifurtimox and benznidazole should not be prescribed to pregnant women and elderly patients or patients with any disease associated with severe Chagas' disease such as heart and respiratory diseases, and in cases of renal or hepatic impairment, systemic infection or malignancies (Coura & Castro, 2002). Since the inefficacy of current chemotherapies has grown, mainstream medicine is increasingly receptive to the use of antimicrobials and other drugs derived from plants (Cowan, 1999).

However, the full acceptance of phytopharmaceuticals and the integration of phytotherapy into the concept of classical medicine will arise only if they meet the same criteria of quality as synthetic pharmaceuticals and if they are submitted to the major pharmacological and toxicological assays for standardization.

The long association between the coexistence of parasites, humans and herbal remedies, has made plants an undeniable source for drug candidates particularly for infectious diseases. Most research efforts into the effects of plants on parasite infections has been undertaken using aqueous or alcoholic extractions; however purified plant essential oils could also be efficacious in treating or preventing parasitic diseases (Anthony et al., 2005). So far, however, most of the studies concerning the biological effects of essential oils have been focused on the bactericidal effects. Only a few studies have addressed the effect of essential oils and their components against trypanosomes including *Trypanosoma cruzi*.

3.1 Essential oils: Characteristics as active agents

Properties such as low density and rapid diffusion across cell membranes (owing to their lipid solubility) can enhance the targeting of active components within an oil to intracellular parasites (Alviano & Alviano, 2009). Table 3 lists some of the essential oils of plant species commonly used in food and folk medicine and whose functional group of their main component presents activity against *T. cruzi*.

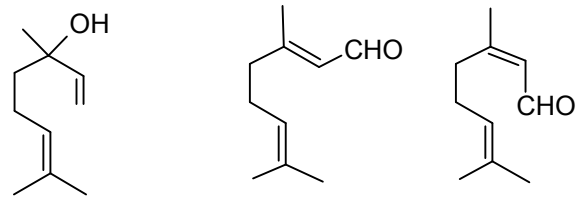
Botanical name	Common name	Part used	Main or active component / Main functional group	Reference
Allium sativa	garlic	garlic cloves	ajoene / disulfide bond	Urbina et al., 1993
Chenopodium ambrosioides	mexican tea	leaves	(−)-(1S,4S)-p-mentha- 2,8-dien-1-hydroperoxide / peroxide	Kiuchi et al., 2002
Laurus nobilis	bay	leaves	(1R,4S)-1-hydroperoxy-p-menth-2-en-8-ol / peroxide	Uchiyama et al., 2002
Cymbopogon citratus	lemongrass	leaves	citral / aldehyde	Santoro et al., 2007a Cardoso & Soares, 2010
Achillea millefolium	yarrow	leaves and flowers	chamazulene / C-C double bonds	Santoro et al., 2007b
Syzygium aromaticum	clove	flowers	eugenol / phenol	
Ocimum basilicum	basil	leaves	linalool / alcohol	
Origanum vulgare	oregano	leaves	3-cyclohe n-1-ol / alcohol	Santoro et al., 2007c
Thymus vulgaris	thyme	leaves	thymol / phenol	
Croton cajucara	white sacaca	leaves	linalool / alcohol	Rodrigues, 2010

Table 3. Plant essential oils and main constituents with trypanocidal activity against *Trypanosoma cruzi*.

3.2 Essential oils: Mode of action against *Trypanosoma cruzi*

Most studies of new agents with anti-protozoal activity are based on the direct action of these drugs on parasites and their immunomodulatory effects, particularly on macrophage responses. In fact, promising results have been obtained by some groups in the search of essential oils and related compounds with activity against pathogenic microorganisms, including *T. cruzi*.

Some plant essential oils have immunomodulatory effects that are useful for treating infectious diseases, particularly in cases where the oil has no direct adverse effect on the host. Linalool-rich essential oils extracted from many plants are reported as antimicrobials for bacteria, fungi and protozoal species (Rosa et al., 2003; Alviano et al., 2005; de Almeida et al., 2007). In fact, the effects of linalool-rich essential oil from *Croton cajucara* (white sacaca) on *Leishmania amazonensis* have been successfully investigated. The median lethal doses and absolute lethal doses of the essential oil and purified linalool (Figure 10) from *C. cajucara* for promastigotes and amastigotes are very low (Rosa et al., 2003). The same essential oil is active against *T. cruzi* infection, promoting parasite damages such as mitochondrial swelling and important alterations in the organization of the nuclear and kinetoplast chromatin. Along these lines, the essential oil from *C. cajucara* could be a useful source of novel drugs. *T. cruzi* is also susceptible to the action of the *Cymbopogom citratus* essential oil (Santoro et al., 2007a; Cardoso & Soares, 2010). The essential oil is effective in killing *T. cruzi* with a low 50% inhibitory concentration (IC_{50}) at 15 µg/mL for bloodstream trypomastigotes. Furthermore, this essential oil also inhibits epimastigote growth at low concentrations, inducing ultrastructural alterations on parasite morphology. The alterations caused by *C. citratus* can be observed in Figure 11. In addition, the treatment of *T. cruzi* with citral (Figure 10), the main constituent of *C. citratus* essential oil, results in epimastigote growth inhibition and decrease of viable trypomastigotes at 42 and 14.2 µg/mL, respectively, showing its high microbicidal activity (Santoro et al., 2007a). This monoterpene also exerts inhibitory effects on *T. cruzi* metacyclogenesis (Cardoso & Soares, 2010). Although no effect is observed at concentrations lower than 20 µg/mL, metacyclogenesis is almost totally abolished at 40 µg/mL after 24, 48 or 72 h of incubation in TAU3AAG (differentiation) medium. Higher concentrations (60, 80 or 100 µg/mL) induce 100% cell lysis.



Linalool **Citral**

Fig. 10. Linalool and citral structures.

The essential oils of *C. cajucara* and *C. citratus* have no toxicity for macrophages, the main cells involved in the early steps of infection. In fact, the number of internalized parasites (amastigote forms of *T. cruzi*) drastically decreases when infected macrophages are treated with the essential oil of *C. citratus*. Interestingly, the *C. cajucara* can not only reduce the number

of internalized parasites such as *L. amazonensis* and *T. cruzi* amastigotes in infected macrophages, but it can also promote macrophage activation with a consequent production of nitric oxide. However, how these oils cause macrophage activation is still poorly understood.

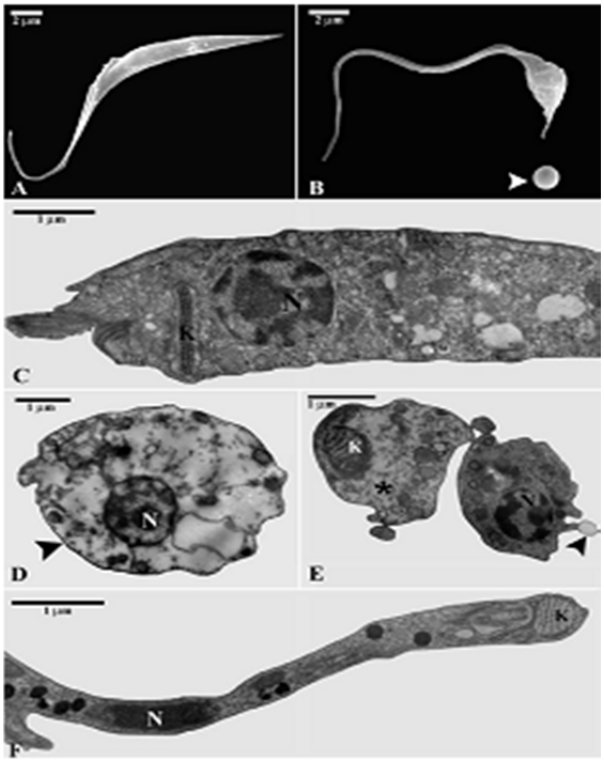
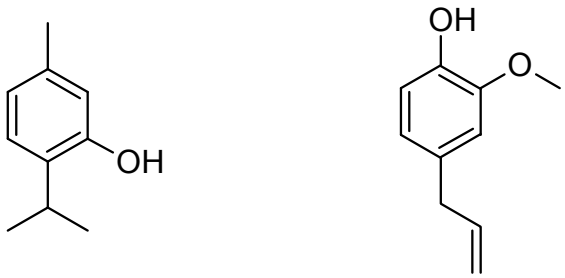


Fig. 11. Effect of lemongrass essential oil on epimastigotes and bloodstream trypomastigotes, as observed by scanning (SEM) or transmission (TEM) electron microscopy, after treatment with IC_{50}/24 h. (A) Untreated, control parasite observed by SEM. (B) SEM of lemongrass-treated epimastigote after incubation with 126.5 µg/mL essential oil. Note the rounding of the parasite body and a small vesicle (arrowhead) that appears to have been detached from the parasite plasma membrane. (C) Untreated epimastigote showing normal organelles by TEM. (D) Treated epimastigote, showing cytoplasmic extraction. The plasma membrane and the subpellicular microtubules remain unaltered (arrowhead). (E) Bloodstream trypomastigotes treated with IC_{50} (15.5 µg/mL) of lemongrass essential oil as observed by TEM, showing intense cytoplasmic extraction (asterisk) and formation of a membrane bleb (arrowhead). (F) Control, untreated bloodstream trypomastigote showing typical organelles. **K**, kinetoplast; **N**, nucleus (Santoro et al., 2007). Image reproduced with permission from Memórias do Instituto Oswaldo Cruz editor in chief.

The trypanocidal activity of the essential oils of *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme) demonstrated that epimastigotes treated with different concentrations of these essential oils resulted in growth inhibition and dose-dependent inhibitory

concentration. Trypomastigotes proved to be more susceptible to the action of the essential oils and especially to the action of thymol (Figure 12), the active phenolic component of the thymus essential oil with 50% inhibitory concentration, approximately 60 µg/mL. Different pathways may account for these variations in sensitivity to the oils evaluated, given that epimastigotes and trypomastigotes are adapted to survive in different environmental conditions (Santoro et al., 2007c).

Trypomastigotes treated with *Syzygium aromaticum* (clove) essential oil demonstrated swelling of the parasite body, cytoplasmic extraction and nuclear alterations, whereas the plasma membrane remained unchanged. Similar observations were made in parasites treated with essential oils *Ocimum basilicum* (basil) and *Achillea millefolium* (yarrow) (Santoro et al., 2007b). The phenolic compound eugenol (Figure 12) is the major component of *S. aromaticum* essential oil, but it is also an important chemical constituent of essential oils from many aromatic plants, such as *Dicipelium cariophyllatum*, *Pimenta dioica*, *Croton zehntneri* var. eugenoliferum, and *C. zehntneri* (De Vincenzi et al., 2000). However, incubation of *T. cruzi* with eugenol alone results in a less potent activity with 50% inhibitory concentration values equal to 246 µg/mL for epimastigotes and 76µg/mL for trypomastigotes (Santoro et al., 2007b). The contrast of results obtained with essential oils of *S. aromaticum* and eugenol in the experiments carried out with *T. cruzi* could be explained by synergistic effects of different compounds of the plant essential oil used in this work. The synergistic phenomenon is well known in several other systems (Zee-Cheng, 1992; Alviano & Alviano, 2009). Additionally, comparing the effectiveness of the phenolic compounds, eugenol and thymol, the increased availability of the phenolic group in the latter may be the reason for its higher activity, reinforcing the importance of this group for its mode of action (Ultee et al., 2002).

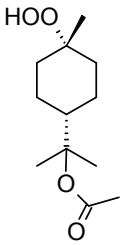


Thymol **Eugenol**

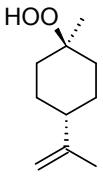
Fig. 12. Thymol and eugenol structures

Many essential oils are known to exert antimicrobial activity but the mode of action is often not entirely understood. The large number of constituents in essential oils makes it possible to have more than one cellular target. Perhaps this could explain why some essential oils present higher activity than their isolated compounds. However, some characteristics of essential oils can help us to understand their efficacy. First, the lipophilic constituents from essential oils may be involved in antimicrobial mechanisms by permeating cell membranes and disrupting the structure of the different layers of membrane polysaccharides, fatty acids

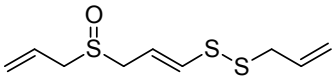
and phospholipids, which permeabilize the membranes (Bakkali et al., 2008). The membrane permeabilization leads to the leakage of macromolecules and lysis. Second, essential oil metabolic targets could lead to serious cellular damages. Alterations such as mitochondrial damage, with membranous arrangements within this organelle and in the flagellar pocket, and the formation of autophagic structures observed in some parasites are associated with depletion of ergosterol and the alteration of the physical properties of the membranes (Santa-Rita et al., 2004). Also, the alcohol sesquiterpene nerolidol possessess inhibitory activity under biosynthesis of isoprenoids (dolichol, ergosterol and ubiquinone) from *Leishmania* species (Arruda et al., 2005). In this context, the alcohol sesquiterpene linalool could exert the same activity based on ultrastructure alterations cited previously and the same functional groups. Parasites of the genera *Leishmania* and *Trypanosoma* have a strict requirement for specific endogenous sterols (ergosterol and analogs) for survival and growth and can not use the cholesterol supply present in their vertebrate hosts. Another example of metabolism inhibition is the growth inhibition of *T. cruzi* epimastigote caused by ajoene (Figure 13), a derivative of garlic that can be attributed to changes in the phospholipid composition of the parasite membrane. One of the evidences for these alterations is the low levels of phosphatidylcholine detected, followed by high levels of its immediate precursor, phosphatidylethanolamine, suggesting that ajoene inhibits the final stage of phosphatidylcholine biosynthesis, altering the phospholipid composition of the cell membrane (Urbina et al., 1993).



(1R, 4S)-1-hydroperoxy-p-menth-2-en-8-ol acetate



(-)-(1S, 4S)-p-mentha-2,8-dien-1-hydroperoxide



Ajoene

Fig. 13. Ajoene, (1R,4S)-1-hydroperoxy-p-menth-2-en-8-ol acetate and (−)-(1S,4S)-p-mentha-2,8-dien-1-hydroperoxide structures.

The peroxide group present in some monoterpenes is important for the activity of these compounds. The transformation of peroxide into the hydroxyl group ends the activity of the substances. For example (1R,4S)-1-hydroperoxy-p-menth-2-en-8-ol acetate isolated from leaves of *Laurus nobilis* showed minimum lethal concentrations at 1.4 µM against

epimastigotes of *T. cruzi* (Uchiyama et al., 2002). Other similar substances isolated from *Chenopodium ambrosioides*, ascaridole and related monoterpenes (−)-(2S,4S)- and (−)-(2R,4S)-p-mentha-1(7),8-dien-2-hydroperoxide, (−)-(1R,4S)- and (−)-(1S,4S)-p-mentha- 2,8-dien-1-hydroperoxide and (−)-(1R,4S)-hydroperoxy-p-menth-2-en-8-ol acetate (Figure 13) were active against epimastigotes at concentrations of 23, 1.2, 1.6, 3.1, 0.8 and 1.4 μM, respectively (Kiuchi et al., 2002). The mode of action of these monoterpene peroxides is associated to reactive oxygen species derived from the hydroperoxy group.

Only a few studies evaluating the activity of essential oils on *T. cruzi* have been presented so far, however many of them with promising results. Besides the features described here, it should be noted that up to the present no resistance or adaptation by parasites has been reported in the literature. This may be due to multiple targets that essential oils can achieve, disabling many of the parasite modes of resistance. Much more can be done along these lines to discover new and effective therapies for the control and treatment of Chagas' disease.

4. Cysteine peptidase: A target for drug development

4.1 Peptidases

Peptidases, also known as proteases or proteinases, are hydrolases able to hydrolyze peptide bonds in proteins or peptides. They act as processing enzymes taking part in regulatory or catabolic processes in the cell as extracellular enzymes and play an important role in the degradation of proteic substrates serving as carbon or energy sources. Peptidases are important in several pathological conditions and one of them, well documented in the literature, is their role in parasitic invasions that occur in parasitic diseases such as Chagas disease. Some *Trypanosoma cruzi* peptidases are thought to play central roles in diverse processes such as, differentiation, cell cycle progression, proteins degradation and evasion of the host immune response (Klemba, 2002; Kosec, 2006; McKerrow, 2008; Vermelho, 2010; Alvarez, 2011).

The MEROPS database is an Internet resource containing information on peptidases, their substrates and inhibitors including details of cleavage positions in substrates, both physiological and non-physiological, natural and synthetic and their mechanism of catalysis. The enzymes are classified using three different approaches including the chemical mechanism of catalysis, the catalytic reaction and the molecular structure and homology. Based on these criteria peptidases are grouped into protein species, which in turn are grouped into families, and then grouped into clans (Rawlings, 2010).

Peptidases are druggable targets and peptidases inhibitors have proven to be effective drugs for hypertension (Hoover, 2010), HIV infection (Moyle, 1998), human cancers (Hoekstra, 2006) and infectious diseases (Renslo, 2006). Especially in infectious diseases peptidases play an important role in the life cycle of protozoan parasites and in the pathogenesis of these diseases, such as in the inactivation of host immune defence mediators, the processing of host or parasite surface proteins for invasion of host cells, as well as in the digestion of host proteins for nutrition (Franke de Cazzulo, 1994; McKerrow, 2008).

Peptidases, such as, serine, threonine, aspartyl, metallo and cysteine peptidases have been detected in *Trypanosoma cruzi* and they have been shown to be involved in numerous roles in the physiology of the parasite. The inhibition of some of these enzymes has shown high anti-*T. cruzi* activity *in vitro* and *in vivo* (Capaci-Rodrigues, 2010). The serine peptidases described in *Trypanosoma cruzi* include oligopeptidase B, a member of the prolyl

oligopeptidase family involved in Ca^{2+} signaling during mammalian cell invasion; a 80-kDa prolyl serine oligopeptidase (POP Tc80), belonging to the prolyl oligopeptidase family (EC 3.4.21.26) against which inhibitors are being developed, and a lysosomal serine carboxypeptidase that probably hydrolyzes human collagen (Types I and IV) and fibronectin that has been implicated in the parasite adhesion to host cells and cell entry (Burleigh, 1995; Burleigh, 1997; Caler, 1998; Grellier, 2001; Bastos, 2005; da Silva-Lopez, 2008). The threonine (proteasome) peptidase in the parasite has properties similar to those of other eukaryotes, and its inhibition by lactacystin blocks some differentiation steps in the life cycle of the parasite (Nkemgu-Njinkeng, 2002; Cardoso, 2008; Gutiérrez, 2009).

Recently, in *T. cruzi*, two aspartic peptidases were isolated from the epimastigote forms, named cruzipain-I and cruzipain-II. One from the water soluble fraction and the second from the 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) fraction. Both peptidase activities were inhibited by the classic pepstatin-A inhibitor and the aspartic active site labelling agent, 1,2- epoxy-3-(phenyl-nitrophenoxy) propane (EPNP). However, no coding genes for aspartic peptidases have yet been observed in the *T. cruzi* genome. The authors explained that they could not rule out the possibility that aspartic peptidase homologues are present in *T. cruzi*, because this genome has not been completely mapped (Pinho, 2009).

Metallopeptidases homologous to the gp63 of *Leishmania* spp. are present in *T. cruzi*, as well as two metallocarboxypeptidases belonging to the M32 family, before found only in prokaryotes (Bonaldo, 1991; Lowndes, 1996; Burleigh, 2002; Cuevas, 2003; Nogueira de Melo, 2004; Gutierrez, 2008; Niemirowicz, 2007; Niemirowicz, 2008; Kulkarni, 2009).

The most abundant among these enzymes is cruzipain (Cz), also known as GP57/51 or cruzain (the recombinant catalytic domain of cruzipain) a cysteine peptidase (CP) expressed as a mixture of isoforms. This CP is located in clan CA, being a member of the papain C1 family of CP with an intermediate specificity between cathepsin L and cathepsin B (Cazzulo, 1989).

Although Cz is found in different compartments, it is expressed in all stages of the *T. cruzi* lifecycle. In the epimastigote stage, cruzipain is probably involved in the degradation of proteins. It may also play an important role in nutrition of the parasite, at least in the gut of the hematophagous insect vector. Cruzipain is stored in the pre lysosomal organelle called reservosome, in the posterior portion of the cell. In the intracellular amastigote stage, this peptidase can still be detected in the lysosome-related compartment, but most cruzipain is found fixed on the surface of the parasite, presumably through a GPI anchor, directly in contact with host cell cytoplasm. Trypomastigotes are able to secret some isoforms into the medium. This is extremely significant for the role of cruzipain as a virulence factor in Chagas disease (Souto-Padron, 1990; Engel, 2000; Vieira, 2005; McKerrow, 2009; Alvarez, 2011).

Cruzipain is involved in a number of cellular processes and in important virulence factors in the pathogenesis of parasitic diseases, such as: in the cell invasion phenomenon, facilitating the proteolytic degradation of host tissues and triggering the evasion mechanism (Franke de Cazzulo, 1994; Costales, 2007; Capaci-Rodrigues, 2010; Nogueira de Melo, 2010).

In addition, this CP has demonstrated an ability to induce the production of the proinflammatory peptide Lys-bradykinin by the proteolysis of kininogen or by activation of plasmatic pre-kallikrein (Del Nery, 1997; Scharfstein, 2000; Benítez-Hernández, 2010; Capaci-Rodrigues, 2010).

Although some other minor CPs have been described in *T. cruzi*, such as 30 kDa cathepsin B-like CPs and more recently *Tc*CPmet (secreted by metacyclic trypomastigotes), it is not yet known how many different enzymes of this type are present and their possible functions (Garcia, 1998; Yong, 2000; Duschak, 2006; Capaci-Rodrigues, 2010).

The structure of the active site, of the cruzipain , has been reported through X-ray crystal structures of the enzyme in complex with reversible and irreversible inhibitors. The enzyme is composed of one polypeptide chain of 215 amino acid residues and the catalytic triad of Cys25, His159, and Asn175 , as well as the highly conserved Trp177 of cruzipain, which is contained within a cleft between the two structural domains of the enzyme (McGrath, 1995). This domain is formed by seven substrate binding sites, four (S4, S3, S2, and S1) on the acyl side and three (S1′, S2′, and S3′) on the amino side of the scissile bond. The interaction of the S2 site of the enzyme with the corresponding P2 inhibitor residue is the key specificity determining factor (Chen, 2010).

Although Cz has a catalytic domain that is highly homologous to human cysteine peptidases (HCP) (Brinen, 2000), studies have indicated that there is a preferential inhibition with cruzipain compared to HCP. The preferential inhibition is possibly due to the fact that human cathepsins are located in the lysosomes, a less accessible sub-compartment. The parasite, Cz is found in the host cell cytoplasm and therefore, more exposed to the action of inhibitors (McKerrow, 1995; McKerrow, 1999; Brak, 2008). Moreover, the parasite has a specific mechanism for the uptake of small molecules such as peptidase inhibitors that could facilitate the entry of these inhibitors into the parasite (McKerrow, 1999; Mallari, 2008; McKerrow,2008).

Recently there has been great interest in developing new cruzipain inhibitors as chemotherapeutic targets. As proof of this more than 50% of the 51 patents in the 2000-2006 period aiming at different targets for the treatment of Chagas disease, were cruzipain inhibitors (targets included: ergosterol biosynthesis inhibitors, inhibitors of polyamine and trypanothione formation) (Duschak, 2007).

In an attempt to obtain a new lead, scaffolds for cruzipain inhibition with good efficacy and minimal toxicity for several classes of cruzipain inhibitors have been developed. Knowledge of the Cz crystal structure has promoted the design of a variety of cysteine peptidase inhibitors including peptidyl and non-peptidyl inhibitors. Among peptidyl inhibitors, the irreversible inhibitor, *N*-acylhydrazone has been studied (Ifa, 2000; Lima, 2009; dos Santos Filho, 2009), as well as the halomethyl ketone based (Ashall, 1990; Harth, 1993), diazomethane ketones (Shaw, 1994; Lalmanach, 1996), vinyl sulfones (Roush, 2001; Engel, 1998; Barr, 2005; Kerr, 2009; Jacobsen, 2000), and reversible inhibitors such as oxadiazoles (Ferreira,2009), and aryl ureas (Du, 2000). In the non-peptidyl group thiosemicarbazones (Du, 2002), triazole, triazine nitriles (Brak, 2010; Mott, 2010) and non-peptidic vinylsulfones (Bryant, 2009) can be found some of which are presented in Table 4.

Among all chemical classes of inhibitors of cruzipain (Cz) researched until now, vinyl sulfone can be highlighted as it is in an advanced stage clinical trial.

Peptidyl vinyl sulfone inhibitor compounds are irreversible inhibitors of cruzipain via a Michael addition by active site Cys25. According to Palmer's (Palmer, 1995) hypothesis, the Michael addition is facilitated through hydrogen bonding between the protonated His159 residue and one of the vinyl sulfone oxygens. Thus, the vinyl group would be polarized, and consequently the β-vinyl carbon would become positively charged. This would then facilitate a nucleophilic attack by the active site thiolate. The other sulfone oxygen could participate in hydrogen bonding with the active site glutamine (Figure 14) (Powers, 2002).

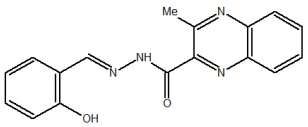
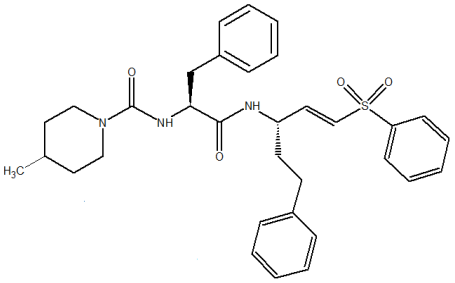
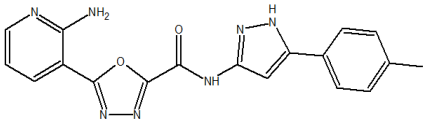
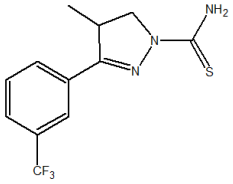
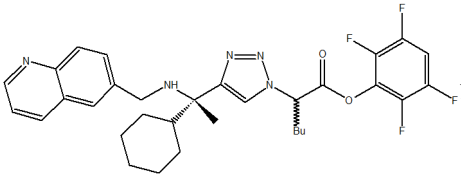
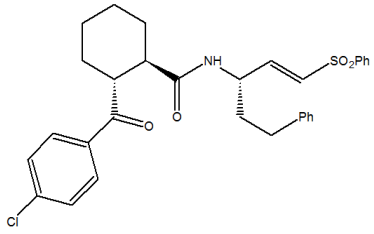
Chemical class of cruzipain inhibitor			Chemical Structure	Reference
Peptidyl inhibitor	Irreversible	*N*-acylhydrazones		Lima, 2009
		Vinyl sulfones		Kerr, 2009
		Oxadiazoles		Ferreira, 2009
		Thiosemicarbazones		Du, 2002
Non-peptidyl inhibitors	Reversible	Triazole, triazine nitriles		Brak, 2010
		Non-peptidic vinyl sulfones		Bryant, 2009

Table 4. Some cruzipain inhibitors

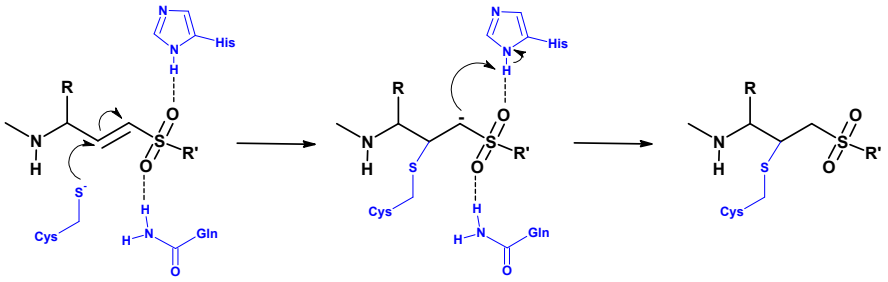


Fig. 14. Mechanism of irreversible inhibition of cysteine peptidase by vinyl sulfones (Palmer, 1995; Powers, 2002; Capaci-Rodrigues, 2010)

The most important peptidyl vinyl sulfone derivative is K11777 (*N*-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl), also known as K777 (compound **6**, Figure 15) that is currently in late-stage preclinical trials. K11777 is a second generation vinyl sulfone (Roush, 2001). The compound was developed by Jim McKerrow's team at the Sandler Center for Drug Discovery in San Francisco in the United States(McKerrow, 2009). K777 is non-mutagenic, has an acceptable PK profile, anis the most extensively characterized in efficacy and safety studies in rodents, dogs and primates **(Clayton , 2010)** . Studies with K11777 have proven that blocking cruzipain is enough to cure Chagas disease in all of its stages and disrupts amastigote intracellular development. (McKerrow, 2009, Doyle 2011). This compound has a wide spectrum of activity against different *T. cruzi* strains (e.g., Y, Tulahuén, CL, CA-I/72), even against nifurtimox and benznidazole-resistant *T. cruzi* strains (McKerrow, 2009). In addition, studies have indicated that concomitant use of a cruzipain inhibitor with benznidazole might be beneficial for the treatment of Chagas disease, as well as, the possibility of reducing the benznidazole dosage and thus decrease the side effects of this drug (McKerrow, 2009).K777 has now progressed into IND (Investigation New Drug) - enabling studies in collaboration with the DNDi (Drugs for Neglected Diseases Initiative) **(**Clayton , 2010).

This vinyl sulfone was developed by a rational design aimed to enhance specificity and *in vivo* stability and minimizing toxicity (Engel, 1998) from the predecessor K11002 (compound **7**, Figure 15) by the replacement of a morpholine-urea ring by a *N*-methylpiperazine ring (P3 subsite). This change was intended to increase the oral bioavailability, and increase the solubility in intestinal fluids. The *in vitro* assay showed an improvement of 3 to 19.9% (Jacobsen, 2000) and in murine models of acute and chronic Chagas disease, showed prolonged survival and eradicated the parasite infection with minimal toxicity (Engel, 1998; Barr, 2005; Urbina, 2003; Kerr, 2009; McKerrow, 2009).

Therefore, the P3 substituent of the vinyl sulfones has been of great interest, and the modification of this position has influenced several properties, such as lysosomotropism, hepatotoxicity, and pharmacokinetics (Kerr, 2009; Jacobsen, 2000; Zhang, 1998). Similar to most other papain-like cysteine peptidase, the interaction of the S2 subsite of the enzyme with the corresponding P2 residue is the key specificity determining factor. Cruzipain is a dual-specific peptidase that is able to accommodate phenylalanine or arginine in the P2 residue of the ligand due to the presence of Glu208 found at the base of the S2 subsite, however with a preference for phenylalanine over arginine (Chen, 2010). In this way, a new analogue of K11777 was synthesized, e.g.: WRR-483 (compound 8, Figure 15), replacing

phenylalanine (K11777) with arginine (WRR-483) in the P2 substituent**.** This compound showed sensitivity to pH conditions, being more potent at higher pH levels, but it was still relatively weak when compared to K11777 in the inhibition of cruzipain. Probably the Glu208 in the S2 pocket of the cruzain-WRR-483 is not fully anchored to the arginine residue. However, in the *in vitro* cell assay, this compound showed trypanocidal activity comparable to the lead compound, K11777, and surprisingly was effective in curing acute *T. cruzi* infection in an *in vivo* assay. This difference could be explained by the possibility that WRR-483 is targeting an as yet unknown cathepsin L-like cysteine peptidase in *T. cruzi*. Another possibility is that the WRR-483 inhibits cruzipain either located on the cell membrane or released by the parasite, and not in the lysosome. The inhibitor is hydrophilic in nature, primarily due to the guanidine group, and is more active at physiological pH, making it very favourable in the extracellular environment. In the light of these results, studies have shown that WRR-483 has potential to be developed as a treatment for Chagas' disease (Chen, 2010).

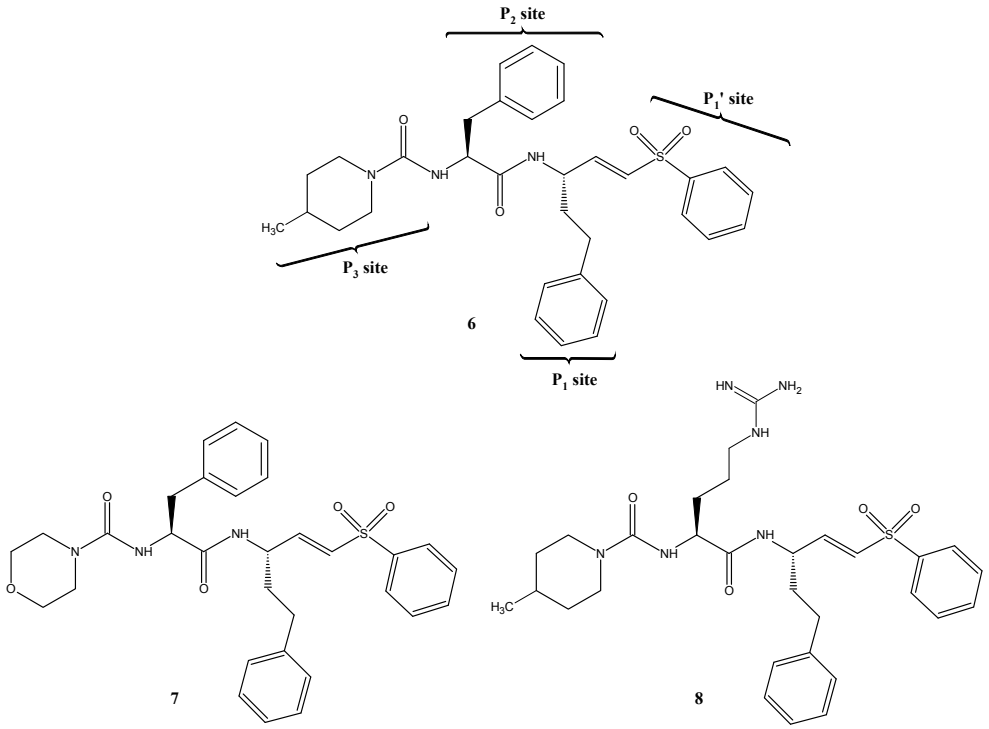


Fig. 15. Chemical structure of K11777 (**6**), K11002 (**7**) and WRR-483(**8**) vinyl sulfone derivatives inhibitors of cruzipain. The P1 –P3 subsites of K11777 are labelled (McKerrow, 1999; Chen, 2010).

5. Conclusion

This chapter focused in the action mechanism and limitations of the drugs currently used for Chagas disease and described the importance of the study of Megazol and natural products development for Chagas disease chemotherapy that it is active against different strains of the parasite and has great effectiveness in monkeys even in the chronic stage. For this reason, megazol was considered as an alternative lead-compound for the treatment of the Chagas' diseas. Megazol is a highly active compound against *Trypanosoma cruzi*, and has become a core structure for the design of new trypanocidal agents. Another point presented is about the use o natural products in Chagas disease. The knowledge of using natural products is of great value, especially the use of medicinal plants, as the countless number of species reflects an inestimable number of biologically active molecules against different illnesses, including infectious diseases. In the last decades the interest in natural compounds extracted from plants with anti-*Trypanosoma cruzi* activity has been renewed, and laboratories across the world have shown promising results. Moreover the use of peptidases, mainly cysteine peptidases as a target for drug development was discussed showing the importance of these enzymes development of new drugs for Chagas disease. A special focus was given for K777 currently in late-stage preclinical trials

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The Gut Microbiota as Target for Innovative Drug Development: Perspectives and a Case Study of Inflammatory Bowel Diseases

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1. Introduction

For many centuries scientists have been intrigued by the function of the intestine in human health and have tried to explain the role of the intestinal tract in our body, with sometimes rather original interpretations of its function. Leonardo Da Vinci for instance, who described the anatomy of the human body in great detail, concluded that the digestive system is in fact a part of the respiratory system, supporting its functioning. Indeed, he stated the following, giving a quite original interpretation on the function of the intestines: "The compressed intestines with the condensed air which is generated in them, thrust the diaphragm upwards; the diaphragm compresses the lungs and expresses the air" (O'Malley & Saunders, 1982). So gasses produced in the intestine would help to breath… In general, until quite recently it was believed that the main function of the intestine would be to dispose waste materials and reabsorb water from the intestine. Since the work of Antonie Van Leeuwenhoek it is known that the intestine contains an extensive microbial community (Smit & Heniger, 1975).

Nowadays, it's clear that the intestine is much more than an organ for waste material and absorption of water, salts and drugs, and indeed has a very important impact on human health, for a major part related to the specific composition of the complex microbial community in the colon. This microbial community composition is governed by age, diet, environment and phylogeny (Ley et al., 2008; Zoetendal et al., 1998; Zoetendal et al., 2001) and contains all three domains of life: Bacteria, Archaea and Eukarya (fungi, yeasts and protozoa). The human colon harbors a highly complex microbial ecosystem of about 200 g living cells, at concentrations of 10^{11} microorganisms per gram gut content, in total numbers which outnumber the amount of somatic and germ cells in the human body with a factor 10. Together, all 6.5 billion humans on earth represent a gut reservoir of 10^{23}-10^{24} microbial cells, which is just five orders of magnitude less than the world's oceans (10^{29} cells) (Ley et al., 2006). Therefore, the human gut constitutes a substantial habitat in our biosphere and we can in fact consider the human body as a mix of human and bacterial cells. Despite such high numbers, the microbial diversity is however relatively limited. Although 55 and 13 divisions of respectively Bacteria and Archaea have been described, only 8 bacterial

divisions have been identified so far in the gastrointestinal tract (GIT) and the gut microbiome is dominated by only 2 bacterial divisions, Firmicutes and Bacteroidetes, that make up over 90% of the intestinal microbiota. The remainder consists of Actinobacteria (Turnbaugh et al., 2009) and, to a lesser extent, Proteobacteria, Verrucomicrobia, and Cyanobacteria (Backhed et al., 2005; Eckburg et al., 2005; Ley et al., 2006). Further, only two archaeal species have been described with *Methanobrevibacter smithii* being more predominant than *Methanosphaera stadtmanae* (Eckburg et al., 2005). At this level, the intestinal communities of all humans therefore appear quite similar. However, within these divisions, a limited number of lineages terminate in broad, shallow radiations comprising hundreds of species and thousands of strains, making the microbiota of an individual as personalized as a fingerprint (Backhed et al., 2005; Ley et al., 2006).

In addition to the very high numbers of microbes in our intestines, it is estimated that the collection of all microbial genomes comprises 2 to 4 million genes, which is 70 to 140 times more than that of their host. This 'microbiome' encompasses all genes that are responsible for numerous processes such as substrate breakdown, protein synthesis, biomass production, production of signaling molecules, antimicrobial compounds and encodes biochemical pathways that humans have not evolved (Egert et al., 2006). The microbiota in the large intestine can therefore be seen as a separate organ encompassing a broad range of specific additional activities, which provide great opportunities for its host and which is capable of even more conversions than the human liver.

Summarized, the intestinal microbial ecosystem not only constitutes a major part of the body's cell numbers, its extensive gene pool represents an almost unlimited functionality in our body. Whereas typical examples of such activity relate to the extraction of energy from otherwise indigestible plant polysaccharides, it has become clear that the mutualistic interaction between the host and its microbiota, has a major influence on the host's health. This includes the stimulation of the gut immune system (Salminen et al., 1998), the regulation of cell proliferation (Dethlefsen et al., 2006), the synthesis of essential vitamins K and B (Conly & Stein, 1992), and providing resistance to colonization by pathogens (Hopkins & Macfarlane, 2003). However, recent insights in the composition of the intestinal microbiota and host-microbiota cross-talk have shown that the balance in this mutualistic interaction is very fragile and a dysregulation in specific community assemblages is now considered as a risk factor contributing to a disease state. This is shown by recent reports linking intestinal bacteria with diseases ranging from allergies (MacDonald & Monteleone, 2005) to bowel inflammation (Elson et al., 2006), and obesity (Backhed et al., 2007).

If we better understand the possibilities of the intestinal microbiome and the importance of optimal host-microbiota cross-talk, this will off course open up completely new perspectives to exploit this role of the microbiota to improve host health and for innovative drug development (Possemiers et al., 2009). Into a major extent, such strategies can relate to the potent, almost unlimited metabolic potential of the intestinal microbiome. Whereas metabolic processes in the body which affect the final bioactivity of drug compounds have typically been associated with the hepatic metabolism, microbial drug biotransformation may also dramatically affect drug bioavailability and a possible lack thereof (Possemiers et al., 2011a). As current evaluation of drug bioavailability and biological activity is typically based on the ADME principle (Absorption, Distribution, Metabolism and Excretion), inclusion of microbial metabolic processes in drug bioavailability screening should indeed become standard practice. There is therefore a clear need to better understand the microbiota and its metabolic capability, which will be discussed in this book chapter.

Drug development may reach far beyond only understanding intestinal metabolic processes. Indeed, innovative strategies which actively make use of bacterial cells and their metabolic functionalities to improve drug bioavailability have been developed over the last decade. Several specific examples will be provided in the book chapter, ranging from the use of intestinal microbial metabolism for targeted prodrug activation up to the use of microorganisms as production facilities inside the intestine for innovative drug development.
An important challenge towards such new drug development strategies is the high complexity of intestinal (microbial) processes. Not only is the lack of direct accessibility to study intestinal processes in the gut itself a strong limitation for using animals or humans in the screening process, the intestinal microbiota is also characterized by a dramatic interindividual variability, ultimately leading to a strongly varying response between individuals. This interindividual variability together with the need for a suitable environment for mechanistic mode-of-action studies create the necessity for suitable *in vitro* and *in silico* models to predict metabolic fates of drugs. Complex multi-stage *in vitro* models together with *in silico* models can provide us with very valuable information and will therefore also be discussed in this chapter.
Finally, several of these aspects will be handled with inflammatory bowel diseases (IBD) as case study, a set of inflammatory diseases in the intestine for which a bacterial role has been described.

2. The microbial metabolic perspective

A wide range of metabolic reactions can be catalyzed by the large diversity of microbial enzymes. Ilett et al. (1990) have already indicated that gut bacterial metabolism is reductive, hydrolytic and even of degradative nature with a strong potential for both bioactivation and detoxification of xenobiotics. The latter is in contrast to the oxidative and conjugative reactions from the phase I and II enzymes in the enterocytes and hepatocytes (Ilett et al., 1990). Moreover, the intestinal microbiota interferes with the human biotransformation process through the enterohepatic circulation of xenobiotic compounds what may reverse the detoxification cycle of the liver. Additionally, the microbial metabolic potency can be addressed for the local release of the active compounds in the colon and as production facility for specific active compounds.
Figure 1 gives a schematic overview of the microbial metabolic potential and its interference in the enterohepatic circulation and Table 2 gives an overview of the described drug development approaches making use of bacterial cells and their metabolic functionalities throughout the book chapter.

2.1 Direct microbial metabolism influences final activity profiles

Chemical components in food, either as food component or as contaminant, represent a significant source of both positive and negative influences on the health and function of the human body. For example, polyphenol mixtures represent one such class and within a much wider realm of application, there is now substantial evidence to support the hypothesis that these may be beneficial in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, or osteoporosis risk (Scalbert et al., 2005). It is now well investigated that the majority of polyphenol compounds reach the colon unaltered. There, they undergo extensive metabolism by the resident gut microbiome prior to

absorption. This is a key step in understanding the fate of many xenobiotic compounds and their final clinical effects and yet this process has remained almost completely unexplored.
An important field of research dealing with gut microbial conversion of food compounds is that of a specific group of polyphenols: the phytoestrogens. Phytoestrogens are plant components that structurally resemble 17β-estradiol. Depending on the target organ and the endogenous estrogen levels, these phytoestrogens may elicit an estrogenic or anti-estrogenic effect, by which they may play a role in the prevention and treatment of hormone-dependent diseases such as breast- and prostate cancer, or osteoporosis and menopausal complaints. The most important classes from nutrition are isoflavones (primarily from soy), coumestans (fruit, cereals, leguminosae), prenylflavonoids (hops), and lignans (fruit, cereals, leguminosae). Hop contains the most powerful phytoestrogen known thus far, namely 8-prenylnaringenin (8-PN). Rowland et al. (2003) discussed the role of the gut microbial community in the bioavailability and metabolism of estrogens in general and phytoestrogens more in particular. Intestinal bacteria contribute to the bioavailability and biological activity of phytoestrogens in a number of ways. Firstly, intestinal bacteria produce β-glucosidases thereby cleaving the naturally occurring glycosylated phytoestrogens into an aglycon. Secondly, the gut bacteria elicit glucuronidase and sulfatase activity, thereby deconjugating phase II metabolites from the liver and releasing the aglycons again. Thirdly and most importantly, intestinal bacteria are capable of transforming the original components to metabolites that have a higher biological activity. For example, equol and 8-PN are bacterial metabolites from daidzein (soy) and isoxanthohumol (hop), respectively and have a much higher biological activity than their precursor product. A remarkable aspect of the microbial conversion of daidzein into equol and of isoxanthohumol into 8-PN is the large interindividual variability in the conversion efficiency. This was illustrated for equol production (Bolca et al., 2007a) and 8-PN production (Bolca et al., 2007b).
A second example of a natural product used for its medicinal properties are anthranoid-containing laxatives. Anthranoids are obtained from the dried leaflets and pods of plants such as senna plants, *Cascara sagrada*, *Frangulae cortex* and rhubarb. In plants, anthranoids are mostly present as sugar derivatives and due to this β-glycosidic linkage the molecule is carried unabsorbed into the large intestine where the microbial metabolism starts and the active aglycon anthrone is released (de Witte & Lemli, 1990). Changes in the colonic motility, absorption, and secretion result in an increased intestinal transit rate and fluid accumulation. Chronic use of these laxatives causes melanosis coli, a deep black pigmentation of the colon mucosa but whether there is an association with colorectal cancer, is still controversial (Van Gorkom et al., 1999; Nusko et al., 2000).
Besides the interest in the microbial conversion of health-promoting components from ingested foodstuffs, much attention is also given to the role of intestinal bacteria in the conversion of ingested drugs, environmental or food contaminants or xenobiotics in general. The ability of the gut bacteria to metabolize drugs came under the attention of pharmaceutical companies since an accident in 1993 with the antiviral drug orivudine which revealed that its gut microbial metabolite (E)-5-(2-bromovinyl)uracil interfered with the clearance of a co-administered anti-cancer drug 5-fluoro-uracil. This resulted in the death of 18 patients (Okuda et al., 1998). Recently, Sousa et al. (2008) reviewed the conversion of over 30 drug compounds by gut microorganisms and the related consequences for their biological effect in the human body. Examples are the reduction of omeprazole and digoxin, hydrolysis of lactulose and sorivudine, acetylation of 5-aminosalicylic acid, proteolysis of

insulin and calcitonin and N-demethylation of methamphetamine. Additionally, demethylation, deamination, decarboxylation and dehalogenation reactions have also been described, next to other reactions such as aromatization, esterification and N-nitrosation (Ilett et al., 1990). Microbial metabolism is not only confined to drugs, but also targets contaminants. The heterocyclic aromatic amine, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) – a compound which is produced during the cooking and grilling of meat – is converted by intestinal bacteria into the direct acting mutagen 7-hydroxy-IQ (van Tassell et al., 1989). IQ incubation studies of intestinal microbiota suspensions from different individuals showed a large interindividual variability between the potency to form the 7-OH-IQ. Apparently, this reaction can be carried out by bacteria belonging to the predominant populations of the human gut, such as *Bacteroides thetaiotaomicron*, *Clostridium clostridiiforme*, and *Escherichia coli* (Humblot et al., 2005). As a third example of microbial metabolism, oxidative reactions were reported by Van de Wiele et al. (2005) who described the hydroxylation of polycyclic aromatic hydrocarbons (PAHs) by *in vitro* cultured intestinal microbiota. Hydroxylation of PAHs gives these compounds an affinity for the human estrogen receptor. In that way, hydroxylated PAHs may act as pseudo-estrogens and interfere with normal hormone-driven process in the body. A final example targets the conversion of metal(loid)s, both in an inorganic as an organic chemical form. Michalke et al. (2008) reported that human gut microbes actively volatilize bismuth and other metal(loid)s, including arsenic (As), through methylation and hydrogenation. Moreover, Meyer et al. (2008) postulated that gut methanogens play a crucial role in metal(loid) volatilization, thereby exerting toxic effects to the human body, not only by direct interaction with the host but also by disturbing the endogenous gut microbiota composition and metabolism. Further, a thorough *in vitro* exploration with the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®), a dynamic human gastrointestinal simulator, revealed a high microbial metabolic potency toward metal(loid)s (Diaz-Bone & Van de Wiele, 2009). This was demonstrated by the finding of significant volatilization of arsenic (As), selenium (Se), bismuth (Bi), tellurium (Te), and antimony; the formation of highly toxic AsH_3 (arsine) and $(CH3)_2Te$ (dimethyl telluride); and the discovery of two new As–sulfur metabolites. Van de Wiele et al. (2010) and Pinyayev et al. (2011) later showed the contribution of the gut microbiota, presumable sulfate reducers, in the formation of methylated thioarsenicals from which the toxicity is thought to be equivalent to that of trivalent inorganic arsenic.

2.2 Microbial metabolism interferes with the enterohepatic circulation

Compounds that have been absorbed in the intestine and subsequently detoxified are usually conjugated with polar groups (glucuronic acid, glycine, sulfate, glutathion, and taurine) in the liver prior to secretion with the bile (Ilett et al., 1990). After released in the intestinal lumen, these phase II biotransformation products may be hydrolyzed again by bacterial enzymes such as β-glucuronidase, sulfatase, and other glycosidases. This would negate the detoxification cycle and delay the excretion of many exogenous compounds since the original compounds or phase I metabolites are more prone to intestinal absorption than their phase II conjugates. Several studies have already shown the biotransformation capacity of gut bacteria towards hydrocarbons. For example, bacterial β-glucuronidase activity hydrolyzes the glucuronidated form of IQ, a food-borne carcinogen, thereby increasing the colonic genotoxicity of this compound in rats (Humblot et al., 2007). Additionally, guinea pig experiments showed that the hydrolysis of benzo(a)pyrene conjugates by the intestinal

microbiota resulted in *de novo* production of toxic benzo(a)pyrene intermediates what caused the formation of DNA adducts in the colon (Bowes & Renwick, 1986). Other research studies have described similar deconjugation reactions for many other compounds.

2.3 Colon targeted drug release using the microbial metabolism

The enormous metabolic potency from the gut microbiota can also be employed for colon-targeted drug delivery. The targeted delivery of drugs in the colon provides certain benefits, or is even necessary in certain therapeutic scenarios. Depending on the nature of the administered molecule, drugs can be highly sensitive to the stringent conditions of the upper GIT (low gastric pH, high enzymatic activity), which makes it necessary to postpone their intestinal absorption to the lower GIT (distal ileum or colon). In addition, patients that suffer from diseases that are more confined to the colon region (f. ex. IBD, carcinoma or bacterial infection) may benefit from a local rather than a systemic therapy. Such local drug release would optimize the therapeutic efficiency or minimize possible side effects from a systemic (intravenous) drug administration. Two strategies can be used to involve gut microorganisms.

A first strategy makes use of prodrugs, which are pharmacologically inactive derivatives of the parent drug compound (reviewed by Patel et al., 2007). These prodrugs are designed to be more resistant to breakdown processes in the upper digestive tract (f. ex. acid hydrolysis, protease activity) and require conversion to the bioactive molecule by enzymatic activity. This bioactivation process often relies on gut microbial enzymatic activity, which dramatically increases due to the high microbial load in the colon environment (10^{10}-10^{11} microorganisms per gram versus 10^{6}-10^{7} microorganisms per gram in the ileum). Azo-bond prodrugs and glycoside prodrugs are considered the most important classes of prodrugs, while glucuronate, dextran, cyclodextrin and polypeptide prodrugs are also manufactured (Patel et al., 2007).

A second strategy that requires gut microbial activity to deliver drugs in the colon is the coating of drugs with biodegradable polymers. In this case, the polymer is resistant to the digestive processes from the upper GIT and thereby protects the encapsulated drug molecule. The polymer is however subjected to microbial biodegradation in the colon and subsequently releases the drug molecules, which can then be taken up across the colon epithelium. To illustrate, azo-containing polyurethane molecules have been used as a coating material for drugs. The molecule is degraded by gut microbial azoreductase activity and subsequently releases the active drug compound (Chavan et al., 2001; Kimura et al., 1992).

Colon-specific drug delivery systems making use of these microbial properties have been successfully applied for both local as systemic drug therapy (Patel et al., 2007).

2.4 Bacteria as production facilities

Bacteria may furthermore be used as actual 'production facilities' that produce active compounds. The most well-known example is probably the production of antibiotics by microorganisms. Due to the selection of resistant pathogens, there is a contuining need for new antibiotics. This need has been largely met by the production of synthetic antibiotics but due to advances in technology there is an increasing interest in the production of natural antibiotics from bacteria. Soil actinomycetes have been a major source of

antibiotics and recently, searches of underexplored ecological niches (f. ex. deep-sea sediment samples, bacterial symbionts of insects or fungi, Mycobacteria) have revealed new molecules. These molecules produced by the bacteria are essential to combat resistant pathogens, which are increasingly prevalent in the community (Clardy et al., 2006; Fischbach & Walsh, 2009).

As a second example, we will discuss here in detail the case of carotenoids in which *Bacillus* spores are used as 'production facilities' producing active ingredients directly in the intestine. Mammals cannot synthesize carotenoids *de novo* and must acquire them from the diet. These are key components that have a role in nutrition (Vitamin A), vision and its development (retinoic acid). Moreover, the antioxidant properties of carotenoids protect the cells from environmental stresses and are also able to prevent the onset of chronic disease states in mammals (Duc et al., 2006; Giovannucci, 2002; Mares-Perlman et al., 2002). In order to exert these beneficial effects in the GIT, ingested carotenoids must reach a concentration that is sufficient to act as a scavenger of oxygen radicals (Agarwal & Rao, 1998; Fuhrman et al., 1997; Witztum, 1994). Finally, after intestinal absorption, carotenoids can also have anti-inflammatory or anti-carcinogenic effects (Ben-Dor et al., 2005). The problem is that pure carotenoids are rapidly degraded in the stomach. It is possible to increase their resistance by incorporating high doses of carotenoids into a food matrix in order to guarantee a recommended daily intake of about 800 $mg.day^{-1}$. However, this enrichment procedure is expensive and therefore limited (Duc et al., 2006). Recently, scientists at the Royal Holloway University of London have isolated carotenoid-producing spore-forming *Bacillus* spp.. These pigmented *Bacillus* species (yellow, pink, orange…) have been characterized and the pigments have been shown to be due to one or more carotenoids (Duc et al., 2006; Hong et al., 2009; Khaneja et al., 2010; Perez-Fons et al., 2011). More specifically, the presence of 1-HO-demethylspheroidene, ubiquinone and phytoene was identified in some strains by means of a combination of HPLC analysis and UV/VIS spectral data. The carotenoids contained within the spores appeared to be gastric stable and could therefore provide a good source of carotenoids in the small intestine where - following the germination of the *Bacillus* strains - they are released. The released carotenoids or their metabolites may be absorbed from the gut and reach systemic circulation. Alternatively, a part of the carotenoids and their derivatives may reach the colon, where they can be subjected to further microbial metabolism and/or absorbed through the colonic epithelium. This is particularly appropriate for the carotenoids still contained in the spores, as the release before the beginning of the colon may not be complete. Moreover, if the bacteria are able to survive and grow in the colonic microbial community, they may produce and release additional carotenoids into the colonic environment. It has been shown that the *Bacillus*-derived apocarotenoids antioxidant properties are 10 times higher than lycopene. Next to acting as an optimal carrier for the improved delivery of gastric-stable carotenoids, the *Bacillus* strains may exert probiotic properties in the colon, resulting for instance in an improvement of the gastrointestinal environment, immune stimulation, antimicrobial activities and competitive exclusion (Cutting, 2011). A final added value is that, compared to other vegetative bacteria which are typically used as ingredient/probiotic, *Bacillus* spores are particularly attractive because they can be stored at ambient temperature in liquid or dried form indefinitely without the need for refrigeration, thereby ensuring better control of the administered doses.

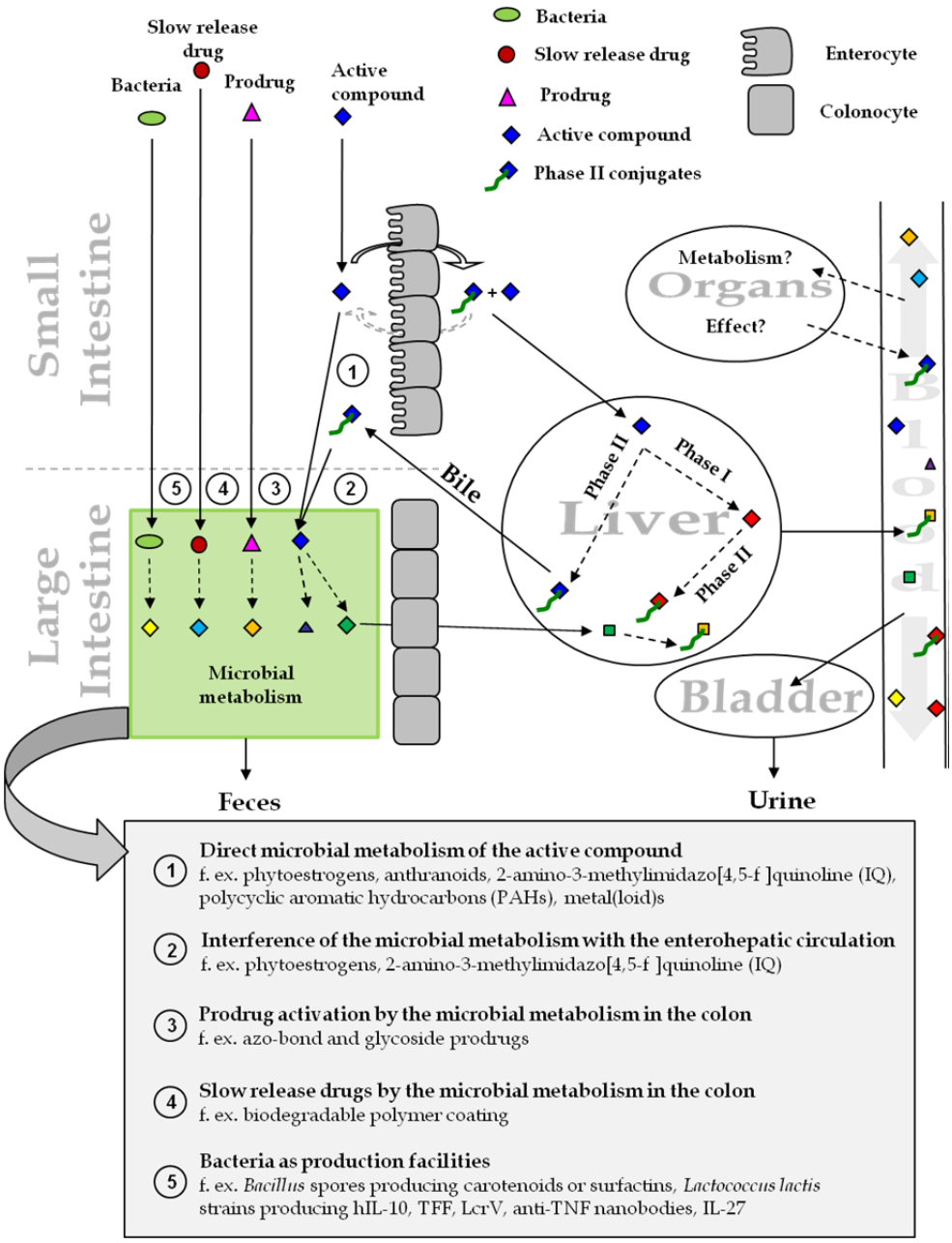

Fig. 1. Schematic overview of the microbial metabolic potential and its interference with the enterohepatic circulation. Adapted from Possemiers et al. (2011a)

3. Gut models for studying microbial modulation of human health

As mentioned in the previous paragraphs, the high complexity of intestinal microbial processes is a main challenge in new drug development strategies. There is a clear necessity of creating suitable *in vitro* and *in silico* models to predict metabolic fate of drugs. These models need to take into account the dramatic interindividual variability of the gut microbiota (both in terms of composition and activity) that ultimately leads to a strongly varying response between individuals. When using animals or humans in the screening processes, the lack of direct accessibility to study intestinal processes in the gut itself represents a serious obstacle to elucidate the importance of intestinal processes for a specific drug candidate. Moreover, whereas final *in vivo* testing is off course required to confirm formulation performance, this approach holds a number of drawbacks to be used in early stages of drug development such as ethical concerns, lack of information on the mechanism of action, analytical difficulties related to dilution of the active compounds and their metabolites in the plasma and the rest of the body as well as doubts on the representativeness of animal models for the human situation (Pieper & Bertau, 2010).

In vitro simulation studies may offer many unique advantages, even if they suffer from the absence of a complete physiological environment. They are easy to set up and sample, and have a high reproducibility. They offer the possibility of performing mechanistic studies and there are no ethical constraints. However, it is clearly of key importance to evaluate the specific properties of a candidate active compound under conditions that are relevant to *in vivo* situations. In fact, the better an *in vitro* system can simulate the real gut situation, the higher is the physiological significance of the obtained information (Marzorati et al., 2009).

In this respect, simple batch experiments, single stage reactors or the conventional dissolution systems do not replicate the rapidly changing dynamic environment of the gut lumen and the physiological processes occurring therein. In the last decade, the need of having systems that could better simulate *in vivo* conditions led to the creation and perfectioning of dynamic *in vitro* simulators that attempt to reproduce all or part of the physiological parameters that could influence the gastrointestinal microbial community and its metabolic activity (Macfarlane & Dillon, 2007; Minekus et al., 1999; Molly et al., 1993). These systems should allow recreating *in vivo*-like conditions in relation to the different sections of the GIT, i.e. stomach (secretion of gastric juice and simulation of fasted and fed condition); small intestine (secretion of pancreatic juice and bile salts, absorption of nutrients/electrolytes and high shear forces); large intestine (presence of a representative microbial community – in terms of activity and composition – in the ascending, transverse and descending colon); and finally, host-microbiota interaction (simulation of the final effect on the host).

Among the several options available on the market, the TIM model (TNO, Delft, The Netherlands) and the SHIME® (ProDigest and Ghent University, Gent, Belgium) are considered as being the most accurate imitations of the GIT both on a structural and a functional level (Pieper & Bertau, 2010).

The TIM model is composed of two separate computer-controlled units - TIM 1 and 2 - running independently (Minekus & Havenaar, 1996; Minekus et al., 1999). The TIM 1 system mimics the stomach and small intestine (i.e. duodenum, jejunum and ileum), while the TIM 2 is a simulation of the proximal colon of monogastric animals (pH = 5.8). Fluid transportation from vessel to vessel is executed by peristaltic valve-pumps and there is a constant absorption of water and of small lipophilic and hydrophilic compounds by means

of hollow fiber membranes. This system can be used to study drug-nutrient interactions, molecule bioconversion and nutrient compound bioavailability (Anson et al., 2009b; Blanquet et al., 2003). For example, the bioavailability of ferulic acid, an antioxidant, was studied with the TIM 1 system. It was found to be very variable and dependent on the food source (bran, flour, aleurone). However, supplementation of free ferulic acid to flour significantly increased its bioavailability. The authors concluded that the TIM model was a valid model to predict the *in vivo* bioavailability of ferulic acid (Anson et al., 2009b). In a TIM 2 simulation, the model is first inoculated with a frozen-conserved cell culture derived from a fecal inoculum. During the experiment, the system is fed with a 'Standard Ileal Efflux Medium' and samples can be taken both from the lumen of the simulator and from the dialyzed liquid during the simulation. It was shown by the TIM 2 model that bioprocessing of wheat bran (fermentation treatment or enzymatic- in combination with fermentation treatment) improves the colonic metabolism of ferulic acid (Anson et al., 2009a).

The prototype of the SHIME® was originally developed by Molly et al. (1993) (Figure 2). Nowadays, it is a computer-controlled system consisting of a succession of five reactors representing the complete GIT of the adult humans. The first two reactors are of the fill-and-draw principle and simulate the physiological processing occurring in the stomach and in the small intestine (i.e. different sigmoidal decrease of pH under fasted or fed conditions; addition of gastric enzymes, pancreatic and bile liquid). A dialysis filter is used to simulate the absorptive processes occurring in this area of the GIT. The last three compartments are continuously stirred reactors inoculated with a fresh dilution of a fecal sample (the characteristics of the donor can be decided according to the aim of the study). Retention time and pH of the different vessels are chosen in order to resemble *in vivo* conditions in the different parts of the GIT (pH in the range 5.6-5.9, 6.2-6.5 and 6.6-6.9 in the ascending, transverse and descending colon, respectively) (Possemiers et al., 2004). No absorption is simulated in the large intestine. In a TWINSHIME®, in which 2 systems are run in parallel, long-term (up to 3 weeks) placebo-controlled *in vitro* studies or direct comparison of two different treatments are possible without interference of external parameters (Grootaert et al., 2009; Marzorati et al., 2009; Possemiers et al., 2008). The possibility of performing long-term studies with the SHIME® is of special interest. In fact, it has to be taken into account that bacteria may need to adapt their metabolism in order to be able to degrade a specific active compound. In this respect, a study conducted with a single dose of the active product may results in a wrong interpretation of the possible effect of the microbial metabolism on the product itself. These long-term studies are of special relevance when investigating the chronic exposure to a given compound. The SHIME® model was found to be an ideal model to study the bioavailability of isoxanthohumol, a phytoestrogen, allowing to study the microbial metabolism in the different parts of the intestine (Possemiers et al., 2006). Moreover, using the TWINSHIME® model, the probiotic effect of the 8-PN producing strain *Eubacterium limosum* could be compared for high and low 8-PN producing individuals (Possemiers et al., 2008).

Whereas the TIM system appears nowadays as the most suitable simulator for the processes occurring in the upper GIT, the SHIME® can provide more reliable results in terms of simulation of processes at the level of microbial metabolism.

It has to be acknowledged that the simulation of absorption, which takes into account only diffusion, is limited in both systems, as well as the absence of a direct prediction of the possible effect of the treatment on the host (Pieper & Bertau, 2010). The coupling of these dynamic simulators with an "off-line" test system that makes use of Caco-2/HT 29 cell lines

has been proposed as a possible approach to increase the scientific outcome of the *in vitro* simulation (Deat et al., 2009; Possemiers et al., 2011a). Indeed, cellular models are a complementary tool to mimic the active uptake of active compounds and their metabolites. Moreover, the exposure of these cells to the complete luminal content of the GIT, containing both the active compound, its potential metabolites and the rest of the intestinal environment, creates a situation closer to the *in vivo* condition as compared to those studies where the cells are only exposed to the active compound as a pure product. This approach was recently used to study the immune modulating properties of a dried fermentate derived from *Saccharomyces cerevisiae*. The SHIME® experiment confirmed quantitative increases in lactobacilli, qualitative modulation of both general and specific populations, reduction of pathogens, and even showed an increase in the production of the immune-protective short-chain fatty acid, butyrate. Moreover, treatment of Caco-2 cell lines with the intestinal suspension significantly decreased the production of the pro-inflammatory cytokine IL-8 (Possemiers et al., 2011b).

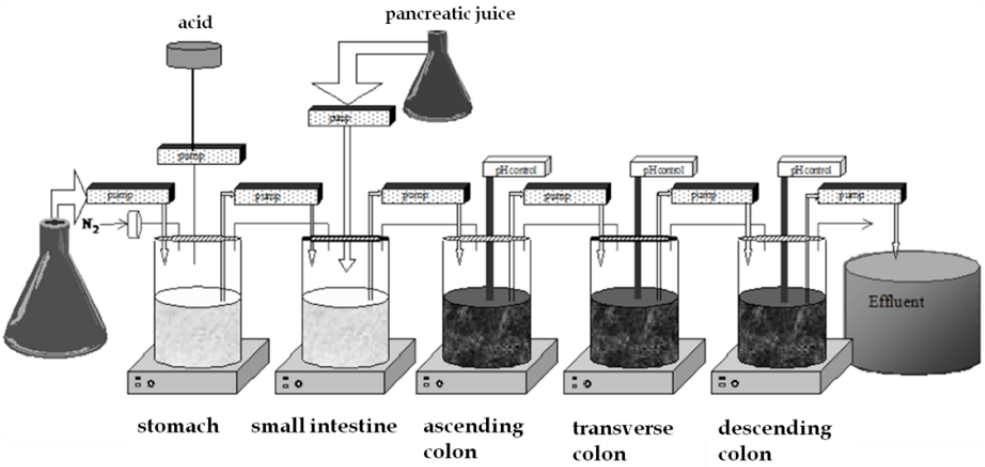

Fig. 2. Scheme of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®)

As compared to the state of the art, two further developments have been recently conducted in order to improve the simulation power of the SHIME®: the M-SHIME (Mucus-SHIME) and the HMI (Host-Microbiota Interaction) module. In the so-called M-SHIME, a mucosal compartment (mucin-covered microcosms) has been introduced in each colon vessel in order to reproduce the bacterial adhesion to the gut wall mucus layer (Van den Abbeele et al., 2011). This improvement allows evaluating the colonization and development of specific microorganisms that benefit from mucosal adhesion, microorganisms that would otherwise be washed out in those systems that only simulate the gut lumen. These are those microorganisms whose metabolism can have profound health effects in consideration of the fact they live in strict contact with the host surfaces.

Finally, the HMI module is a two-compartment model which allows to investigate in one anaerobic compartment the development of the mucosa-associated microbiota under realistic conditions of shear stress and to culture eukaryotic cells in the lower aerobic compartment for up to 48 h (Marzorati, 2010). The two compartments are separated by a

semi-permeable membrane that allows to simulate oxygen diffusion (micro-aerophilic conditions at the base of the biofilm) and bi-directional transport of molecules (i.e. absorption of microbial metabolites and excretion of host defence molecules). This new module has been tested in combination with the above-mentioned SHIME® in order to perform 'on-line' continuous experiments but, in principle, it can also be combined with other GIT simulators available on the market (Marzorati et al., 2011). Also in this model, the dried *Saccharomyces cerevisiae* fermentate was found to have immunomodulatory effects by decreasing the production of pro-inflammatory compounds, IL-8 and IL-1β (Marzorati et al., 2011). At the moment the system is conceived to evaluate the effect of microbial processes on the host cells and the effect of host cells on microbial processes. However, a simple addition of a third compartment would provide also the possibility of performing studies of bioavailability through cell lines (Figure 3).

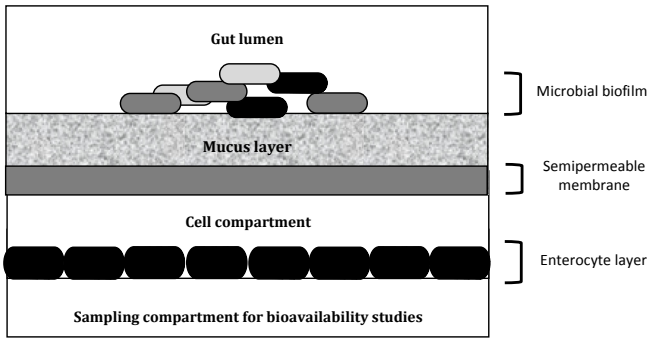

Fig. 3. Scheme of the Host-Microbiota Interaction (HMI) module. Adapted from Marzorati et al. (2011)

Table 1 summarizes the main characteristics of the two GIT simulators with respect to: i) the possibility of performing studies related to new drug development, and ii) the possibility to investigate the role of the microbial metabolism on the biotransformation of active compounds.

Another tool that can be useful in the cost-intensive process of a new drug development is the use of *in silico* models for biosimulation. These are normally considered as an alternative to the classical *in vivo* and *in vitro* studies. They make use of mathematical models to assess drug absorption, distribution, metabolism and excretion (Pieper & Bertau, 2010). The most commonly used models are the physiologically-based pharmacokinetics (Leahy, 2003), the systems biology-based drug metabolism simulation (Bugrim et al., 2004), the quantitative structure-activity relationship modelling (Chang & Swaan, 2006), and the computational oral absorption simulation (Sugano, 2009). More specific information on the topic has been recently reviewed by Bertau et al. (2008) and Pieper & Bertau (2010). A common trait of all these models is the fact they mainly focus on (hepatic) host metabolism, thereby omitting the role of the gut microbiota due to its extreme complexity and the lack of knowledge related to several metabolic processes occurring in the GIT. Even if the potential role of bacteria in metabolizing the active compound into other intermediates of degradation – that could have a deleterious effect for the human health or simply hinder the efficacy of the product itself – is clearly acknowledged, it is still not possible to model the complex

bacterial network within the gut. Currently on-going metagenomic and metatranscriptomic studies will provide us with further information with respect to this topic and, in a later stage, it will be possible to complement the already available models with a new set of data that will allow a simulation of the processes closer to the reality.

Characteristic	TIM	SHIME®
Simulation of the upper GIT	Yes	Yes
Simulation of the lower GIT	Yes	Yes
Full GIT in a single system	No	Yes
Simulation of the different regions of the small intestine	Yes	No[1]
Simulation of the different regions of the colon	No	Yes
Fecal inoculum	Frozen culture	Fresh inoculum[2]
Peristaltic movement	Yes	No[3]
Simulation of the absorption in the colon	Yes	No
Possibility of performing bioavailability studies (passive diffusion)	Yes[4]	Yes
Possibility of using the fluid from the system on Caco-2 cell lines to study active transport	Yes	Yes
Possibility of combining the simulator with the HMI module	No	Yes
Possibility of performing long-term studies[5]	No	Yes

[1] The pH increase along the small intestine can be simulated during the incubation
[2] Possibility of choosing a donor with specific characteristics
[3] Magnetic stirring
[4] TIM1 was specifically designed for this purpose
[5] To specifically follow up the adaptation of the microbial metabolism to the active compound

Table 1. Comparison of the TIM and SHIME® model

4. Case study: The gastrointestinal microbiota and inflammatory bowel diseases

In the next paragraph, the importance of the gut microbiome and its metabolism in drug development will be further discussed by means of a specific case study, i.e. IBD. The microbial metabolic potential as a therapy for IBD has been exploited and has provided a successful and widely used treatment for IBD which will be futher discussed in detail. Moreover, as no cure is available, there is an ungoing search for new therapies with the microbial potential as a very interesting target and tool.

4.1 The role of gut bacteria in immune homeostasis

Optimal immune functioning is crucial for the health and normal performance of humans and animals. The immune system operates at the systemic as well as at the local level, the latter which includes the mucosal tissue in the gut and upper respiratory tract. Whereas the systemic

immune system protects us from infections and disease in general, mucosal immunity has the important function of first line defence against penetrating allergens and pathogens.

Due to its unique function, about 70% of the body's immune system is found in the digestive tract. Indeed, the GIT is the site where the divergent needs of nutrient absorption and host defence collide. Whereas nutrient absorption requires a large surface area and thin epithelium, such design has the potential to compromise host defence. The body therefore needs an extensive immune protection in the gut to counteract this potential threat. The immune system of the gut divides into two parts, the physical barrier of the intestine and the active immune components. The physical barrier is central to the protection of the body to infections and the excessive penetration of allergens. Acid in the stomach, active peristalsis, mucus secretion and the tightly connected monolayer of the epithelium each play a major role in preventing microorganisms from entering the body. The cells of the immune system are organized in a complex pattern within the intestine, i.e. the gut-associated lymphoid tissue (GALT) (Gaskins, 1997). GALT comprises cells from both the innate and adaptive immune system. The innate immunity is responsible for the recognition of endogenous microorganisms, which is essential to maintain intestinal immune homeostasis. A schematic overview of the recognition, activation and response of the innate immune system is shown in Figure 4. Innate immune recognition is based on the detection of molecular structures that are unique to both pathogenic and non-pathogenic microorganisms, called microbe-associated molecular patterns (MAMPs), like lipopolysaccharide (LPS) or lipoteichoic acids (LTA) (Medzhitov, 2007). The main classes for the detection of MAMPs are pattern-recognition receptors (PRRs) including transmembrane Toll-like receptors (TLRs) and cytosolic nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) (Kelly et al., 2005). GALT has the constant challenge of responding to pathogens, while remaining relatively unresponsive to food antigens and commensal bacteria, the normal inhabitants of the gut (Sanderson & Walker, 2007). After the detection of the ligands, intracellular signaling results in the transcription of pro-inflammatory cytokines (Kawai & Akira). In response, the production of antimicrobial peptides (Salzman et al., 2010), tight junctions associated proteins (Su et al., 2009) and mucus that forms a protective polysaccharide glycocalyx bilayer on top of the epithelial cells (Johansson et al., 2008) is induced. While maintaining the capacity to eliminate infection and induce proper tissue repair, the host has to activate specific negative regulators and regulatory pathways to reduce the response to tissue injury.

Besides the activation of antimicrobial defence processes, the innate immune system can stimulate the adaptive immune response. The latter is activated by antigens that cross the epithelial barrier and are engulfed by antigen presenting cells (APCs) resulting in an antibody-mediated or cell-mediated immune response. The receptors on the immune cells provide a system by which infections with the same pathogen are remembered. This so called long-term memory is characteristic for the adaptive immune system (Carroll, 2004; Cooper & Alder, 2006).

Suboptimal functioning of the immune system in the gut may have important consequences for the gut environment itself (overstimulation may lead to excessive inflammation and inflammatory bowel diseases, whereas insufficient activity opens the way for pathogen infections) but may also affect the rest of the body (pathogen translocation or leakage of bacterial fragments from the gut into the blood may cause both acute and chronic inflammation).

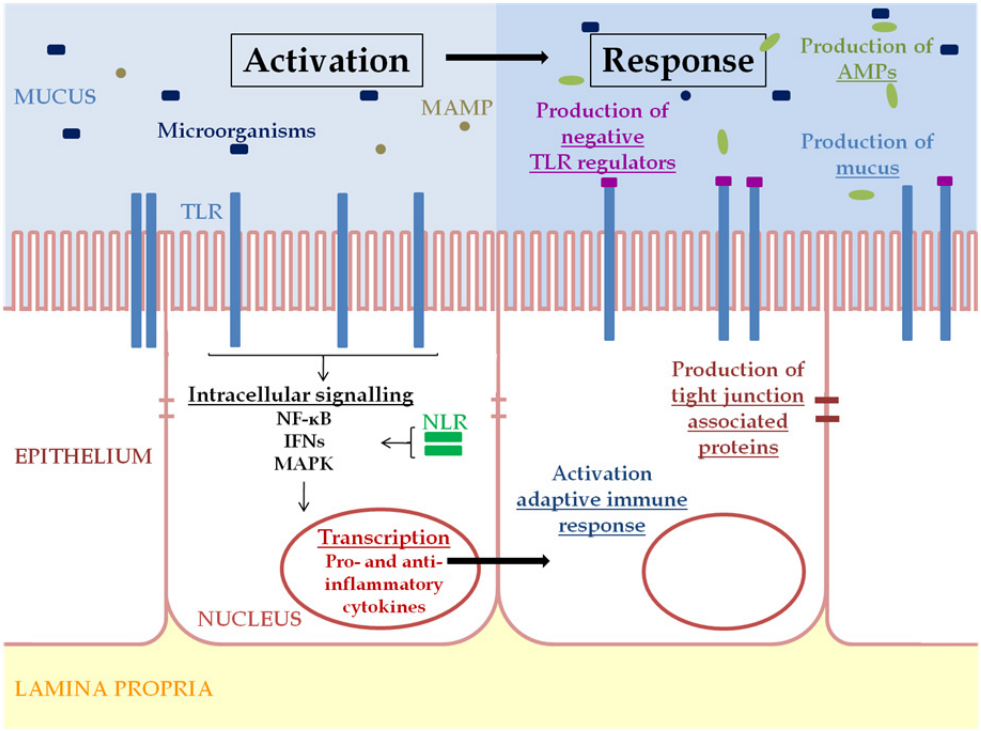

Fig. 4. Schematic overview of the activation and response of the innate immune system. AMP: antimicrobial peptides, IFNs: type I interferons, MAMPs: microbe-associated molecular patterns, MAPK: mitogen-activated protein kinase, NF-κB: nuclear factor-κB, NLR: nucleotide-binding oligomerization domain-like receptors, and TLR: Toll-like receptor

4.2 The role of gut bacteria in inflammation

IBD is a collective term for idiopathic and chronic inflammatory disorders of the intestinal tract. The best known forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Research from the last decade has given us new insights into the etiology of IBD although the cause still remains speculative. The most widely accepted theory is that a genetically dysregulated host immune response is over-aggressive against the commensal microbiota (Cho, 2008; Sartor, 2006; Strober et al., 2007).

Genome-wide association (GWA) studies have become a useful and powerful tool to identify disease-associated genes involved in the immunopathogenesis of IBD. The most well-known CD associated gene is NOD2 (also designated CARD15) (Hugot et al., 1996). Three single nucleotide polymorphisms (SNPs) are located within or near the LRR domain of the NOD2 protein, the domain that senses the bacterial products. Studies in primary human cells carrying the 3 major NOD2 SNPs have consistently demonstrated a deficient signalisation pathway what makes an intestinal immune response against the bacteria impossible. In contrast, in mice, NOD2 polymorphisms were shown to potentiate the NF-κB activity (Maeda, 2005; Watanabe et al., 2004). It is clear from these studies that the role of NOD2 mutation in the innate immune signaling is very complex and not completely

understood. Other genes that were associated with CD are two autophagy-related genes ATG16L1 and IRGM (Cho, 2008). GWA studies have further revealed that significant association of numerous SNPs throughout the IL23R gene region are associated with CD and UC (Duerr et al., 2006). Although genetic defects are apparent, they cannot explain completely the increase in the prevalence of IBD.

Several lines of evidence suggest a role for the microbiota in the development of IBD. The most compelling is probably the study by Garrett et al. (2007) in which T-bet deficient mice were used. T-bet regulates the response of the mucosal immune system to the commensal bacteria by controlling TNF-α production. T-bet deficient mice develop a disease that resembles UC. Remarkably, the transmission of the gut microbiota from T-bet deficient mice induced a colitogenic process in T-bet sufficient pups (Garrett et al., 2007). More evidence comes from animal models in which the presence of gut microbiota is required in order to develop intestinal inflammation (Nell et al., 2010; Saleh & Elson, 2011) and antibiotic treatments being effective in subsets of patients with CD (Perencevich & Burakoff, 2006).

One of the theories states that the pathogen *Mycobacterium avium* subspecies paratuberculosis (MAP) is the causative agent for IBD. However, studies based on the detection of MAP by PCR or ELISA in blood samples or biopsies have shown conflicting results (Autschbach et al., 2005; Baksh et al., 2004; Bentley et al., 2008; Clancy et al., 2007; Rowbotham et al., 1995; Wu et al., 1991). More recently, a newly defined *E. coli* pathovar, adherent-invasive *E. coli* (AIEC) was found to be highly associated with ileal mucosa of CD patients (Darfeuille-Michaud et al., 2004).

Other studies focused on a dysbiosis between the commensal microbial communities. Using a wide range of different techniques, changes in the population diversity of both the luminal and mucosa-associated microbiota have been demonstrated. Luminal changes were mainly associated with a decrease in the diversity of the Firmicutes, in particular Lactobacilli and Clostridia, and Bacteroidetes (Manichanh et al., 2006; Marteau, 2009; Scanlan et al., 2006; Sokol et al., 2009), while an increase in the Enterobacteriaceae population was reported for CD (Seksik et al., 2003). All reports described a reduced diversity of the mucosa-associated microbial communities in IBD. The reduction in diversity was comparable to the changes found in the luminal microbiota, i.e. a loss of Firmicutes and Bacteroidetes, particularly Lactobacilli and Eubacterium, with an increase in Enterobacteriaceae species for CD samples (Frank et al., 2007; Nishikawa et al., 2009; Ott et al., 2004; Tamboli et al., 2004). In addition, microscopy studies found an increased bacterial load in the mucosa of IBD patients (Swidsinski et al., 2005). Considering the different studies, no consensus has been reached concerning differences in the diversity of the fecal or mucosa-associated microbiota of IBD patients and healthy volunteers as etiological factor in IBD.

Finally, products of bacterial activity, such as butyrate are also known to have a regulatory effect on inflammation in IBD (Sanderson, 2004; Segain et al., 2000).

4.3 Innovative approaches for drug development in IBD

As dysbiosis in the microbiota is believed to be involved in IBD, strategies to deliberately modulate the microbiota have been developed. These strategies can be of nutritional origin using pro-, pre-, and synbiotics to induce directed changes in the microbial communities leading to health benefits for the host. Probiotics are live microorganisms that confer health benefits when administered in adequate amounts. Prebiotics are indigestible food compounds that selectively stimulate the growth and/or activity of one or a limited number of microbial species in the gut. Synbiotics are a combination of pro- and prebiotics. More

than 25 individual bacterial species (f. ex. *E. coli* Nissle 1917, *Saccharomyces boulardii* 750, *Lactobacillus rahmnosus* LGG), a few formulations containing multiple species (f. ex. VSL#3), short-chain carbohydrates (f. ex. fructooligosaccharides, galactooligosaccharides and arabinoxylans), germinated barley foodstuff or a combination of those have been studied using experimental models of colitis. Evidence from these animal models indicated that probiotics can alter the intestinal microbiota and ameliorate disease (Sartor, 2004) while prebiotic supplementation has been shown to enhance luminal immunoregulatory bacteria, reduce the risk of intestinal infections and the activity of pro-inflammatory transcription factors, and attenuate inflammation and mucosal damage (Cavin et al., 2005; Hedin et al., 2007; Holma et al., 2002; Kanauchi et al., 2008; Komiyama et al., 2011; Sartor, 2004). In addition, prebiotics can stimulate the production of short-chain fatty acids, such as propionate and butyrate, which are believed to improve colonic health. Probiotic therapy with *E. coli* Nissle 1917, bifidobacteria and bifidobacteria fermented milk showed efficacy and safety in maintaining remission of UC and had possible preventive effects on the relapse (Cui et al., 2004; Ishikawa et al., 2003; Kruis et al., 2004). Effectiveness in the induction of remission in UC patients was shown for combined therapy of VSL#3 with balsalazide and direct delivery of *E. coli* Nissle 1917 enemas to the colon (Matthes et al., 2010; Tursi et al., 2004). Germinated barley foodstuff showed a significant decrease in the mean clinical activity of patients with mild- to moderately-active UC (Mitsuyama et al., 1998). Moreover, the combination of oligofructose, inulin, and a probiotic strain (*Bifidobacterium longum*) showed an increase in mucosal-associated bifidobacteria concentrations associated with a decrease in both pro-inflammatory cytokines and antimicrobial defensins. However, the observed alterations in the mucosal cytokine balance was not translated into clinical changes (Furrie et al., 2005). Maintenance of remission in CD patients was reported to be effective when *S. boulardii* combined with the antibiotic mesalazine was administered (Guslandi et al., 2000). However, more studies report that pro-, pre-, and synbiotics are not effective in the induction and maintenance of remission in CD patients (Hedin et al., 2007). To summarize, some human trials indicate the effectiveness of pro-, pre-, and synbiotics in UC but they are scarce in CD. Due to variation in the populations studied, in the distribution of the disease, in the prevalence of genotypes, the small numbers of patients and the lack of details on the diet of the patients (Hedin et al., 2007), there is a marked heterogeneity in the performed studies making a consensus on the treatment of IBD with pro-, pre-, and synbiotics difficult and controversial.

As described above, a strategie which makes actively use of bacterial metabolism to improve drug bioavailability and which is used in the therapy of IBD, is linkage of the drug to a conjugate. Due to the metabolic activity of one or a few species of the microbiota, the release of the active compound is specific for the colon. By using this approach, side effects due to systemic release of the compound can be avoided. The most prevalent example in this category is sulfasalazine, a drug originally designed for the treatment of rheumatoid arthritis that was later discovered to also benefit patients with inflammatory disorders and which is widely used now. Due to the azoreductase activity of the colonic bacteria, the azo-bond connecting the active compound 5-ASA to sulfapyridine, is hydrolyzed. As the azoreductase enzyme is specific for colonic bacteria, 5-ASA is mainly released in the colon (Azad Khan et al., 1982) leading to a highly specific delivery of the active compound. While there is solid data supporting 5-aminosalicylic acid in the induction and maintenance of remission for UC, its efficacy in the treatment of CD is not as clear. One of the aspects involved is the effectiveness of the release of 5-ASA in the small intestines as the colonic release has not occurred yet in this

region. Limitations of sulfasalazine include allergic reactions and side effects, largely attributed to the sulfapyridine moiety. In response, two non-sulfapyridine-containing 5-ASA agents, balsalazide and olsalazine were developed (Patel et al., 2007). In addition, inhibition of sulfide production by 5-aminosalicylic acid-containing drugs was reported and may contribute to their therapeutic effect in UC (Edmond et al., 2003).

Furthermore, bacteria may be used as actual production facilities for local delivery of drugs as described above for carotenoid-producing spore-forming *Bacillus* spp.. A first example for IBD is the strain *Bacillus subtilis* PB6 that has been found to secrete surfactins and cyclic lipopeptides and was investigated for its therapeutic effect. Surfactins have exceptional emulsifying but also antibacterial, antiviral and antitumoral properties. As inhibitors of cytosolic phospholipase A2 (PLA2), surfactins may also function as anti-inflammatory agents. PLA2 is the key enzyme in the production of diverse lipid mediators and is involved in the pathophysiology of IBD. Oral administration of *B. subtilis* PB6 to TNBS-treated rats suppressed the inflammation which was seen on several parameters, i.e. mortality rate, weight gain, colon pathology and weight, and plasma levels of pro- and anti-inflammatory cytokines (Selvam et al., 2009).

The production capacity of a bacterium for a specific compound does not necessarily have to be an intrinsic property of the strain. For example, a specific strain of *Lactococcus lactis* has been genetically modified to secrete human interleukin-10 (IL-10), an anti-inflammatory cytokine. IL-10 is a good candidate for the treatment of IBD, yet direct injection of IL-10 induces several undesired side effects. Local delivery of IL-10 in the colon produced by *Lactococcus lactis* offers great advantages over the standard delivery method as the latter is associated with patient discomfort, various systemic side effects and costly production processes (Steidler et al., 2000). However, the use of genetically modified (GM) organisms in healthcare raises legitimate concerns on deliberate release and potential spread of the GM trait. To prevent spreading of the transgene and the GM bacteria into the environment, the thymidylate synthase gene (*thyA*) was replaced with the expression cassette for *hIL10*. *ThyA* codes for an enzyme necessary for the synthesis of the nucleobase thymine and nucleoside thymidine. As a result, the modified strain can only survive when thymidine or thymine are available in the intestinal environment. When deprived of one of these compounds, thymineless death is rapidly induced, preventing the accumulation of the GM bacteria in the environment. Moreover, as thymineless death results in the fragmentation of the DNA, the chances for uptake of *L. lactis* DNA by other strains are very small (Steidler et al., 2003). Several health authorities and biosafety committees have positively evaluated this containment strategy (Rottiers et al., 2009). A phase I trial with the modified *L. lactis* strain (LL-Thy12) showed promising results as maintenance treatment in CD avoiding systemic side effects (Braat et al., 2006). A second example of a bacterium engineered as production facility is a genetically modified *L. lactis* able to secrete the low calcium response V (LcrV) protein from the enteropathogenic species *Yersinia pseudotuberculosis*. Oral administration of this *L. lactis* induced the expression of IL-10 in the colon and decreased inflammation in 2 murine models of colitis, i.e. TNBS and DSS (Foligne et al., 2007). As these effects were absent in the IL-10$^{-/-}$ mice model, this study once more confirmed the therapeutic potential of IL-10. Recently, Steidler and colleagues proposed *L. lactis* genetically engineered to secrete anti-TNF nanobodies, trefoil factor (TFF) or IL-27 as promising therapeutics for IBD (Durum et al., 2010; Vandenbroucke et al., 2010; Vandenbroucke et al., 2004). These strains have only been tested in preclinical studies.

Table 2 summarizes the different strategies for drug development in which the microbial metabolism plays a key role.

Approach	Species involved	Specific bacterial activity	Advantages	Progress of development	References
Targeted alterations of the gut microbiota	Probiotics f. ex. *E. coli* Nissle 1917, bifidobacteria fermented milk, VSL#3	Modulation of the host-microbe interactions by the growth of health-promoting bacteria	Inhibition of intestinal pathogens Improve epithelial and mucosal barrier function Alteration of the immunoregulation	Commercialized	(Cui et al., 2004; Guslandi et al., 2000; Ishikawa et al., 2003; Kruis et al., 2004; Matthes et al., 2010; Tursi et al., 2004)
	Prebiotics f. ex. germinated barley foodstuff, arabinoxylans			Commercialized	(Komiyama et al., 2011; Mitsuyama et al., 1998)
	Synbiotics f. ex. oligofructose, inulin, and *Bifidobacterium longum*			Commercialized	(Furrie et al., 2005)
Microbial metabolism for colon targeted drug release	Colonic bacteria	Azo-reductase and others for the conversion of the prodrug to the active compound	Specific release of the active compound in the colon	Commercialized f. ex. sulfasalazine, olsalazine, balsalazide	(Azad Khan et al., 1982)
	Colonic bacteria	Azo-reductase and others for the degradation of the polymer capsule		Commercialized f. ex. mesalazine, budesonide	(Chavan et al., 2001; Kimura et al., 1992)
Bacteria as production facilities-	Cyanobacteria, *Burkholderia* spp., Actinomycetes, Myxobacteria	Production of new classes of antibiotics	Natural product to combat resistant pathogens	Preclinical studies	(Donia et al., 2008; Partida-Martinez & Hertweck, 2005; Scott et al., 2008; Wenzel & Muller, 2009)
	Carotenoid producing *Bacillus* spores	Production of carotenoids	Resistant to gastric conditions but germination under colonic conditions Storage at ambient temperature	Preclinical studies	(Duc et al., 2006; Hong et al., 2009; Khaneja et al., 2010; Perez-Fons et al., 2011)
	Bacillus subtilis PB6 spores	Production of surfactins		Preclinical studies	(Selvam et al., 2009)
	Lactococcus lactis	Production of hIL-10	Oral administration Efficient local delivery More favorable side effect profile Cost-efficient manufacturing process	Ongoing large-scale, double-blind, placebo-controlled phase IIA trial	(Braat et al., 2006; Rottiers et al., 2009; Steidler et al., 2000; Steidler et al., 2003)
		Production of Trefoil factor (TFF)		Preclinical studies	(Vandenbroucke et al., 2004)
		Production of low calcium response V (LcrV)		Preclinical studies	(Foligne et al., 2007)
		Production of anti-TNF nanobodies		Preclinical studies	(Vandenbroucke et al., 2010)
		Production of IL-27		Preclinical studies	(Durum et al., 2010)

Table 2. Innovative approaches for the development of biopharmaceuticals making benefit of specific microbial functionalities

5. Conclusion

This chapter has highlighted the enormous potential of the gut microbial metabolism in the modulation of nutritional compounds, drugs and environmental contaminants. We have shown that the intestinal microbiome can be involved in different levels of the ADME characteristics of active compounds. The microbiota can interfere in the absorption of drugs, f. ex. by slow release of the active compound. They can interfere in the metabolism as illustrated by many examples and they may prolong the action of drugs by allowing their enterohepatic circulation to continue and to inhibit excretion. Moreover, the microbial metabolism from ingested compounds can have varying responses, which can be beneficial but can cause some serious risks as well. Due to their interference in ADME and the resulting health effects, a complete understanding of the full metabolic potency of the gut microbiome to predict its modulating effect on xenobiotics is emerging. Gut microbial processes therefore need to be incorporated in pharmacokinetic models.

To achieve full understanding of the microbial potential, the development of suitable *in vitro* models is essential. Two dynamic GIT simulators, the TIM and SHIME® model have been developed for this purpose, each with their own specific advantages an disadvantages to investigate the role of the microbial metabolism on the biotransformation of active compounds. Incorporation of absorptive processes in these models drastically improves the simulation of the bioavailability of the active compounds and by incorporating host cells in the SHIME® model, this model will be a useful tool to evaluate the effect of microbial processes on the host cells and vice versa.

I*n vitro* models do not merely offer opportunities to understand the biotransformation of active compounds but they offer the possibility to investigate the metabolic fate of newly developed drugs. Moreover, new strategies making use of the microbial metabolic potential to improve drug efficacy were discussed going from the local release of active compounds from prodrugs to engineering of strains to secrete specific health promoting compounds. Inflammatory disorders offer the perfect case for the application of these strategies. What has been described until now is only the beginning of a new generation of drugs making use of the enormous potential of the intestinal microbiome.

Finally, given the potential implications the microbiota may have on the stability, bioavailablity and safety of xenobiotics, assessment of the activity of the intestinal microbiome should become a standard process in pharmaceutical drug development. The microbial potential should be further exploited to improve drug development and develop new strategies. By the ongoing technical improvements of *in vitro* models, these offer a valid tool to evaluate the bioavailability of new compounds and their therapeutic effect on host cells. Moreover, as personalized health care is becoming more and more integrated in modern medicine, interindividual variability in the gut microbiome should be an integral part of this process.

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Endophytic Fungi of Tropical Forests: A Promising Source of Bioactive Prototype Molecules for the Treatment of Neglected Diseases

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1. Introduction

Endophytic fungi are ubiquitous fungi that reside inter- or intracellularly in plant parts for at least a portion of their lives without causing apparent symptoms of infection (Petrini, 1991) and represent a large portion of fungal species. Endophytic fungi can be biotrophic mutualists, benign commensals, decomposers or latent pathogens (Promputtha et al., 2007). According to Rodriguez et al. (2009), all plants in the natural environment can shelter endophytic fungi, including algae, mosses, ferns, conifers and angiosperms. This fungal group appears to significantly influence the lifestyle of its host. Taxonomically, most of the endophytic fungi belong to the phylum *Ascomycota* and its associated anamorphs, while some species belong to the phyla *Basidiomycota* and *Zygomycota* (Huang et al., 2001). There have been many studies on the diversity, ecology and biotechnological applications of endophytic fungi in grasses and wood plants in temperate environments. However, there is limited information about the diversity of endophytic fungal communities in tropical forests, which are endowed with a rich biodiversity of flora. Dreyfuss & Chapela (1994) have estimated that approximately 1.3 million species of endophytic fungi remain to be discovered. This diverse fungal group could impact the ecology, fitness and shape of plant communities, conferring resistance to abiotic (temperature, pH, osmotic pressure) and biotic (from bacteria, fungi, nematodes and insects) stresses (Rodriguez et al., 2001). Endophytic fungi are an important source of bioactive molecules. These bioactive metabolites have a broad range of biological activities and could be the starting materials for pharmaceuticals or lead structures for the development of pharmaceutical or agrochemical products. The substances produced by endophytic fungi originate from different biosynthetic pathways, including isoprenoid, polyketide and amino acid and belong to diverse structural groups, such as terpenoids, steroids, xanthones, quinones, phenols, isocoumarins, benzopyranones, tetralones, cytochalasins and enniatins (Schulz et al., 2002). Indeed, these bioactive

molecules represent a chemical reservoir for discovering new compounds, such as antibiotic, antioxidant, immunomodulating, anticancer and antiparasitic compounds, for use in the pharmaceutical and agrochemical industries.

2. Neglected tropical diseases

The neglected tropical diseases (NTDs), a group of chronic, debilitating and poverty-promoting parasitic, bacterial, viral and fungal infections, are among the most common causes of illness in the poorest people living in developing countries (Hotez et al., 2008). NTDs cause over 500,000 deaths annually and are estimated to result in a greater number of lost disability-adjusted life years than malaria and tuberculosis (Hotez et al., 2006, 2009). These diseases flourish in areas where water supply and sanitation are inadequate, nutrition is poor, literacy rates are low, health systems are rudimentary, and insects and other disease vectors are constant household and occupational companions. Not surprisingly, these diseases cluster together and frequently overlap where these conditions occur. In fact, the link with poverty is so strong that the prevalence of these diseases serves as an indicator of the level of a country's socioeconomic development (WHO, 2006).

The NTDs have some common features. They occur in impoverished settings and are chronic conditions; victims can harbour NTDs for years or decades, frequently resulting in disability, disfigurement and stigmatisation. Not only are these diseases of poverty, but they also promote poverty because of their effects on child development, cognition and education as well as on adult agricultural worker productivity (Hotez & Yamey, 2009). These diseases have also been neglected by research and development. There is little incentive for industries to develop drugs and vaccines for markets that cannot pay. When inexpensive and effective drugs already exist and are available, delivery fails because patients cannot pay and health systems are weak or non-existent (WHO, 2006).

The most important bacterial NTDs are trachoma, leprosy, and some of the bacterial zoonoses, especially leptospirosis (Hotez et al., 2008). Interruption and default of therapies against bacterial NTDs are still important obstacles to disease control in many endemic countries, with consequences for both patients and control programs; low adherence results in potential remaining sources of infection, incomplete curing and irreversible complications and may lead to multidrug resistance (Heukelbach et al., 2011).

Several mycoses, such as paracoccidioidomycosis (PCM), are also responsible for major public health and economic hardships in Latin America (Hotez et al., 2008). The drugs most commonly used for treating patients with paracoccidioidomycosis (PCM) are sulphonamides, ketoconazole, itraconazole and amphotericin B. Extended periods of treatment are necessary, and there are increasing concerns about drug toxicity, the cost of treatment, and unacceptable rates of noncompliance with these therapies (Travassos et al., 2008).

The most important viral NTDs are dengue and yellow fevers (Hotez et al., 2008). Tropical climates have experienced a great resurgence in dengue fever in recent years, and it appears to be spreading to new areas (Carroll et al., 2007). The WHO reports that two-fifths of the world's population is at risk of dengue infection, with an increase in the annual number of cases (Murrell et al., 2011). There is no specific treatment for dengue fever. Dengue fever is an increasing concern because of the lack of a licensed vaccine that protects against all four dengue serotypes (WHO, 2006). The increase in dengue infections and the prevalence of all four circulating dengue serotypes has contributed to a rise in the incidence of dengue haemorrhagic fever (Murrell et al., 2011).

Yellow fever originated in Africa and was imported to Europe and the Americas as a consequence of the slave trade between these continents (Gardner & Ryman, 2010). An inexpensive live attenuated vaccine against yellow fever (the 17D vaccine) has been effectively used to prevent yellow fever for more than 70 years. Interest in developing new inactivated vaccines has been spurred by the recognition of rare but serious and sometimes fatal adverse events following live virus vaccination (Hayes, 2010).

Leishmania (*Trypanosomatidae*) are protozoan parasites that cause high morbidity and mortality levels and are recognised by the WHO as a major tropical public health problem (Asford, 1997). There are currently no vaccines for leishmaniasis; although the drugs available for leishmaniasis treatment are toxic, expensive and sometimes ineffective, they are the only effective way to treat all forms of the disease (Croft & Coombs, 2003). Chagas disease (American *Trypanosomiasis*) is caused by the haemoflagellate protozoan *Trypanosoma cruzi* and is transmitted to humans either by blood-sucking triatomine vectors, blood transfusion or congenital transmission. The geographical distribution of human *T. cruzi* infection extends from the southern United States and Mexico to southern Argentina (WHO, 1991). According to Reyes & Vallejo (2005), there is evidence that trypanocidal drug treatment with nitrofuran and imidazole compounds can treat acute *T. cruzi* infection, but further studies are needed to develop new trypanocidal drugs.

Helminths are parasitic worms that are the most common agents of human infection in developing countries and include the NTDs schistosomiasis, cysticercosis and onchocerciasis. There are two major phyla of helminths, which include the major intestinal worms, filarial worms that cause lymphatic filariasis and onchocerciasis and platyhelminthes such as the schistosomes and the agent of cysticercosis (Hotez, 2008). Only the drugs albendazole, oxamniquine, praziquantel and ivermectin are available to treat helminthiasis (Hotez, 2008). New advances in helminth biology, particularly molecular techniques, have led to the identification of new targets for the discovery and development of anthelmintic drugs.

3. Diversity of tropical endophytic fungi

Dreyfuss & Chapela (1994) have estimated that there are 1.3 million species of endophytic fungi alone, the majority of which are likely found in tropical ecosystems. This estimate is supported by various studies that have sought to characterise the fungal communities associated with tropical plants. Fungal endophytic communities are divided into two basic groups: generalists (which are found in high abundance among different plant species) and singletons (which are found in low abundance and in a specific plant host). Tropical plants are expected to shelter a highly diverse population of endophytic fungi, but few tropical plants have been screened for their presence. Studies have shown that tropical plants shelter a great diversity of singleton species.

According to Hawksworth (2004), the magnitude of fungal diversity in tropical forests is unclear, and new species remain to be described. The greatest fungal diversity probably occurs in tropical forests, where a highly diverse population of angiosperms is present (Arnold et al., 2000). In support of this proposal, a large number of fungal endophytic species have been described in association with plants in Asia, Australia, Africa, Central and South America, Mexico and some Pacific and Atlantic Islands. However, the diversity of endophytic fungi can vary across different biomes of a tropical forest. Suryanarayanam et al. (2002) showed that the endophytic fungal assemblage of a dry tropical forest had much less

endophyte diversity than a wet tropical forest. Endophytic fungi can be passive residents or act as an assemblage of latent pathogens in their host (Ganley et al., 2004). Arnold et al. (2000) suggested that endophytic fungi are hyperdiverse and that 1.5 million species may be an underestimate of their magnitude. In addition, the taxonomic placement of tropical fungi has been confounded by misidentifications made in comparison with temperate fungal communities, including the endophytic fungal community present in the leaves of tropical plants (Arnold et al., 2001).

In general, endophytic fungi have been categorised into two main groups based on differences in evolution, taxonomy, plant hosts and ecological functions: clavicipitaceous, which are able to infect only some species of grasses, and nonclavicipitaceous, which are found in the asymptomatic tissues of bryophytes, ferns, gymnosperms and angiosperms (Rodriguez et al., 2001). Clavicipitaceous endophytes belong to the family *Clavicipitaceae* (*Hypocreales*; *Ascomycota*), many species of which are known to produce bioactive molecules (mainly of the genera *Cordyceps*, *Balansia*, *Epichloë*/*Neotyphodium*, *Claviceps* and *Myriogenospora*). In contrast, nonclavicipitaceous endophytes are a large group that have not been well-defined taxonomically, but the majority of the species belong to the phyla *Ascomycota* and *Basidiomycota*, represented by the genera *Alternaria*, *Arthrobotrys*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Coprinellus*, *Curvularia*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phoma*, among others. Different species of these two endophytic groups have been investigated for their ability to produce various molecules, and species living in association with tropical plants have been shown to be significant producers of bioactive metabolites.

4. Hosts of tropical bioactive endophytic fungal communities

Tropical and temperate forests are considered to be the most diverse terrestrial ecosystems, with the greatest number and diversity of endophytic fungi (Strobel, 2002). The constant innovation present in ecosystems where the evolutionary race to survive is the most active may result in the production of a plethora of chemical molecules (Strobel, 2006). Tropical rainforests are an important example of this type of environment: there is great competition, resources are limited, and selection pressure is at its peak. Consequently, there is a high probability that fungi associated with tropical hosts may be a source of novel molecular structures and compounds that are active against neglected diseases.

Endophytic relationships may have begun from the time that higher plants first appeared hundreds of millions of years ago. Evidence of plant-associated fungi has been discovered in fossilised tissues of stems and leaves (Taylor et al., 1999). As a consequence of these long-term associations, some of these microorganisms may have developed genetic systems that allow the exchange of information between themselves and the higher plant. This exchange would allow the fungi to more efficiently cope with the environmental conditions and perhaps increase compatibility with the plant host. Moreover, the dependent evolution of endophytic fungi may have allowed them to better adapt to the plant such that the fungi could contribute to the relationship by performing protective functions against pathogens and insects (Petrini et al., 1992; Strobel & Daisy, 2003; Gunatilaka, 2005). To make these contributions to their plant hosts, endophytic fungi may produce secondary metabolites that have potential uses in agriculture, medicine and industry (Strobel & Daisy, 2003).

Each of the approximately 300,000 known plant species may host at least one endophytic fungus. As tropical and subtropical regions harbour most of the world's plant diversity,

endophytic fungal diversity in this climatic zone is also higher, and all vascular plant species examined to date possess an endophytic fungus. According to Strobel (2003), reasonable guidelines should govern the plant selection strategy for the discovery of bioactive endophytic fungi, which would include plants that are found in unique environmental settings, have ethnobotanical histories, or are endemic or growing in regions of high diversity. All of these selection strategies are applicable for the isolation of endophytic fungi from tropical and subtropical hosts, and these microorganisms can be obtained from the leaves, stems, petioles, barks and roots of many tropical angiosperms.

5. Techniques for the isolation and identification of endophytic fungi

The methods used to isolate endophytic fungi vary in the technique used for surface-disinfection of the host plant tissue (leaves, stems, roots, bark, flowers, fruits and seeds) and the choice of culture media. The disinfection process can influence the detection of endophytic fungi; in general, the plant surface is disinfected with a strong oxidant or disinfectant agent for a specific period of time. The most commonly used agents include 1-4% detergent, 3% H_2O_2, 2-10% NaOCl, or 70-95% ethanol. The culture medium is another important parameter. Commonly used media include potato dextrose agar, malt extract agar, yeast malt agar and Sabouraud agar, supplemented with antibacterial agents (chloramphenicol, penicillin, ampicillin, tetracycline and streptomycin, among others) to suppress contaminating bacteria and isolate endophytic fungi.

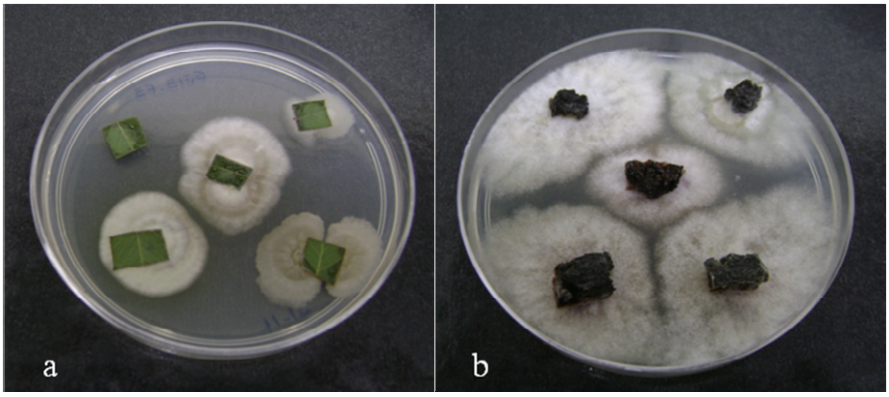


Fig. 1. Mycelium of endophytic fungi emerging from the (a) leaves and (b) bark of the medicinal tropical Brazilian plant *Stryphnodendron adstringens*.

After isolation, the endophytic fungi, including the bioactive species, must be identified correctly. Macro- and micromorphological cultural characteristics, molecular analyses and metabolite profiles are the main criteria that are used to identify endophyte fungal taxonomy. The identification of endophytic fungi relies significantly on the taxonomic expertise of the mycologist and frequently requires polyphasic taxonomy. In tropical regions, multiple endophytic fungal species are recovered and are commonly grouped based on similar culture characteristics into morphospecies (Figure 2), which represent a functional taxonomic unit for endophytic fungal species (Arnold et al., 2000). After characterisation as a morphospecies, endophytic fungi are submitted to molecular grouping

using microsatellite markers that are detected with $(GTG)_5$, M13 or EI primers based on PCR-fingerprinting methods that amplify genomic segments different from the repeat region itself (Lieckfeldt et al., 1993).

Fig. 2. Morphospecies of endophytic fungal isolates associated with the tropical plant *Vellozia graminea*.

However, most endophytic fungi (about 50%) do not produce conidia or spores when cultured on common mycological media. In these cases, endophytic fungi can frequently be identified based on the sequence of the Internal Transcribed Spacer (ITS) region of the large subunit of the rRNA gene. Molecular techniques are a powerful tool for identifying the endophytic genera and species of non-sporulating fungi. After sequencing the ITS1–5.8S–ITS2 region, the sequence of the endophytic fungus is compared with the sequences of other taxa deposited in public databases. The GenBank database is a major source of nucleotide sequences.

In addition, endophytic fungi produce a large number of metabolites, and certain molecules are very consistent at the species level in some genera when cultured under standardised conditions. According to Larsen et al. (2005), fungal isolates of different species have different chemotypes, which can be differentiated or grouped by modern methods for dereplication analysis. The chemotaxonomic analysis begins with the preparation of the fungal extract, which typically requires the media potato dextrose agar (PDA), Sabouraud agar, malt extract agar (MEA), yeast extract sucrose agar (YES) or Czapek yeast autolysate agar (CYA). The chemical analysis includes techniques such as Thin Layer Chromatography (TLC), Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS), and Nuclear Magnetic Resonance (NMR), alone or in combination, in association with informatics tools.

6. Fermentation techniques and crude extract production

Secondary metabolites are compounds with varied and sophisticated chemical structures that are usually produced only during the stationary phase of growth (Robinson et al., 2001). These compounds do not have a physiological role during exponential phase, and their production starts when a key nutrient source, such as carbon, nitrogen or phosphate, is

exhausted (Barrios-González & Mejia, 1996). The last two decades have been a period of rapid discovery of new biological activities of these compounds, and appropriate modern strategies for identifying metabolites are essential (Petrini et al., 1992).

Endophytic fungi must first be acquired in pure culture, and optimal media and growth conditions must be determined to begin microorganism fermentation and production of crude extracts. These extracts can then be separated by various chromatographic procedures to yield the substance of interest. Factors that can quantitatively and qualitatively affect the production of secondary metabolites include temperature, pH, medium composition, culture duration and the degree of aeration. These parameters can be manipulated and modified to improve the production of compounds of bioactive significance (Barrios-González & Mejia, 1996).

There are several media that can be used to cultivate endophytic fungi, such as potato dextrose agar (PDA), corn meal agar (CMA), oatmeal agar (OMA) and Czapek yeast autolysate agar (CYA). The medium is chosen based on the purpose and the species under investigation. Liquid media are usually used for physiological studies, but agar media are more convenient and practical for the rapid screening of plentiful isolates (Hölker et al., 2004). To isolate endophytic fungal secondary metabolites, fermentation techniques such as Submerged fermentation (SmF) (or Liquid fermentation) and Solid-state fermentation (SSF) have become widely used (Table 1) (Barrios-González & Mejia, 1996; Pandey, 2003; Hölker et al., 2005).

Characteristics	**Solid-State**	**Submerged**
Microorganism, substrate	Static	Agitated
Water usage	Limited	Unlimited
Oxygen supply by	Diffusion	Aeration
Volume of fermentation	Smaller	Larger
Energy requirement	Low	High
Capital investment	Low	High
Concentration of the end product	High	Low
Sterility demands	Low	High
Simulation of the natural environment	Better	Worse

Table 1. Comparison of the main characteristics of Solid-state fermentation and Submerged fermentation.

Although the SmF and SSF techniques differ, both can be used to identify secondary metabolites produced by endophytic fungi. Figure 3 illustrates examples of SmF and SSF processes for obtaining these substances for screening programs. However, the appropriateness of a given technique should be evaluated based on the aim of the study and the available resources. In addition, optimal parameters for both techniques, such as incubation conditions, medium composition, agitation, temperature and pH, must be standardised to improve process efficiency and maintain reproducibility.

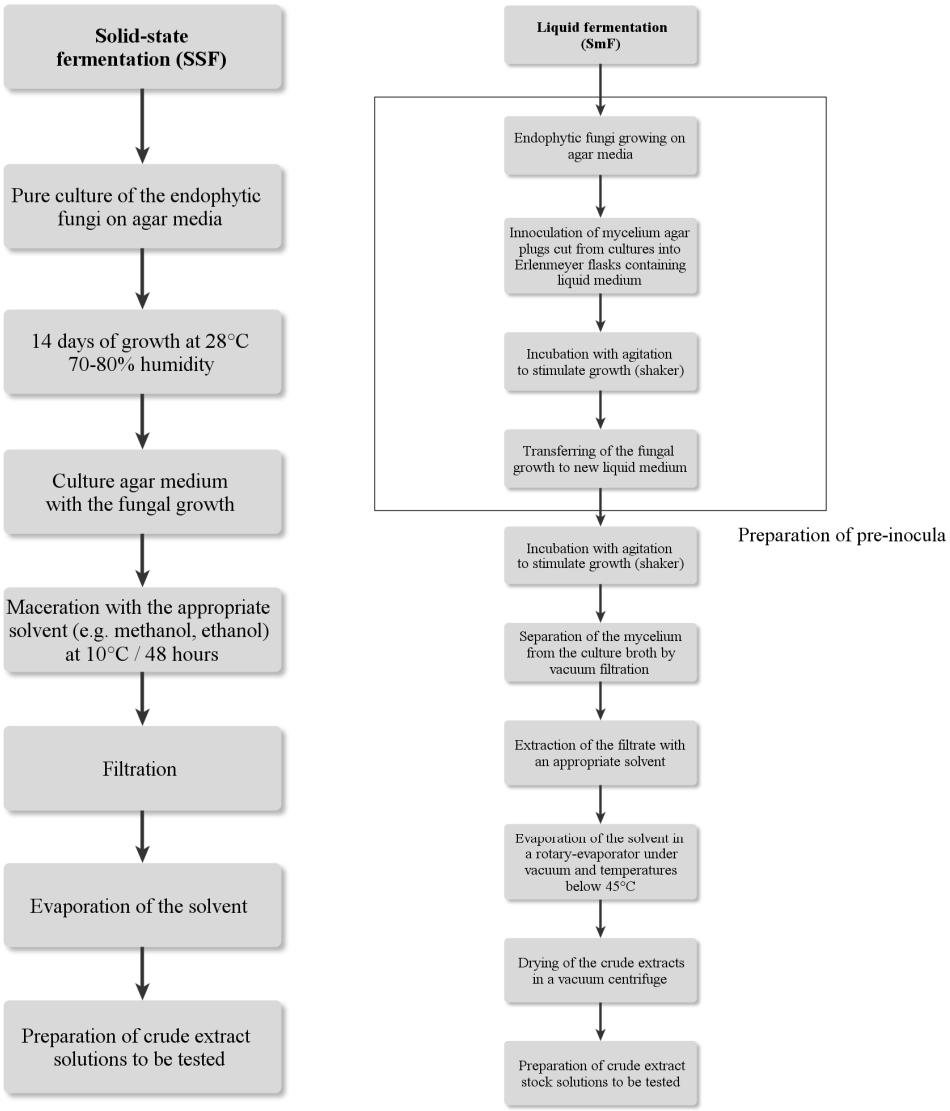


Fig. 3. Liquid fermentation and Solid-state fermentation processes for obtaining endophytic fungal secondary metabolites.

7. Bioactive compounds against neglected diseases

To date, fungal metabolites have primarily served as lead structures for the development of anticancer, antifungal and antibacterial agents. Although new drugs are needed to treat all aspects of leishmaniasis, the scientific literature on the bioprospecting of endophytic fungi of tropical rainforests is limited. Brazilian ecosystems are a potential source of endophytic

fungi that are able to produce bioactive prototype molecules for developing drugs to combat NTDs. *Penicillium janthinellum* was isolated as an endophytic fungus from the fruit of *Melia azedarach* (*Meliaceae*), a plant collected in Brazil. Methanol extract fractionation furnished the known polyketide, citrinin (Fig. 4), which was previously found in *Penicillium citrinum* and several *Aspergillus* species (Vrabcheva et al., 2000) and inhibited 100% of *Leishmania mexicana* at a concentration of 40 µg/mL (Marinho et al., 2005).

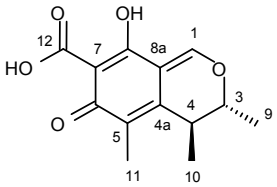


Fig. 4. Citrinin.

The endophytic fungus *Edenia* sp. was isolated from a mature leaf of *Petrea volubilis* (*Verbenaceae*), which was collected from the Coiba National Park in Panama. Bioassay-directed fractionation of organic extracts of *Edenia* sp. led to the isolation of the antileishmanial compounds preussomerin EG_1 [IC_{50} 0.12 µM (Fig.5, 2)], palmarumycin CP2 [IC_{50} 3.93 µM (Fig. 5, 3)], palmarumycin CP17 [IC_{50} 1.34 µM (Fig. 5, 4)], palmarumycin CP18 [IC_{50} 0.62 µM (Fig. 5, 5)], CJ-12,37 [IC_{50} 8.40 µM (Fig. 5, 6)], palmarumycin CP19 [IC_{50} 11.6 µM (Fig. 5, 7)] and 5-methylochracin (IC_{50} 33.4 µM), which inhibited the growth of amastigote forms of *Leishmania donovani*. Preussomerin EG1 was the most active substance and inhibited growth of *L. donovani* with a potency similar to that of amphotericin B (IC_{50} 0.09 µM) (Martínez-Luis et al., 2009).

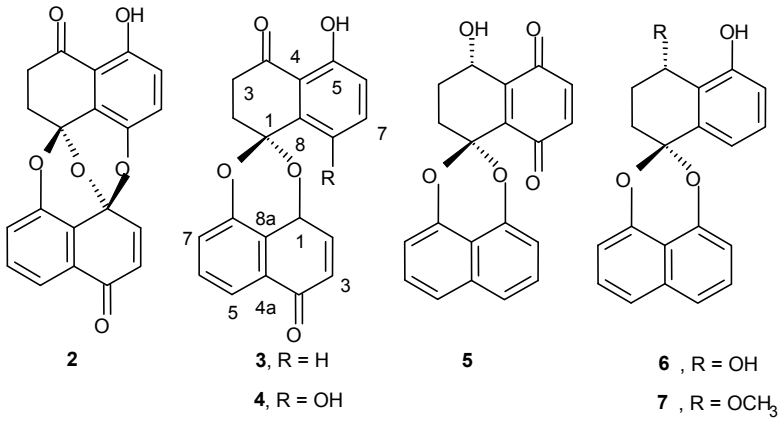


Fig. 5. Preussomerin EG_1 (2), palmarumycin CP2 (3), palmarumycin CP17 (4), palmarumycin CP18 (5), CJ-12,37 (6) and palmarumycin CP19 (7)

The isolate UFMGCB 555, obtained from the plant *Piptadenia adiantoides* and identified as *Cochliobolus* sp., produces cochlioquinone A (Fig.6, 8) and isocochlioquinone A (Fig. 6, 9). Both compounds were active in an assay against *L. amazonensis*, with EC_{50} values of 1.7 µM and 4.1 µM, respectively (Campos et al., 2008).

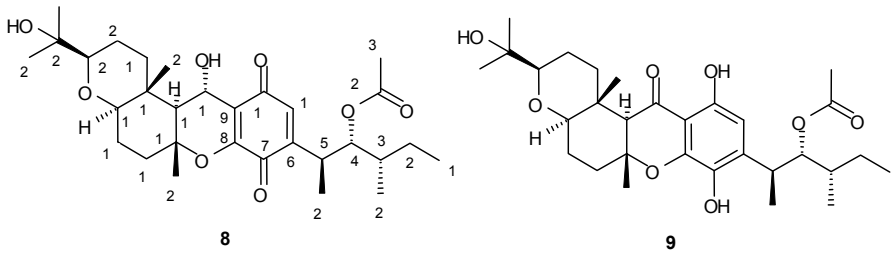


Fig. 6. Cochlioquinone A (8) and isocochlioquinone A (9)

Grandisin (Fig. 7, 10), a tetrahydrofuran lignan isolated from *Piper solmsianum* (*Piperaceae*) (Martins et al., 2003) and *Virola surinamensis* (*Myristicaeae*) (Lopes et al., 1996), has potent trypanocidal activity against the trypomastigote form of *T. cruzi* at 5 µg/mL (Lopes et al., 1998). Biotransformation of this compound by the endophytic fungus *Phomopsis* sp., obtained from *Viguiera arenaria*, yielded a new compound, 3,4-dimethyl-2-(4′-hydroxy-3′,5′-dimethoxyphenyl)-5-methoxy-tetrahydrofuran (Fig. 7, 11). The metabolite had trypanocidal activity (IC_{50} 9.8 µmol/mL) similar to the natural precursor (IC_{50} 3.7 µmol/mL) (Verza et al., 2009).

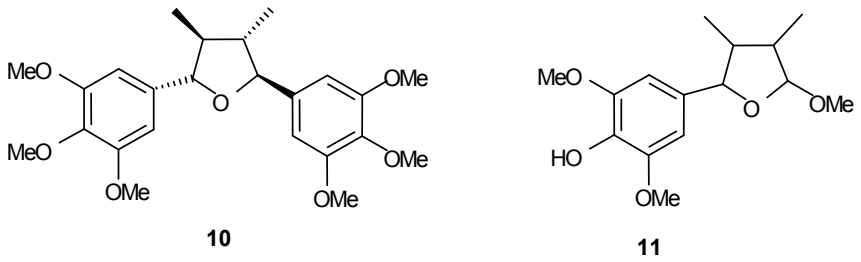


Fig. 7. Grandisin (10) and 3,4-dimethyl-2-(4′-hydroxy-3′,5′-dimethoxyphenyl)-5-methoxy-tetrahydrofuran (11).

Altenusin (Fig. 8) is a metabolite obtained from the organic extract of a broth culture of the endophytic fungus *Alternaria* sp. UFMGCB 55, which was isolated from a plant known to contain trypanocidal compounds, *Trixis vauthieri*. This fungus inhibited TryR enzymatic activity with an IC_{50} value of 4.3 mM (Cota et al., 2008). The endophytic fungus *Diaporthe phaseolorum*, recovered from *Viguiera arenaria*, displayed promising results by inhibiting the parasitic enzyme gGAPDH (95%) at 100 µg/mL (Guimarães et al., 2010).

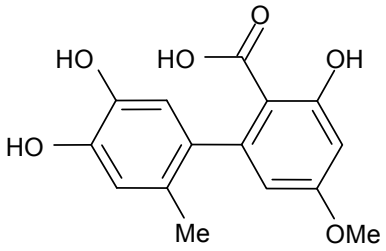


Fig. 8. Altenusin.

An organohalogen natural product (2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione) (Fig. 9, 13) and a quinone derivative (7-hydroxy-8-methoxy-3,6-dimethyldibenzofuran-1,4-dione) (Fig. 9, 14) were obtained from the organic extract of *Xylaria* sp. PBR-30. This endophytic fungus was isolated from healthy leaves of *Sandoricum koetjape* (*Meliaceae*). These natural products had *in vitro* activity against *P. falciparum* (K1, multidrug-resistant strain), with IC_{50} values of 1.84 and 6.68 µM (Tansuwan et al., 2007).

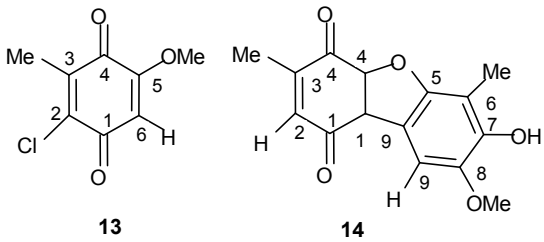


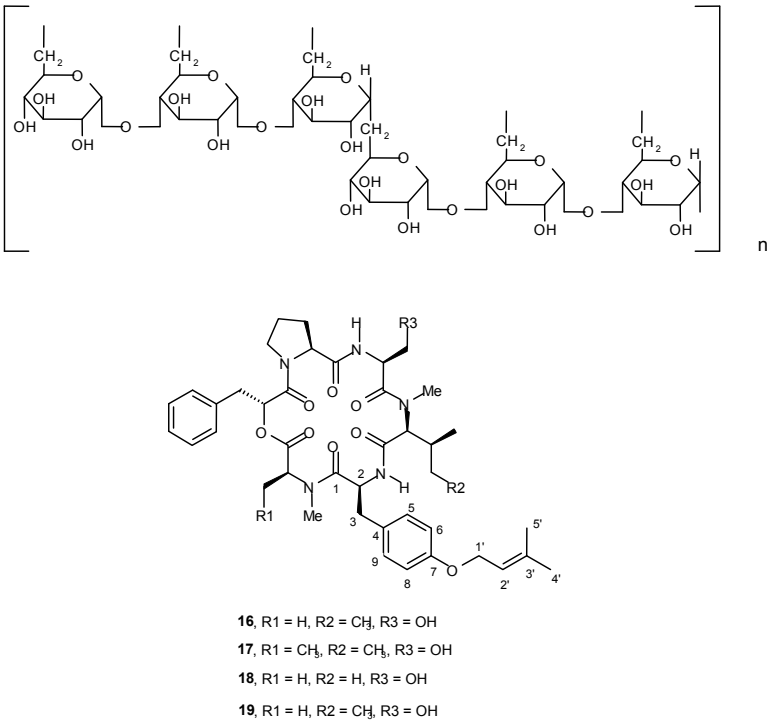
Fig. 9. 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (13) and 7-hydroxy-8-methoxy-3,6-dimethyldibenzofuran-1,4-dione (14).

The yeast-like fungus *Aureobasidium pullulans*, which was isolated from a leaf of *Culophyllum* sp. collected in Narathiwat Province, Thailand (Isaka et al., 2007), produces the cyclohexadepsipeptides pullularins A-D (Fig. 10, 16-19). Pullularin A exhibited antimalarial activity (IC_{50} 3.6 µg/mL) and moderate antituberculosis activity (MIC 25 µg/mL). Pullularin B exhibited considerable antimalarial activity (IC_{50} 3.3 µg/mL), but this substance and pullularin C exhibited weaker activities in other assays when compared with pullularin A. The low lipophilicity of a deprenyl analogue of pullularin A may explain the inactivity of this substance in all of the assays (Isaka et al., 2007).

Codinaeopsis gonytrichoides was isolated from *Vochysia guatemalensis* (*Vochysiaceae*), a white yemeri tree collected in Costa Rica. A new tryptophan-polyketide hybrid named codinaeopsin (Fig. 11, 20), which contains an unusual heterocyclic unit linking indole and decalin fragments, was isolated from the crude extract of this endophytic fungus. Codinaeopsin is active against the 3D7 strain of *P. falciparum* with an IC_{50} value of 2.3 µg/mL (4.7 µM). Codinaeopsin has the same scaffold as the HIV integrase inhibitor

equisetin (Fig. 11, 21), the antifungal agent cryptocin (Fig. 11, 22), and the telomerase inhibitor UCS1025A (Fig. 11, 23). These compounds have a linear fragment joined to amino acids or N-methyl amino acids (Kontnik & Clardy, 2008).

Stems of *Melaleuca quinquenervia* (*Myrtaceae*), collected from Toohey Forest, Australia, were examined for fungal content. Chemical investigations of a fermentation culture from the endophytic fungus *Pestalotiopsis* sp. yielded three caprolactams, which were named pestalactams A–C (Fig. 12, 24-26). Pestalactams A (Fig. 12, 24) and B (Fig. 12, 25) displayed modest *in vitro* selectivity against chloroquine-resistant (IC_{50} 41.3 and 36.3 μM, respectively) and chloroquine-sensitive (IC_{50} 16.2 and 20.7 μM, respectively) cell lines of the malaria-causing parasite *P. falciparum* versus neonatal foreskin fibroblasts (NFF, IC_{50} 20.2 and 12.8 μM, respectively), with both compounds yielding ~16–41% parasite growth inhibition at 25 mM and ~12–64% NFF inhibition at 100 mM (Davis et al., 2010).



16, R1 = H, R2 = CH_3, R3 = OH

17, R1 = CH_3, R2 = CH_3, R3 = OH

18, R1 = H, R2 = H, R3 = OH

19, R1 = H, R2 = CH_3, R3 = OH

Fig. 10. Pullularins A-D (16-19)

Chalara alabamensis, an anamorphic fungus, was isolated from the host plant *Asterogyne martiana* (*Arecaceae*), which was collected in Costa Rica. The dichloromethane extract of this fungus inhibited PfHsp86, an essential protein-folding chaperone from *P. falciparum*, with an EC_{50} value of 24 μg/mL. The only active compound isolated from the extract was viridiol (Fig. 13), a steroidal furan with an EC_{50} value of 1.2 μg/mL (Cao et al., 2010).

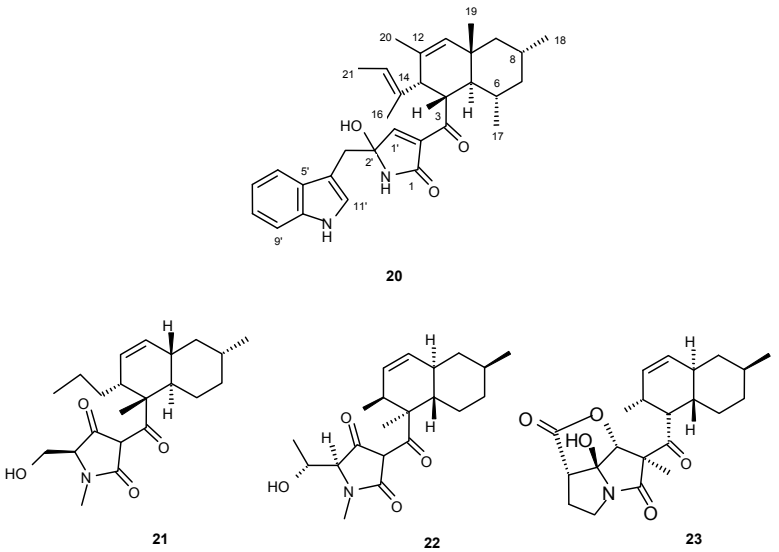


Fig. 11. Codinaeopsin (20), equisetin (21), cryptocin (22) and UCS1025A (23).

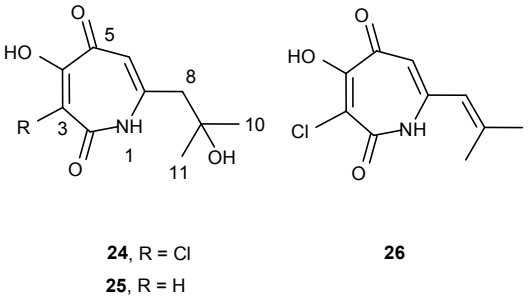


Fig. 12. Pestalactams A–C (24-26).

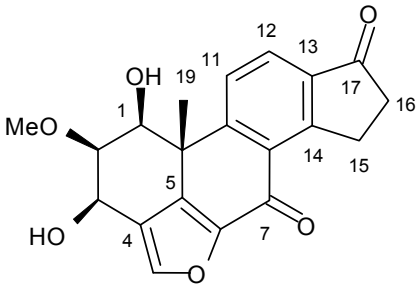


Fig. 13. Viridiol.

Structurally related aromatic sesquiterpenes named phomoarcherins A-C (Fig. 14, 28-30) were obtained from the ethyl acetate extract of the endophytic fungus *Phomopsis archeri*, which was isolated from the cortex stem of *Vanilla albidia* Blume (*Orchidaceae*). The most active compound in the series was amphotericin B, which had antimalarial activity against *P. falciparum* (IC_{50} of 0.79 μg/mL) and contains a ketone function at C-3 and an aromatic lactone ring (Hemtasin et al., 2011).

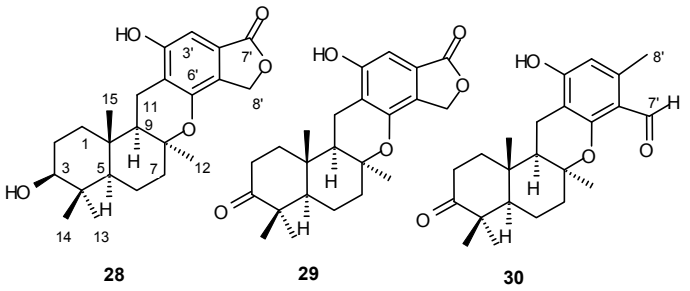


Fig. 14. Phomoarcherins A-C (28-30).

Pestalopyrone (Fig. 15), 6-(1′-methylprop-1′-enyl)-4-methoxy-2-pyrone, which was isolated from a Costa Rican endophytic fungus, *Phomatospora bellaminuta*, had activity against *P. falciparum* in an assay with an IC_{50} value of 37 μM and is a promising candidate for a prototype molecule for antimalarial drugs (Cao & Clardy, 2011).

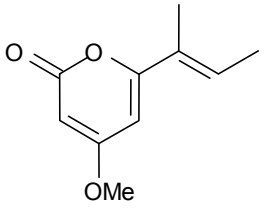


Fig. 15. Pestalopyrone.

8. Conclusion

Few bioactive molecules discovered from tropical endophytic fungi were included in studies for the development of new drugs. However, the compounds alternusin (anti-*T. cruzi*), cochlioquinone A and isocochlioquinone A (anti-leishmania) isolated from tropical fungal endophytes are *in vitro* and *in vivo* studies to maybe became new neglected disease drugs. Indeed, the use the tropical endophytic fungi as novel scaffolds for development of new drugs against neglected diseases represent a challenge to researchers of several scientific areas. The study of this fungal group offer some unique advantages: (i) endophytic fungi have a complex relationship with their host plant, which to survive produce a rich bioactive metabolic profile; (ii) fungi are eukaryotic organisms able to produce complex

molecules, which only few of them can be obtained by chemical synthesis; (iii) there are a lot of different isolates of same species of endophytic fungi, which also have differences in their capability to produce bioactive compounds; (iv) tropical endophytic fungi preserved in culture collections can be growth in different conditions of nutrients, temperature, pH, agitation and aeration to optimize the recover the high amount of crude extracts, as well as bioactive pure compounds; (v) if the crude extract and fractions produced by endophytic fungi do not display toxic activities, they can be used as "mycotherapic" agents. In addition, the metabolites described in this review also may be used against non-neglected diseases, because they are able to act against eukaryotic cells such as cancer cells, immune system cells, cells infected with virus, some human pathogenic fungi, among others. Unexplored natural environments are an excellent source of bioactive compounds that can act as the scaffold for commercial drugs. By taking advantage of new genomic, proteomic and drug design techniques, endophytic fungal communities associated with tropical forest plants, with their high diversity of species and their diverse genetic and metabolic pathways, may be resources for intelligent screening for discovering new drugs to treat neglected diseases.

9. Acknowledgments

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Bioproduction of Depsidones for Pharmaceutical Purposes

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1. Introduction

Lichens are intimate and long-term specific symbioses of photosynthetic algae or cyanobacteria and heterotrophic fungi joined to form a new biological entity different from its individual components (Galun & Kardish, 1995). Mainly chlorolichens produce unique phenolic substances, depside, depsidones and some dibenzofurans, such as usnic acids (Huneck & Yoshimura, 1996), that are not produced by other fungi and plants, and that show some biological activities with interesting applications from a pharmacological point of view, mainly as antiviral agents or for dermatological treatments. Although semi-synthetic organic processes have been attempted for the production of depsidones (Elix et al., 1987), they results very tedious, expensive and inappropriate for industrial applications. Alternatively, extraction from lichen thalli collected in nature implies a very high rate of biomass destruction which cannot be balanced because of the very slow rate of growth of these organisms. This last condition also invalidates the use of transgenic specimens. Thus, the use of bioreactors using very small amounts of lichen biomass seems to be as yet the most viable alternative to rapid and efficient production of depsidones.

Depsidones are organic compounds consisting of two phenolic acids linked together by both ester and ether bonds. So far, type I polyketide synthases (PKSs) are the suggested catalysts for the biosynthesis of these lichen compounds (Muggia & Grube, 2010). It is generally accepted that depsidones come from depsides, formed by two molecules of orsellinic acid derivatives linked by an ester bond, which means that the formation of ether was carried out on previously formed depsides. Thus, despide and depsidones are closely related and biogenetically they all seem to belong to only one group of chemical structures. It seems to be therefore definite that they are evolved from the same primary compound, the variations being brought about by processes of oxidation and reduction, and other simple reactions (Seshadri, 1944).

Polyketide synthases, also known as PKSs, are a family of enzymes or enzyme complexes that produce polyketides, a large class of secondary metabolites in bacteria, fungi, plants, and a few animals lineages. The biosyntheses of polyketides share striking similarities with fatty acid biosynthesis (Khosla et al., 1999; Jenke-Kodama et al., 2005). The pathway is

started by an acetyl-CoA carboxylase which produces malonyl-CoA, the main substrate of PKSs. These enzymes assemble structurally diverse products from simple acyl-CoA substrates by using a catalytic cycle involving decarboxylative Claisen condensations and variable modifications, such as reduction and dehydration. The PKS genes for a certain polyketide are usually organized in one operon in bacteria or in gene clusters in eukaryotes. Each type I polyketide-synthase module consists of several domains developing specific, catalytic functions, separated by short spacer regions. From N- to C-terminus, domains are arranged as: acyltransferase (AT), acyl carrier protein (ACP), keto-synthase (KS), ketoreductase (KR), dehydratase (DH), enoylreductase (ER), O- or C- methyltransferases (MT), sulfhydrolase (SH) and thioesterase (TE). Further optional accessory domains are represented by cyclase (CYC) (Fujii et al., 2001) and methyl transferase (MT) (Hutchinson et al., 2000) activities. In contrast to "post-PKS" O- and N-methylation reactions, which are catalyzed by distinct enzymes after polyketide assembly, methylation of the polyketide carbon backbone takes place during chain formation by means of the intrinsic fungal C-MT domains (Nicholson et al., 2001).

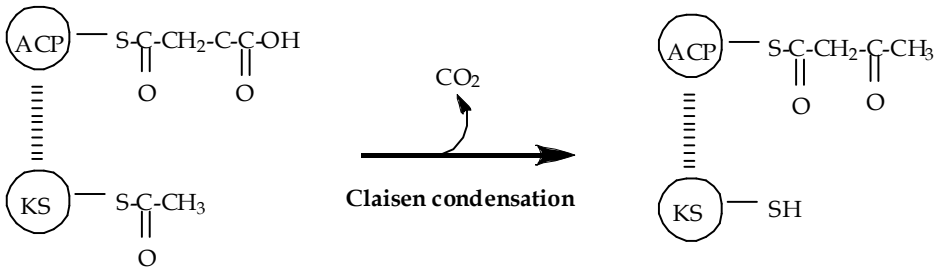

Fig. 1. Claisen condensation catalyzed by PKSI in which a 4C units suffers decarboxylation before receiving a metyl group of an acetate residue bound to KS subunit.

The occurrence of the Claisen condensation (Fig. 1.) is the main step in polyketide biosynthesis (Schümann & Hertweck, 2006) since it permits the production of a phenolic acid with a methyl group as substituent, such as orsellinic acid or 6-methylsalicylate, whereas the absence of this condensation reaction only produces polihydroxy derivatives such as tetrahydroxynaphtalene. After Claisen condensation, a new acetyl-CoA molecule binds on KS subunit to be newly transferred on elongating chain to form a final, lineal precursor of 8C before cyclisation (Fig. 2).

Products of PKS1 action are orsellinic acid or methyl-3-orsellinate when PKS contains an intrinsic methyl transferase subunit, although many derivatives can be synthesized by introducing into orsellinate molecule several chemical motifs through post-PKS modifications defined as tailoring reactions by Rawlings (1999). In some occasions, organic procedures of semi-synthesis or synthesis are used as good models to the experimental approach to the biosynthesis of lichen compounds. However, the biosynthetic pathway is very different from the synthetic way of methyl-3-orsellinate production since methyl-3-orsellinate can be generated from methyl-3-orcinol through the nitration of aryl function to be then reduced to amine group. This last derivative could be the substrat of a diazotonization for the corresponding diazonium salt. After this, a cyanide group could be introduced by nucleophilic substitution using potassium cyanide and then, the carboxyl

function could be produced after hydrolysis (Xavier Filho et al., 2004). For other derivatives, organic synthesis has been successfully used to a model to elucidate the biosynthetic way of polyketide production.

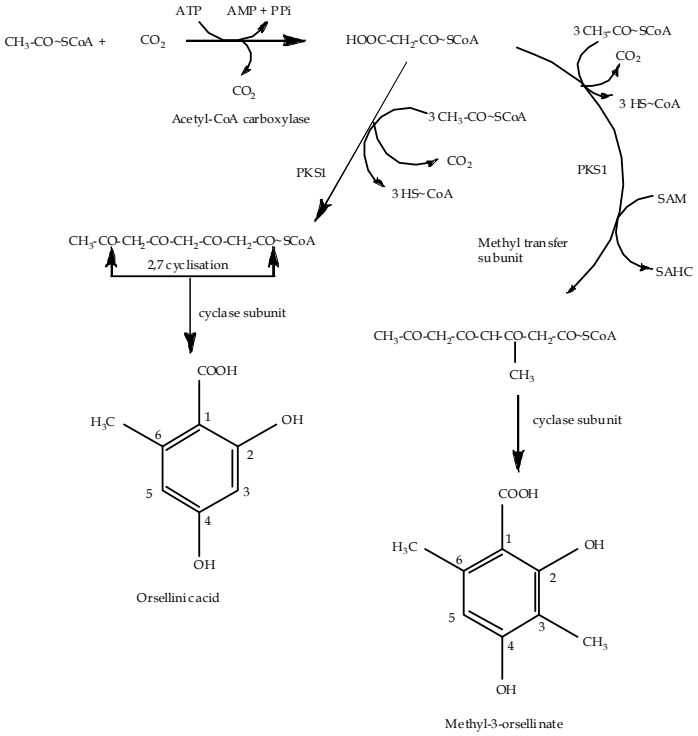

Fig. 2. Orcinol (left) or β-orcinol (right) cyclisation catalyzed by the cyclase subunit of two different PKS without or with methyl transferase subunit.

Based on the presumed catalytic domains required for the synthesis of the key intermediates β-orsellinic acid and methylphloroacetophenone, two pairs of degenerate primers were designed to target specifically the β-ketoacylsynthase (KS) and C-methyltransferase (CMeT) domains of fungal non-reducing polyketide synthase (NR-PKS) genes with CMeT domains. These primers were used to explore the genome of the lichen *Xanthoparmelia semiviridis*, which produces β-orcinol depsidones and usnic acid.

One of the two KS domains amplified from genomic DNA of field-collected *X. semiviridis* was used as a probe to recover the candidate PKS gene. A 13 kb fragment containing an intact putative PKS gene (xsepks1) of 6555 bp was recovered from a partial genomic library (Chooi et al., 2008).

Type III polyketide synthases do not use the acyl carrier protein domain and a simple homodimer of 40-45 kDa proteins performs the complete series of decarboxylation, condensation and cyclisation reactions (Parsley et al., 2011). Type III PKSs are represented by the gene families of chalcone synthases (CHSs) and stilbene synthases (STSs), which until recently have been regarded as typical for plant secondary metabolism (Figure 3). The

discovery of type III PKS genes in a screening of more than 50 fungal genomes and their presence in lichen mycobionts suggest that these polyketide synthases are widely distributed in ascomycetes (Muggia & Grube, 2010). Recent evidence supports the hypothesis of an ancient horizontal gene transfer of type I PKS genes from bacteria to fungi (including lichen mycobionts). Schmitt & Lumbsch (2009) suggested that homologues of these genes could be involved in the production of orsellinic acid, due to their relationship with enzymes producing 6-methylsalicylic acid in bacteria and fungi.

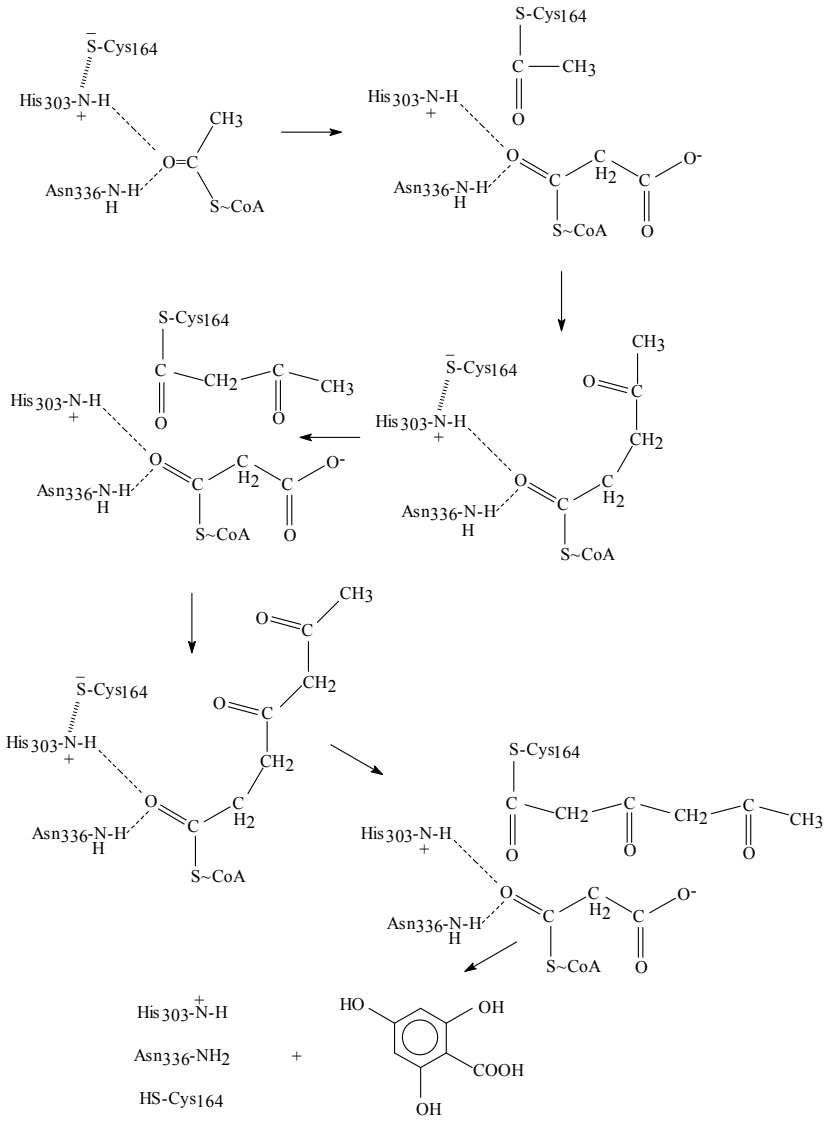

Fig. 3. Biosynthesis of a polyketide chain catalyzed by PKS III.

2. Chemical modifications of orsellinate units

A haematommoyl alcohol dehydrogenase has been described for the first time in a lichen species, *E. prunastri*. The enzyme catalyzes the oxidative production of haematommic acid, a phenolic subunit of the depside atranorin, from haematommoyl alcohol using NAD^+ as a cofactor. The enzyme is also able to reduce the haematommic acid subunit in the atranorin molecule using NADH as an electron donor. However,the affinity of the enzyme for haematommic acid is about three times higher than that found for the depside. The enzyme has been located, using adequate histochemical techniques, in the fungal medulla of the thallus whereas it is completely absent from the algal cells. Synthesis of the depside was enhanced by oxygen and NADH. This enhancement suggested the participation of an oxydase and an alcohol dehydrogenase to produce an aldehyde-substituted phenolic acid as the most probable precursor of atranorin (Fig. 4). The participation of both enzymes has been confirmed by loading immobilisates of living lichen cells with sodium azide (inhibitor of several metallo-oxydases) and pyrazole (an inhibitor of alcohol dehydrogenase), that impede the production *in vivo* of atranorin (Millanes et al., 2003).

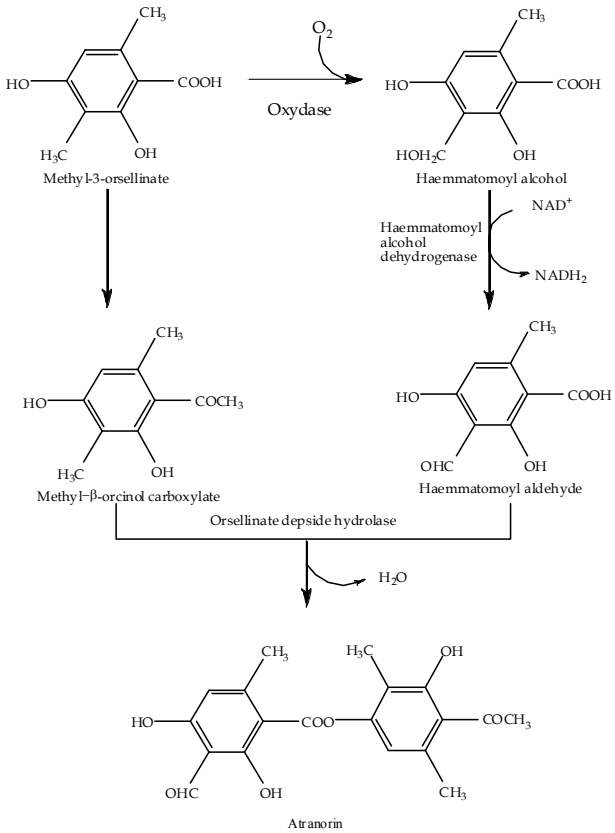

Fig. 4. Biosynthesis of atranorin, a depside of the β-orcinol series, requires the chemical modification of the precursor, methyl-3-orsellinate, by two successive oxidation reactions catalyzed by an O_2-dependent oxidase and a NAD^+-haemmatomoyl alcohol dehydrogenase.

3. Depside biosynthesis

Depsides are synthesized from their monocyclic precursors by an esterification reaction, and it is possible that several esterases exist. Mosbach & Ehrensvård (1966) found that *Lasallia pustulata* contained an esterase which hydrolyzed gyrophoric, umbilicaric and evernic acids. This orsellinate despide hydrolase has been purified and characterized. The molecular mass of a 135-fold purified enzyme was about 42 kDa, as determined by SDS-PAGE. This hydrolase was an exceptionally stable protein, withstanding incubation at 57 °C for 10 min without any loss of activity. In addition, it could be stored frozen for 6 months or at 25 °C for 4 days with practically no loss of activity. The K_m value for lecanoric acid, according to a michaelian kinetic, was estimated as about 56 μM (Schultz & Mosbach, 1971). The enzyme hydrolyzed gyrophoric, lecanoric and evernic acids but no hydrolysis was achieved when *iso*-evernic and digallic acids, or phenylbenzoate, were used as substrates. This indicated that a free hydroxy group in ortho position to the ester bond was required for activity.
A similar esterase has been purified from *Evernia prunastri*. Optimal temperature varied from 30 °C to 50 °C, optimum pH over 8 to 9 and its molecular weigh was estimated as about 120 kDa by SDS-PAGE. The K_m value for evernic acid was estimated at 21.3 mM. Orcinol, as well as both D- and L-usnic acids, behave as competitive inhibitors of the enzyme with K_i values of 0.72, 2.87 and 6.0 mM, respectively. Salicylic acid behaves as a noncompetitive inhibitor with a K_i value of 29.6 mM (González et al., 1984). The enzyme is extremely thermolabile in solution but it remains active at room temperature during several weeks after immobilization in polyacrylamide (García-Junceda et al., 1991a). In addition, orcinol was unable to inhibits immobilized hydrolase in a concentration range from 0.1 to 1.0 mM. *Evernia* hydrolase behaved as a constitutive protein (Vicente & Legaz, 1988) since increases in enzyme activity following incubation of thalli on buffer, 2% (w/v) bicarbonate or 35 μM evernic acid solutions were not impeded by 100 μM cycloheximide. A complete loss of enzyme activity was achieved after drying the lichen thallus at room temperature for 3h. It seems that water content was the major factor affecting hydrolase activity in this lichen species.

4. Depsidone biosynthesis

The existence of coupled depside-depsidone in the same lichen species, such as olivetoric-physodic acids in *Cetraria ciliaris* (Culberson, 1964) indicates that these compounds could be biogenetically related. In fact, it has been proposed that depsidones are formed by dehydrogenative coupling of depsides. Such a synthetic strategy was used when a naturally occurring depsidone was synthesized in the laboratory. In any case, the formation of monocyclic units seems to be a common pathway for both depsides and depsidones. This conclusion was derived from labelling experiments in which the distribution of radiactivity from $^{14}CO_2$ into the depsides evernic acid, atranorin and chloroatranorin, synthesize by *E. prunastri* (Blanco et al., 1984), and into depsidones, physodic and physodalic acids from *Hypogymnia physodes* (Fox & Mosbach, 1967), were almost identical and coincided with that expected from a conventional PKS. Several authors have recognized that the secondary structural differences between known depsides and depsidones need not necessarily have occurred after cyclisation. In other words, the attachment of a fumarate residue on 3-substituted carbon atom of methyl-3-orsellinate to produce the second monocyclic precursor of the depsidone, fumarprotocetraric acid, can be achieved on the monocyclic phenol, on

atranorin (the depside probable precursor of the depsidone) or even on the depsidone itself. Vicente et al. (1984) found that the time course of fumarprotocetraric acid production in *Cladonia sandstedei* floating on urea was inversely related to that ot atranorin accumulation. This may imply a biogenetical relationship between both compounds, but it does not provide any evidence about the structural change described above. However, norstictic or salazinic acids are produced by *C. sandstedei* thalli floating on ammonia, whereas fumarprotocetraric acid and atranorin are completely remobilized. This may be considered as evidence supporting the suggestion that depsidones can be modified after the formation of the ether bond.

To explain the occurrence of iso-structural depside-depsidone pairs, Elix et al. (1987) and Culberson & Elix (1989) suggested that the C-hydroxylation of a *p*-depside in the 5′ position would be followed by acyl migration and a subsequent Smiles re-arrangement of the formed *m*-depside would lead to orcinol-depsidones. This hypothesis has been used to explain the biosynthesis of grayanic acid by the mycobiont of *Cladonia grayi* in axenic culture (Culberson & Armaleo 1992, Armaleo 1995). Alternatively, García- Junceda et al. (1991b) have found that *Pseudevernia furfuracea*, containing the depsidone physodic acid, produces a depsidone ether hydrolase which is able to hydrolyze the ether bond of physodic acid to produce 5-hydroxyolivetoric acid. So far, the appearance of substituents at 6 and 6′ positions has not been studied at an enzymatic level. However, Pereira et al. (1999) found that *Cladonia clathrata* cells immobilized on kaolinite and supplemented with acetate are not able to produce fumarprotocetraric acid, the natural depsidone produced by living thalli, but some of their precursors or catabolites, protocetraric, hypoprotocetraric or 4-O-methylhypoprotocetraric acids, are alternatively accumulated. This has been interpreted as a requirement of a redox coenzyme to support the reducing coupling of a succinyl-CoA rest to the alcohol function in C3position to produce fumarprotocetraric acid (Figure 5).

Depsidone ether hydrolase has been purified at homogeneity by ammonium sulfate precipitation, size-exclusion chromatography through Sephadex G-100 and ionic exchange chromatography in DEAE-Sephadex A50. Purified enzyme was immobilized in polyacrylamide and assayed for the hydrolysis of physodic acid. Both soluble and immobilized enzymes showed sigmoidal kinetics for substrate saturation, but their apparent K_m value, K'_m, decreased from 0.4 mM physodic acid for the soluble protein to 0.1 mM for the immobilized enzyme. The value of substrate interaction coefficient, n_H, showed to be 2.0 in both cases, as expected from the sigmodidal kinetics. The unusual decrease of the apparent K'_m value could be explained through a protein-protein interaction that would increase the allosteric acceleration for binding of the substrate to the enzyme molecule, since the inflexion point in the direct curves of substrate saturation, decreased from 0.3 mM physodic acid to 0.2 mM after immobilization. This probable interaction also was indicated by the fact that mixing two fractions obtained from DEAE-Sephadex column developing high and low enzyme activity, respectively, the mixture showed an hydrolase activity higher than the simple sum of the separate activities of each fractions. This protein-protein interaction could imply changes in the spatial structure of the enzyme which would increase its affinity for the substrate rather than an effective dimerization, since n_H value did not vary after immobilization (García-Junceda et al., 1991b).

The soluble enzyme showed an optimum temperature value of 30 °C and was completely inactivated at 60 °C. However, the optimal temperature for the immobilized enzyme was 35°C and retained about 40% of its activity at 70°C. Depsidone ether hydrolase remained almost completely active after 56 days of storage at room temperature.

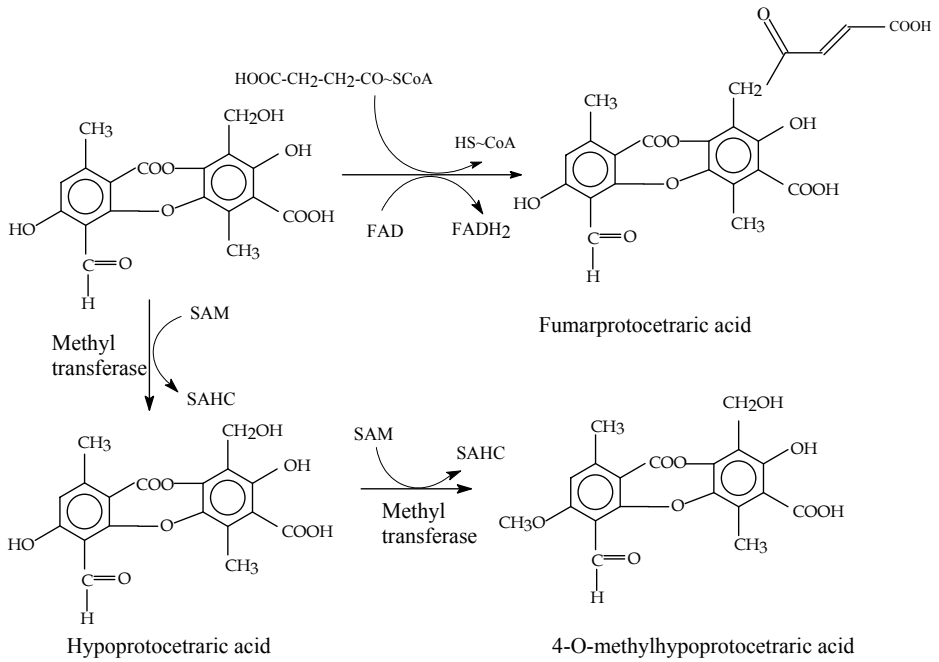

Fig. 5. Enzymatic addition of a fumarate residue, from succinyl-CoA, to the alcohol function at C3′ position of protocetraric acid to produce fumarprotocetraric acid. Alternatively, protocetraric acid could be used to the *in vivo* production of hypoprotocetraric and 4-O-methylhypoprotocetraric acids which are not substrate of the condensating reaction.

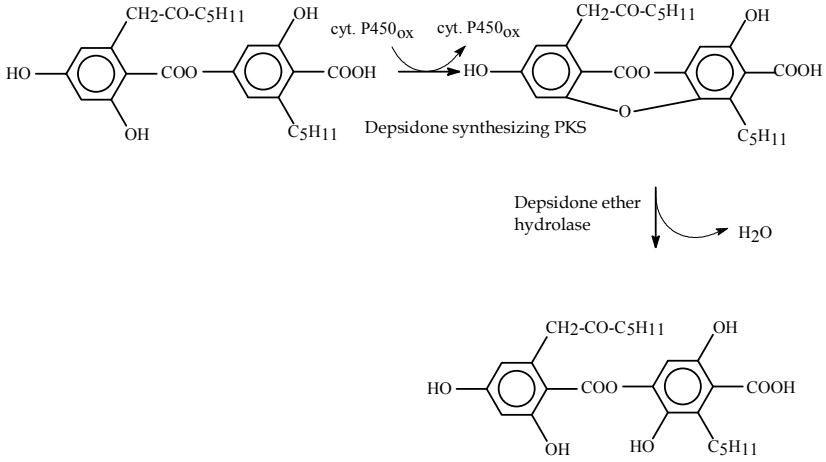

Fig. 6. CgrPKS16 protein, depending on cytochrome P450, catalyzes the oxidative production of a depsidone, physodic acid, from the corresponding depside, olivetoric acid. However, the catabolyzing enzyme depsidone ether hydrolase breaks the ether bond by hydrolysis, producing 5-hydroxyphysodic acid.

The natural occurrence of 5'-hydroxyolivetoric acid strongly suggests that depsidone could be produced by a single dehydration of the depside. This procedure is similar to that proposed by Sala et al. (1981) for the organic synthesis of some depsidones of *Buellia canescens.* However, Armaleo et al. (2011) described the first lichen PKS cluster likely to be implicated in the biosynthesis of a depside and a depsidone. Among the many PKS genes in *Cladonia gray*i they are four, named CgrPKS13 to16, potentially responsible for grayanic acid biosynthesis. CgrPKS16 protein domains were compatible with orcinol depside biosynthesis. Phylogenetically, CgrPKS16 fell in a new subclade of fungal PKSs uniquely producing orcinol compounds. In the *C. grayi* genome, CgrPKS16 clustered with a CytP450 and an O-methyltransferase gene, appropriately matching the three compounds in the grayanic acid pathway. Specifically, authors propose that a single PKS synthesizes two aromatic rings and links them into a depside, and the depside to depsidone transition requires only a cytochrome P450 (Figure 6).

5. Biotechnological production of depsidones

Several genes for lichen phenolics biosynthesis have been as yet identified and cloned. For example, the ketosynthase domains of putative polyketide synthase (PKS) genes from 15 species in the lichenized genus *Lecanora* as well as three representatives of other genera have been amplified and sequenced using conserved primers (Grube & Blaha, 2003). However thee low rate of cell division and the very low rates of vegetative growth have as yet impeded the production of transgenic specimens in which the production of a particular metabolite has been enhanced. Traditionally, biotechnological processes directed to overproduction of lichen phenolics have been related to cell cultures and cell immobilizations.

Aposymbiotically grown mycobionts can also produce these secondary metabolites in culture. However, they also frequently biosynthesize different polyketides than those of the natural lichen, or primary triacylglycerides and fatty acids (Adler et al. 2004; Molina et al. 2003; Reis et al. 2005). The obtained results are often difficult to interpret and factors favoring the production of lichen substances in culture have not been specified for several decades (Stocker-Wörgötter 2008). The production of lichen substances by aposymbiotically grown mycobionts frequently depends on culture conditions. Most experimental works indicate that these key conditions are related to composition of the nutrient medium (Zocher and Stocker-Wörgötter 2005) and stress parameters like osmotic stress (Kon et al. 1997; Hamada 1996), including slow desiccation of the medium (Stocker- Wörgötter 2002; Stocker-Wörgötter et al. 2004), temperature stress, or exposure to high light intensities like UV-C stress (Hager et al. 2008). Manipulations of the culture conditions have shown that a broader variation of metabolites can be obtained in culture than are actually found in natural lichen thalli (Hamada et al. 2001; Boustie and Grube 2005). A strain of the lichen mycobiont isolated from a thallus of *Parmotrema reticulatum* was cultured axenically on different media. Atranorin, the major cortical lichen depside, was produced when the colonies were grown over 5 and 10 months on solid LB medium, combined with a dessication treatment . Colonies grown on MEYE and MY10 with a desiccation treatment did not produce any lichen secondary metabolite. Mycobionts grown for 5 months on solid MEYE medium without a desiccation treatment produced triacylglycerides as the major metabolites, and the fatty acids were characterized as their methyl esters (Fazio et al., 2009).

Tissues from lichen cultures produce several diphenyl oxides, depsides and depsidones. Salazinic acid were detected in cultures of *Usnea flexilis*, *Usnea* sp., and *Ramalina* sp., and protocetraric was produced by *U. flexilis* (Yamamoto et al., 1998) in culture.

Immobilization of lichen cells using different natural or synthetic matrices is the usual procedure to produce lichen phenolics without an excessive use of biomass (Blanco et al., 2002). When cells of *C. verticillaris* were immobilized in calcium-alginate and supplemented with 1.0 mM acetate, a maximum of fumarprotocetraric acid was recovered from the medium at the 10th day. The appearance of this phenolic in the incubation medium could be due to the release of the pre-existent fumarprotocetraric acid from lichen thalli used to produce the immobilisate but a part of this phenolic might be really synthesized by the immobilized cells, since both FMN and CoA impeded, in some extent, the release of fumarprotocetraric released to the media. After this, the amount of fumarprotocetraric acid recovered from the media rapidly decreased for the 15th day and more slowly thereafter, to be practically nullified at the end of the experiment (90 days). Two main differences in the biosynthetic behaviour of *C. verticillaris* immobilisates and those of *C. clathrata*, previously described (Pereira et al. 1999), can be discussed. First, only traces of fumarprotocetraric acid were detected during the first days of immobilization of *C. clathrata* cells whereas *C. verticillaris* produced considerable amounts of the depsidone, although this ability was lost after ten days of beads storage. This loss could be possibly explained as a consequence of the lack of endogenous flavins required to bind succinyl-CoA to a precursor compound and to the impossibility of an effective uptake of exogenous FMN by immobilized cells. Second, *C. clathrata* releases protocetraric acid into the medium whereas this depsidone has not been detected as released from immobilized cells of *C. verticillaris*. However, cells permeabilized with 2-propanol and supplemented with acetate and FMN synthesized and released fumarprotocetraric acid continuously. This fact would confirm that cell permeabilization facilitates that flavin coenzyme enters lichen cells. Fumarprotocetraric acid was also continuously released from immobilized cells supplemented with acetate, but the recovering of the depsidone from bath media slowly decreased from the 7th day of immobilization when cells were stored on acetate only. The addition of 26 μM CoA to the medium containing 40 μM FMN did not enhance the production of fumarprotocetraric acid. This could indicate that CoA cannot get across cell membrane. Small amounts of 4-O-methylhypoprotocetraric acid were only released to the media in parallel to the highest production rate of fumarprotocetraric acid. However, 4-O-methylhypoprotocetraric acid is commonly accumulated by *Xanthoparmelia notata*, whereas hypoprotocetraric acid is a constant component of the chemosyndrom of *Ramalina hypoprotocetrari*a and *R. tumidula*. Release of atranorin from immobilized cells of *C. clathrata* was not influenced by the addition of FMN or CoA to the media, although its release progressively decreased to be nullified at 90 days (data not shown).

Recently, an enzyme capable to esterify an alcohol substituent of protocetraric acid with succinyl-CoA in a complex reaction coupled to dehydrogenation has been pre-purified in our laboratory. The enzyme has been isolated from thalli of *C. verticillaris* whereas protocetraric acid, used as substrate, was purified from *Ramalina farinacea*. The purity of the substrate was established by reverse phase HPLC. The enzyme converts protocetraric acid into fumarprotocetraric acid in a stoichiometric reaction dependent on FAD or FMN but it can also use NAD^+ and $NADP^+$ less efficiently. No reaction was achieved when succinyl-CoA was substituted by succinic acid and HS-CoA. The enzyme did not use hypoprotocetraric or 4-O-methylhyhypoprotocetraric acids as substrates of reaction (Figure

7). This implies that the carboxyl substituent in *meta* position with respect to the alcohol residue, which received the esterifying 4C-organic acid, is absolutely required to the correct formation of the enzyme-substrate complex. Apparently, *C. verticillaris* thalli develop esterifying enzyme activity higher in light than in the dark, since in light fumarprotocetraric acid accumulates in the medulla at high concentration values. Nevertheless, this fact does not imply a positive photo-regulation of the enzyme production but the highest supply of phosynthates from the algal cells to the mycobiont, since enzymes for depsidone production are always fungal proteins. In this way, Armaleo et al. (2008) found that light distribution correlated directly with the amount of atranorin and inversely with that of norstictic acid. We interpret these findings to suggest that sunlight indirectly affects depside and depsidone metabolism by influencing thallus temperature and water potential in the lichen *Parmotrema hypotropum*. *P. hypotropum* accumulates the depside atranorin in the cortex and the depsidone norstictic acid in the medulla and around the algae. A direct correlation was observed between the yearly amount of light reaching the lichen and the amount of atranorin. In contrast, the amount of norstictic acid decreased with increasing light. Authors suggest that depside/depsidone accumulation in lichens is mediated by localized changes in temperature and water potential produced by light absorption within each thallus (Figure. 8).

Although some of the enzymes described here have been successfully immobilized in several inert matrices, such as orsellinate decarboxylase, orsellinate depside hydrolase and depsidone ether hydrolase, always were used for basic research activity (Blanco et al., 2002). Biotechnological production of depsidones requires a process economically sustainable and the previous purification of the adequate enzyme and the chemical preparation of their substrates are very expensive to be used at an industrial scale. However, the use of immobilized cells basically supplemented with acetate is a process sufficiently rapid, accurate and reproducible to produce depsidones at a semi-industrial level.

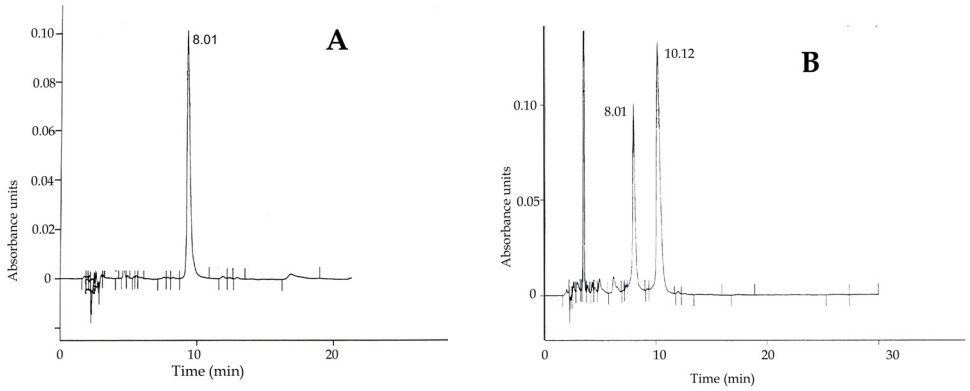

Fig. 7. Chromatographic traces in RP-HPLC of protocetraric acid, isolated from *Ramalina farinacea* (A) that eluted with a retention time value of 8.01 min, and phenolics extracted from a reaction mixture containing protocetraric acid and succinyl-CoA as substrates, the enzyme pre-purified from *Cladonia verticillaris*, and FAD as cofactor. Fumarprotocetraric acid elutes from the column with a retention time value of 10.12 min.

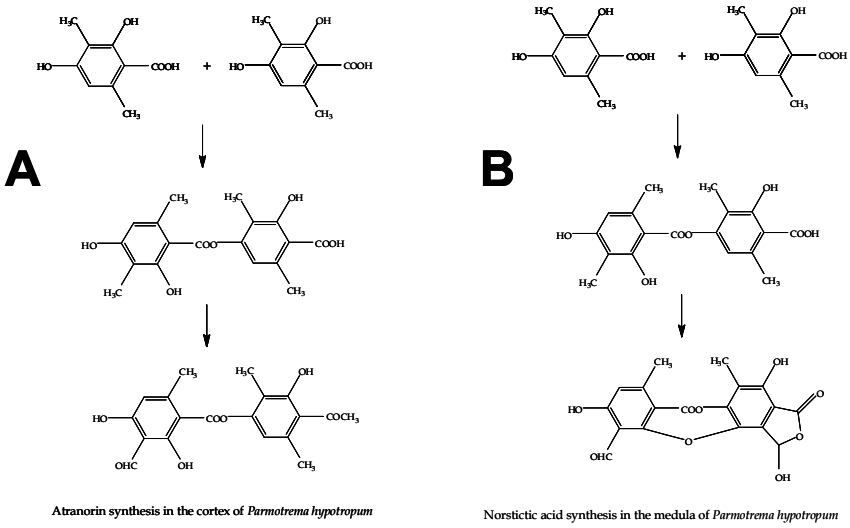

Fig. 8. Alternative production of atranorin (depside) and norstictic acid (depsidone) by thalli of *Parmotrema hypotropum*, according to Armaleo et al. (2008), as influenced by variable values of the ratio temperature/water potential (T/Ψ) along a day. When direct sunlight increases the temperature of the thallus and enhaces evapotranspiration (high T/Ψ values), atranorin is actively synthesized and deposited on the cortex (A). However, the decay of sunlight intensity decreases both thallus temperature and water loss (low values of T/Ψ) and, then, the depsidone norsticitc acid is actively produced and retained in the medulla (B).

6. Clinical and pharmaceutical applications

6.1 Depsidones as allergens

Parfums contains natural compounds used as fixing components. A fixing substance forms stable bonds, generally ester bonds, with those parfum components which easily volatilized, and then, the loss of these odoriferous substances to the air is delayed and they are retained on the skin for long time periods. One of the most popular fixing products if the oak moss, a complex mixture of phenols mainly extracted from three lichen species, *E. prunastri*, *Pseudevernia furfuracea* and *Usnea barbata* some of those containing depsidones (Huneck, 2001). In many cases, certain substances produced by lichens and included in cosmetic preparations cause skin photosensitization (Rademaker 2000). The same or similar substances become part of basic notes in perfumery and they can produce allergic reactions (Joulain & Tabacchi, 2009). Seven patients, from 2000, showed sensitivity to perfums containing oak moss as a part of the basic note. The substances mainly responsible for the allergic reaction are atranorin (4 patients), evernic acid (4 patients) and usnic acid (5 patients) as well as some depsidones, such as acids physodic/phisodalic acids, with low allergenic actitivy, or diffractaic and fumarprotocetraric acids, practically without allergic response. Atranorin is the compound that produces reactions of greater intensity. The clinical changes due to a severe reaction to the allergen were only observed in two patients and consisted of severe skinredness, swelling and interdigital sweating. In all other cases,

the reactions were weak, consisting of erythema and itching. These symptoms appeared associated with intermittent use for parfum (Thune et al., 1982).

6.2 Interactions with photodynamic astringents

The skin exposed to solar radiation leads to the formation of highly reactive intermediates such as singlets and triplets, oxygen singlet ($^1O_2$), hydroxyl radical ($OH^\bullet$) etc, which can damage membranes by lipid peroxidation and oxidation of proteins. These free radicals and singlets also support the photosensitizing properties of some psoralens such as 8-methoxypsoralen, thus being able to act on such reactive intermediates in order to inhibit the photosensitizing action. The 8-methoxypsoralen is able to bind covalently to proteins such as lysozyme and bovine serum albumin (BSA), although the covalent conjugate with SAB, which is formed in the presence of oxygen under UV-irradiation, seems to follow an entirely different mechanism of photo-cycloaddition of the molecule to DNA. When the covalent bond is attempted in the presence of depsidones pannarin or 1'-chloropannarin, the photoprotective capacity of these phenols lichen can be explained on the basis of their ability for inhibiting the photobinding of psoralen to human serum albumin (Fernandez et al., 1998).

Human skin irradiation with both UV-A and UV-B increases the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and this contributes to inflammation, immunosuppression, gene mutation and carcinogenesis. The depsidone pannarin also acts as a potent dose-dependent agent for superoxide scavenging (Russo et al., 2008). Irradiation of pannarin, 1′-chloropannarin and atranorin with 366 nm light leads to significant hemolysis in a red cell suspension. However, their mechanism of action is different. Hemolysis induced by pannarin and 1′-chloropannarin increases in the presence of oxygen, whereas hemolysis induced by atranorin is higher in nitrogen-purged solutions. The effect of free radical scavengers, and the lack of effect of D_2O in the medium, suggest that the hemolysis induced by pannarin and 1′-chloropannarin is not mediated by $^1O_2$. Both the hemolytic and photohemolytic activities of the depsidones, particularly 1′-chloropannarin, increase when the temperature increases from 21 to 37 °C (Hidalgo et al., 1993).

6.3 Antibacterial activity

Lichens have been used for centuries in folk medicine for the treatment of infectious diseases. According to Ingolfsdottir (2002), *Usnea barbata* was used by Hippocrates to treat urinary disorders and *U. longissima* was used in China as an expectorant. Usnic acid isolated from *Cladonia arbuscula* shows high antibiotic activity against *Mycobacterium aurum*, a non-pathogen bacterium with similar sensitivity profile to *M. tuberculosis*. Lobaric acid and atranorin (depsides) isolated from *Stereocaulon alpinum*, salazinic acid (depsidone) from *Parmelia saxatilis*, and protoliquesterinic acid (a cycloaliphatic acid), isolated from *Cetraria islandica*, show four times lower activity against the same and other microorganisms than that detected for usnic acid (Ingolfsdóttir et al., 1997). Usnic acid (a dibenzofuran derivative) also shows antibiotic activity against some Gram-negative bacteria such *as Streptococcus pneumoniae* and *Enterococcus foecalis* and against many anaerobic bacteria such as *Bacteroides fragilis*, *Clostridium perfringens* and *Propionibacterium acnes* (Lauterwein et al., 1995). Some depsidones from *Ramalina farinacea* also develops anitimicrobial activity. Norstictic acid was active against *Aeromonas hydrophila* as well as the above microorganisms except *Yersinia enterocolitica*. Protocetraric acid showed activity only against the tested yeasts *Candida*

albicans and *Candida glabrata* (Tay et al., 2004). Basically, the antibiotic activity of lichen compounds lies to these actions that derive from its phenolic nature, such as membrane lipid peroxidation, uncoupling of oxidative phosphorylation, and inhibition of DNA duplication

Whereas usnic acid has received much attention from researchers as a potential antibiotic, depsidones have sometimes been also studied. Ingólfsdóttir et al (1998) reported that salazinic acid develops antimycobacterial activity. Honda et al. (2010) studied the antibiotic properties of several different depsidones, protocetraric, salazinic, norstictic, hypostictic acids, and five salazinic acid derivatives on *Mycobacterium tuberculosis*. They conclude that the activity against the microorganism depends on the physico-chemical parameters (lipophilicity and pKa) of each compound, that is a function of the substituents in each molecule evaluated and of its structural characteristics. A comparison among these shows that the more lipophilicis more active than the less lipophilic compound. Such features may result in intramolecular interactions among these groups, for example, by hydrogen bonds, which affect the lipophilicity and the pKa.

6.4 Cytotoxicity

Cell exposure to certain xenobiotics can cause cell damage or toxicity. The cytotoxic activity of different lichen polyphenols (9 depsidones, 3 depsides and one tridepside) isolated from various lichen species from South America was tested on cell cultures of rat lymphocytes (Correche et al., 2002). After addition of phenols, the cytotoxicity was analyzed by the addition of tritiated thymidine, a highly sensitive indicator of mitosis. The assay revealed that the majority of the metabolites exhibited a marked cytotoxic activity, in some cases even exceed that shown by colchicine, a potent inhibitor of the achromatric spindle. Comparing the rates of toxicity of the two groups of compounds (depsides and depsidones) also noted that the first turned out to be minor and therefore the depsidones such as salazinic, psoromic, fumatrotocetraric, lobaric, stictic, and variolaric acids could be potentially more harmful than the depsides divaricatic and diffractaic acids, as well as sphaerophorin (depside). In this assay, the toxicity index of depsidones ranged between 1.95 and 34.96. A comparative analysis of the toxicity index and the structure of these compounds showed some correlations. The presence in the ring of both aldehyde and hydroxyl groups positioned into carbons 3 and 4 respectively, was associated to a high rate of toxicity. Chemical species without such radicals showed significantly lower levels of toxicity. Depsides showed lower levels of toxicity that depsidones, reaching the value of cytotoxicity index to a maximum of 7.5. By comparing the chemical structure of these species to the degree of toxicity developed, a relationship between the presence of an acid group in C1 and a hydroxyl in C2 seems to be required but, as noted for atranorin, the occurrence of an aldehyde and an adjacent hydroxyl could explain their high cytotoxicity. It follows that the toxicity is in part conditioned by the ability to establish hydrogen bonds between adjacent radicals, as determinant of the biological response. However, (-)-usnic acid was the only compound to display a moderate cytotoxic activity on various cancer cell lines from a mixture of varios phenolics isolated from the lichen *Cladonia convoluta*, the depsidone 9'-(O-methyl)protocetraric acid and fumarprotocetraric acid. Usnic acid was also shown to induce apoptosis of murine leukaemia L1210 cells in a dose- and time-dependent manner (Bézivin et al., 2004).

By using a human melanoma cells (M14 cell line), Russo et al. (2008) found that sphaerophorin (depside) and pannarin (depsidone) showed a protective effect on plasmid DNA and exhibited a superoxide dismutase like effect. The data obtained from cell culture show that these lichen metabolites inhibit the growth of melanoma cells, inducing their apoptotic cell death, demonstrated by the fragmentation of genomic DNA and by a significant increase of caspase-3 activity, and correlated, at least in part, to the increase of ROS generation.

Alternatively, some non-lichenized fungi are also able to produce depsidones with cytotoxic activity. An endophytic fungus (*Botryosphaeria rhodina*), isolated from the stems of the medicinal plant *Bidens pilosa*, produces four depsidones, botryorhodines A–D, which exhibit potent cytotoxic and antiproliferative effects against several cancer cell lines, such as HeLa cells (Abdou et al., 2010). Ten depsidones, mollicellins, produced by the fungus *Chaetomium brasiliense*, showed also cytotoxic activity against KB, BC1, NCI-H187 lines, and five cholangiocarcinoma cell lines (Khumkomkhet et al., 2009).

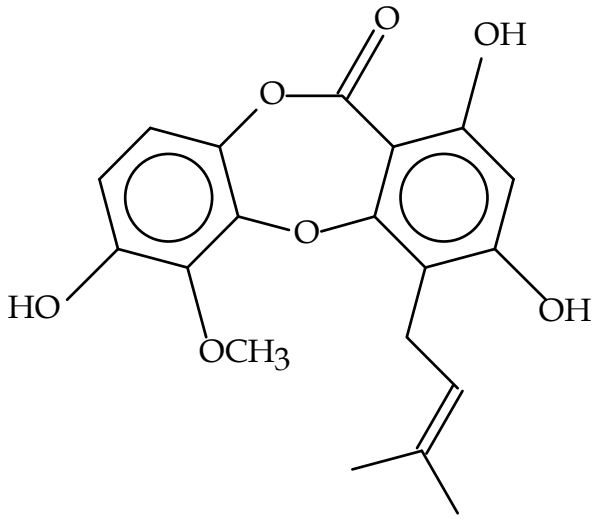

Fig. 9. Chemical structure of garcinisidone F fram *Garcinia assigu*.

Some depsidones have been found in higher plants. For example, *Garcinia assigu* (Gutaceae) produces garcinisidone A-F the chemical structure of which is almost identical to those found in lichens (Fig. 9) . Garcinisidones B to F show inhibitory effects on the Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in Raji cells. Experiments examining the structure-activity relationship, focusing on the garcinisidone nucleus, demonstrated that the 9-prenyl substituent and the dimethylpyran ring on the A and/or B ring were not essential for the activity. In previous studies, it has been found that the prenyl side chain on xanthone, 7-methoxycoumarin, phenylpropanoid, and isoflavonoid nuclei plays an important role in anti-tumor-promoting activity. In view of the present findings taken together, garcinisidones with one or two prenyl side chains on the A-ring might be valuable anti-tumor-promoting agents effective against chemical induced carcinogenesis

6.5 Antioxidant activity

The depsidones norstisctic and fumarprotocetraric acids (Lohézic-Le Dévéhat et al., 2007) showed better superoxide anion scavenging activity (IC50 = 0.566 and 0.580 mM, respectively) than quercetin (IC50 = 0.754 mM). The antioxidant activity of lichenic metabolites, depsides and depsidones, was also assessed by their effects as inhibitors of rat brain homogenate auto-oxidation and β-carotene oxidation (Hidalgo et al., 1994). The results obtained in both systems indicate that lichenic metabolites afford a moderate protection in the micromolar concentration range. The largest effect was measured employing 1'-chloropannarin in the brain homogenate auto-oxidation, where a 66% protection was afforded at 1.7 μM. This protection is very similar to that elicited by addition of the reference antioxidant propylgallate (70% protection at 1.3 μM). Fumarprotocetraric acid from *C. verticillaris* possesed effective antioxidant activity at all concentrations assayed (Xavier Filho, personal communication). This antioxidant activity increased with the concentration used in the assay: 0,1 mg/mL of the product produced a 77,86% inhibition of the peroxidation of human plasma lipids (Fig. 10) and a 33,2% of the peroxidation of linoleic acid emulsion (Fig. 10).

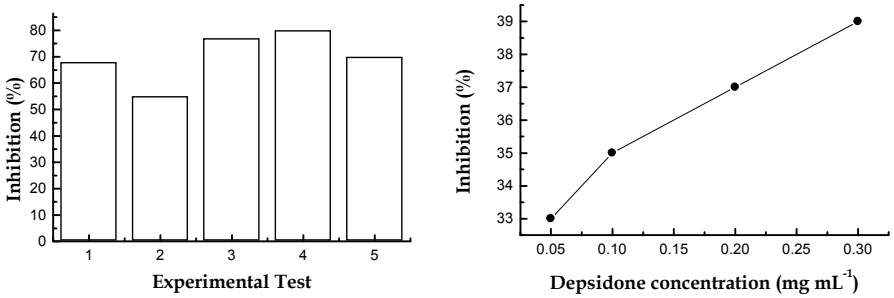

Fig. 10. Left: Inhibition of plasma's lipid peroxidation by 0.1 mg mL^{-1} fumarprotocetraric acid from *Cladonia verticillaris*. Number indicated different assays and number 5 is the average inhibition of four assays of plasma lipid peroxidation. Right: Inhibition of linoleic acid peroxidation by different concentrations of fumarprotocetraric acid.

6.6 Antiviral activity

Some depsidones showed to be active against HIV by inhibiting the viral integrase. During infection, integrase catalyzes two consecutive reactions. Initially, the enzyme processes the viral linear DNA by cutting two nucleotides from each 3 'end, leaving the 3'-OH end free. This is followed by the trans-esterification of phosphodiester bonds, one strand of DNA cutting and joining the 5 'end of the cut to the processed viral 3'-end. These two steps, called processing 3 'and band transfer of DNA can be measured in vitro using a purified recombinant integrase and a 21-mer duplex oligonucleotide corresponding to the end-HIV U5 LTR sequence (Vlietinck et al., 1998).

Using a 3D database of the National Institute of Cancer and the appropiate software (Chem-X), Neamati et al. (1997) have identified two potential architectures of active pharmacophores for virensic acid and other depsidones such as physodic acid. Both compounds, as well as granulatine (the methylester of virensic acid), exhibit high potential

for inhibition of viral integrase processing activity for both 3' processing and for the transfer belt. The common constant of both pharmacophores is to present a fixed distance of 8.37Å between the two neighboring hydroxyl to the carboxyl group of both phenolic units, a dimension possibly related to the topography of the domain receptor of depsidone.
The depside atranorin showed an activity slightly lower than that found for depsidones, but its oxime completely lost its inhibitory activity. Since integrase required to perform its catalytic action a divalent cation (Mg^{2+} or Mn^{2+}) as a metallic cofactor, it was thought that the chelating action of the lichen phenols could be the basis of its inhibitory activity of integrase, although there is still no clear demonstration for this assumption. It does seem to be specifically required that the polycyclic system was rigid and that when this stiffness decreases by introducing a substituent open-chain, significantly decreases the inhibitory activity.
Biotechnological processes consisting of the use of bioreactors with immobilized lichen cells is today the most accurate techniques for depsidone production. It has been used mainly to investigate the enzymatic pathways of depsidone biosynthesis and, in addition, it has been revealed as a very efficient method that produces high yields in product preparation. A bioreactor containing only 0.5 of lichen biomass is able to produce 3.0-5.0 g of the corresponding lichen products after two months with unappreciable loss of biosynthetic activity, never higher than 16 per cent. Moreover, bioreactor can be used to modify the chemical structure of particular phenolic molecules in order to increase a particular biological activity and a decrease of undesirable cytotoxic effect.

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20

Multi-Dose Container for Nasal and Ophthalmic Drugs: A Preservative Free Future?

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1. Introduction

The first nasal spray pumps were developed some 50 years ago and replaced step by step droppers and pipettes. Now they are widely used to moisturize the nasal mucosa using saline solutions (from a regulatory point of view considered medical devices under the EU directive) or as nasal preparations for the administration of topically acting drugs (e.g. nasal decongestants or nasal steroids) or for the non-invasive administration of substances which need to reach systemic circulation (anti-migraine medication, hormones). For chronic diseases like allergic rhinitis, multi-dose devices are very cost effective and convenient and provide the safety and precision regulatory bodies require. To date, most of the medications administered nasally contain a preservative to support long storage times and proper in-use stability.
For ophthalmic drugs, there was a similar development from simple droppers to more sophisticated devices. Squeeze bottles without metering function are still widely used for ophthalmic medications especially for chronic conditions. Artificial tears filled in multi-dose bottles are commonly used to relieve dry eye symptoms, these again being medical devices because the mode of action is purely physical. Looking at active drugs, the most successful in the ophthalmic field are drugs (e.g. beta-antagonists and prostaglandine-analogues) for the treatment of glaucoma and anti-histamines to treat allergic conjunctivitis.
The reader may wonder why nasal spray pumps together with multi-dose ophthalmic droppers are considered in the very same chapter. The answer is that both device classes share the same blueprint: they use a bottle made of plastic or glass attached to a pump or dropper which generates and delivers a more or less metered dose and on top of this serves as a closure. So the pumps or droppers support two general functions:

- Sealing of the container and protection of the content during storage and transportation.
- Delivery of a metered dose of liquid or viscous pharmaceutical formulation.

The major challenge during development is to reach a reliable delivery of the metered dose without having any negative impact caused by drug-device interactions.

2. Intranasal administration

Intranasal administration has a long tradition. Around three thousand years ago intranasal application of dried scabs was used to vaccinate against smallpox in India. Later intranasal administration of psychotropic drugs was and still is used for medical as well as recreational

purposes. Leaving these ancient therapeutic regimes behind, today the topical treatment of allergic rhinitis using anti-histamines (azelastine, levocabastine, olopatadine) or glucocorticoids (e.g. budesonide, fluticasone or ciclesonide) is well established. For this particular indication, drugs should act fast and just locally while systemic absorption should be as low as possible to avoid systemic side effects which are linked with typical oral formulations of comparable drug substances. Since allergic rhinitis is a chronic disease, only multi-dose bottles are used there.

	Preferred profile
Nasal decongestants	
e.g. oxymetazoline, xylometazoline, naphazoline, tetryzoline	Local activity only, low systemic adsorption to minimize side effects (headache and dizziness)
Intranasal steroids	
e.g. beclomethasone, budesonide, fluticasone, mometasone, ciclesonide	Local activity only, low systemic adsorption to minimize systemic side effects (cortisol suppression)
Antihistamines	
azelastine, levocabastine, olopatadine	Local activity only, low systemic adsorption to minimize systemic side effects (sedation)
Triptanes	
sumatriptan, zolmitriptan	Fast onset of action, high systemic adsorption to blunt migraine attacks
Analgesics	
fentanyl, ketorolac, (ketamine)	Fast onset of action but avoid high plasma concentration peaks

Table 1. Established drug classes for intranasal administration

But intranasal administration has much more potential. The nasal mucosa can be used for non-invasive systemic administration of drugs. The nasal mucosa in humans is around 150 cm^2, a surface which is well supplied by blood vessels. This ensures a rapid absorption of most drugs and can generate high blood levels. This administration route avoids the first pass metabolism which needs to be taken into account following oral administration. This bypassing of the gastric system even enables the delivery of peptide hormones. Calcitonin and desmopressin are on the market for years now, insulin and glucagon under clinical development for this administration route (Leary et al., 2008).

Intranasal administration is considered a non-invasive administration route and easy to do for self-administration or for care-givers with low potential for injuries or disease transmission (hepatitis B, HIV). This is of special importance if fast pain relief is required and patient's ability to deal with injections is impaired. Intranasal triptanes for migraine treatment, fentanyl to stop breakthrough pain and ondansetrone to relieve nausea take advantage of this administration route. For these indications, single dose devices or multi-dose devices with counting or lock-out mechanisms are available to reduce the risk of unintended overdosing or misuse.

A controversial discussion is ongoing regarding direct nose to brain drug delivery. This would open the door to a targeted treatment for various CNS diseases like Alzheimer, Parkinson, and epilepsies. Unfortunately, the available literature does not suggest consistent findings, more research is required here (Wen, 2011).

	Indication	Bioavailability [%]
Azelastine	Topical anti-histamine	40
Budesonide	Topical glucocorticoid	34
Fentanyl	Opiate analgesic	80-90
Sumatriptan	Anti-migraine	16
Calcitonin	Bone mineralization, polypeptide (32 amino acids)	~3
Desmopressin	Antidiuretic and anti-heamophilic, cyclic nonapeptide	10-20
Insulin	Antidiabetic (peptide hormone, 51 amino acids)	12-15%

Table 2. Nasal bioavailability of selected drugs (marketed or in late stage development)

For the successful development of a nasal spray it is important to understand the basic mechanisms of such a device to recognize the cliffs which must be avoided. Of course a product intended to be administered as a nasal spray should have no unpleasant smell, should not be irritating and even long term treatment should have no negative effects on the nasal mucosa (e.g. ulceration, loss of sense of smell). There should be also no safety concern, if a dose is unintentionally shot into the eyes.

For most pumps the dispensed volume per actuation is set between 50 and 140 µl, and an administered volume of 100 µl per nostril is optimum in adults. Higher volumes are prone to drip out immediately or to flow down into the throat (post nasal drip) and subsequent swallowing. So the anticipated dose should fit into a volume of roughly 100-200 µl when both nostrils are sprayed. Standard spray pumps will deposit most of the sprayed dose into the anterior region of the nasal cavity. Surface tension of the droplets and mucus layer will cause the immediate spread of the spray. Afterwards mucociliar clearance will distribute the liquid layer within the nasal cavity. Since the nasal mucus layer is continuously renewed and discarded into the throat, the nasal dwelling time of the administered drug depends on how fast it dissolves within the mucus layer and penetrates into the mucosa (Suman et al., 2002).

Although authorities require a lot of data to describe nasal spray devices, for deposition efficiency the plume angle and administration angle are critical factors, while many other spray parameters, including particle size, have relatively minor influences on deposition within the nasal cavity (Foo et al., 2007). These parameters will be discussed in more detail below.

3. Available technology and requirements

3.1 First steps to identify the right spray pump

Developing a suitable formulation with one or more active ingredients is a lengthy and challenging process. Rarely is a formulation just a simple water-based solution. Auxiliary compounds often have to be added, for example to enhance solubility and stability, to increase viscosity or to prevent microbiological contamination. Once the final formulation is available, the next step is to ensure that the ingredients do not affect the function and integrity of the container closure system (CCS). In the ICH guidelines it reads as follows: The suitability of the container closure system used for the storage, transportation (shipping) and use of the drug product should be discussed. This discussion should consider, e.g., choice of materials, protection from moisture and light, compatibility of the materials of construction with the dosage form (including sorption to container and leaching) safety of materials of construction, and performance (such as reproducibility of the

dose delivery from the device when presented as part of the drug product (ICH Guideline, 2005). The two topics we want to focus on are compatibility and performance.
To understand the potential problems an insight into the technology of a nasal spray pump system seems beneficial.
The manufacturer fills the drug formulation into multi-dose bottles made of glass or different plastic materials, which are closed by attaching the spray pump including a dip tube. The pump may be fixed by a screw closure, crimped on or simply snapped onto the bottle. Now the system should be tight and no leakage should be observed during subsequent handling. During shipment and storage, the drug formulation is only in contact with the bottle, the outer side of the dip-tube, the gasket between the pump and the bottle and the outer parts of the pump. It is important to recognize, that in this stage the formulation usually does not have contact with inner parts of the pump nor the actuator. So the bottle will provide the largest surface but also the gasket material should be considered when stability issues (e.g. drug sorption, leachables) occur.
Before someone can use the system, the pump must be primed which is normally done by the patient just before first use. A number of priming strokes are required to purge the air off the system and dip tube and to deliver the product at the targeted dose volume. Nasal spray pumps are displacement pumps. When actuating the pump, a piston moves downward in the metering chamber. A valve mechanism at the bottom of the metering chamber will prevent backflow into the dip tube. So the downward movement of the piston will create pressure within the metering chamber which forces the air (before priming) or the liquid outwards through the actuator and generates the spray. When the actuation pressure is removed, a spring will force the piston to return to its initial position. This creates a vacuum in the metering chamber which pulls the liquid from the container by lifting up the ball from the ball seat above the dip tube at the bottom of the metering chamber. It should be recognized, that the "next dose" may be under normal use conditions in contact with the materials for hours or even days when the pump is not regularly used.

Picture 1. Parts for a standard pump and how it looks like when assembled. On the right side: soaking of parts in formulation to check compatibility. For demonstration just parts for one pump are depicted.

It is recommended to evaluate effects on proper device function and compatibility of all parts which come in contact with the formulation early in the development process. This can be done in a simple way: disassembled pumps are stored for some days in the drug formulation under controlled conditions. This is done because some ingredients may cause swelling of plastic materials which may result in malfunction of the system. After appropriate storage time the parts are checked for discoloration and critical dimensions. Then the components of the pump system are cleaned and assembled and the proper function of the pump is evaluated. If the pump performs according to the agreed specification further problems are unlikely and the evaluation of performance parameters can start. These simple procedures can detect incompatibilities quite early and it is much better than to find out such issues later in the development stage or even after market launch due to complaints from consumers and patients.

3.2 General requirements from authorities

According to EU and FDA guidelines (EMEA guideline, 2006, FDA Guidance for Industry 2002) relevant information should be provided on the characteristics of each of the critical components of the CCS. Critical components are defined as:

- those that come into contact with the patient's mouth or nose or with the formulation
- those that affect the overall performance of the device and
- any additional protective packaging (FDA Guidance for Industry)

Within this chapter we cannot discuss all requirements in detail, but will provide an overview on the most important parameters (also see Table 3). For most parameters you just need to know that they are required and the pump supplier will disclose this information by providing the appropriate files or via referencing a drug master file type III for the US FDA.

Dimensions and used materials	
Dimensions	Critical dimensions for all individual components and drawings of the device and detailed description of all parts, manufacturing and assembly steps
Material used	Documentation on raw material, additives (e.g. colors, stabilizers) processing aids (e.g. lubricants) used for manufacturing including source and quality control
Performance	
Shot weight/dose weight	the quantity of drug substance/spray weight expected to be released from the device during actuation EU: mean shot weight/dose within ±15% of target dose US: mean shot weight/dose within ±10% of target dose For small doses (<20 µg) other criteria may be justified
Priming	Number of actuations needed to prime/re-prime the pump and obtain the full dose
Droplet size distribution	Description of droplet size distribution and quantification of fine particles (particles <10µm diameter)
Spray pattern/plume geometry	Parameters to describe size, shape and pattern of a delivered spray
Actuation force	Minimum actuation force to achieve desired spray characteristics

Table 3. Key requirements for nasal spray pumps

3.3 Overall general description of the device and compatibility issues

The pump manufacturer needs to provide technical drawings of the assembled device as well as for each individual part with critical dimensions which are controlled during production. Also the manufacturing of each part and the assembling process needs to be described in detail, additional operations like washing or coating steps, sterilization procedures and of course detailed quality control measures.

Since nasal sprays and drops are liquid medications, there is a high likelihood of interactions between the immediate packaging and the formulation. Therefore high quality standards for the used materials must be met. Preferably, only material is used which is described in a Pharmacopea or in foodstuff legislation (EMEA Guideline, 2005). Of course other materials may be also used but in such a case an extractable profile and complete documentation on safety and toxicology needs to be generated and submitted.

So all used materials must be identified including the monomers, any additives or colors used and the suppliers for the raw material must be disclosed. To deal with it, it is important, that the manufacturer of the devices has an established quality and change control process which documents all changes and gives notice to customers when required.

> **Definitions**
> **Extractables:** compounds which may be extracted from the container closure system by using stressful conditions.
> **Leachables:** compounds which may leach from the container closure system into the formulation under normal conditions of storage and use.

Even when material of high quality is used for the devices some interactions with the formulation can occur.

To ensure proper function of the pump, steel springs and balls are used which may be in contact with the formulation. Any metal components like balls and springs are prone to cause problems. Even if they are made of non-corrosive material, the surface can discolor the formulation due to impurities or contamination with lower grade material during the manufacturing process. Another risk coming from metal parts are remaining lubricants, which can contaminate the product.

Polyoxymethylene (POM), a widely used material for gaskets and pump parts can release formaldehyde into the drug. Materials like polyethylene or polypropylene are less likely to cause problems, but some monomers or additives can leach into the formulation. This problem of leachables coming from the pump is sometimes overlooked, because during the standard stability studies with non-primed pumps no interaction will be seen with parts inside the pumps. When the pumps are tested for dose consistency, the bottle will be emptied within a few hours. So it is recommended to evaluate compatibility of all parts which come in contact with the formulation as described above early in the development process.

3.4 Performance parameters

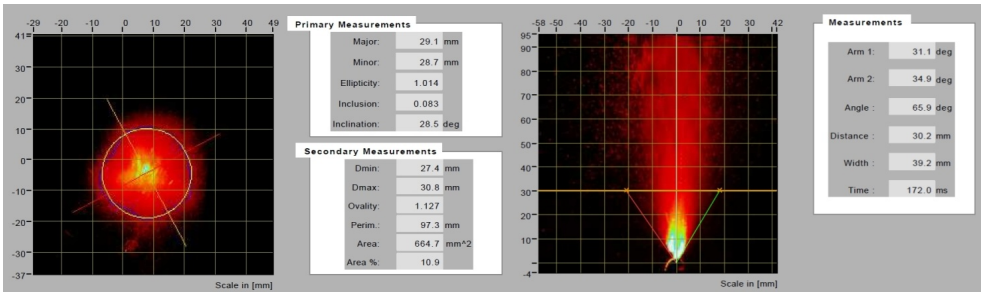
As already mentioned, plume geometry and administration angle have the most significant influence onto nasal deposition but some more parameters are required by authorities for nasal sprays and will be discussed briefly.

It should be clear that the parameters discussed below depend on the experimental conditions and the equipment used for the characterization. So it is important to use well

controlled conditions (e.g. automated actuation at defined speed or force) and procedures for the measurement of performance parameters. From experience it can be told, that the combination of a particular spray pump with a formulation will result in a quite unique performance profile which is hard to meet when considering the use of another pump. The US-FDA recognized that and recommends for generic nasal sprays to use the same brand and model of devices (particularly the metering valve or pump and the actuator) as used in the reference product to get equivalence on the basis of in vitro tests (FDA Guidance for Industry, 2003).

3.4.1 Spray pattern and plume geometry

The spray pattern is best described as a horizontal cut through the spray plume at a defined distance from the orifice, in most cases set at 30 mm. It can be assessed by simply spraying a stained formulation on a sheet of paper or thin layer chromatography (TLC) plates. For TLC plates some chemical reactions may be used to develop the pattern, which is considered more accurately. A higher sophisticated method is the use of a laser beam for the measurements, with systems e.g. from Proveris Scientific, Malvern Instruments, Oxford Lasers or InnovaSystems to name the most widely known providers. Then the shape (e.g. round or ellipsoid shape) and dimensions (e.g. shortest and longest axis) are evaluated and the spray angle may be calculated from this data.



Picture 2. Spray pattern (left) and plume geometry (right) measurements and derived parameters using a laser-based system (Equipment: Proveris Scientific SprayVIEW® and analysed by Proveris Scientific Viota software).

The plume geometry describes the shape and size of the plume (length, width, spray cone angle) at a defined time point following actuation, preferably during the fully developed phase. These parameters are closely linked to a good nasal deposition but are also important for evaluating the performance of the pump. It is recommended to test a range of pumps for a new formulation, because various factors affect the spray pattern and plume geometry, e.g. the size and shape of the nozzle, the design of the pump, and the size of the metering chamber. On the other side, these parameters are quite sensitive to changes in dimensions within the spray pump as well as changes in the formulation. For these reasons, the spray pattern is often used for quality control purposes.

3.4.2 Particle-size distribution

The nose is a very effective filter and most particles and droplets will be caught within the nasal cavity. Only particles less than 10 µm median aerodynamic diameter, so called fine

particles, can reach the lower airways during nasal breathing (Stuart 1984). Most spray pumps will generate an aerosol with a mean particle size in the range of 20-100 µm which are recognized as fine mist and will deposit well in the nasal cavity. During the formation and dissipation phases much larger droplets (>300 µm) can be formed. Droplet size distribution for nasal sprays is assessed by laser diffraction methods. Results are typically expressed as a size in microns below which 10%, 50% or 90% of the volume (D_V or mass D_M) of material exist (e.g. D_V10, D_V50, D_V90, span) and percentage of droplets less than 10 µm. Although a wide range of particles will certainly deposit in the nose, authorities require their characterization because it is a sensitive parameter to detect changes in pump quality or in the formulation.

3.4.3 Actuation force

Nasal spray pumps are normally actuated by a fast movement of thumb, index and middle finger. More recently also pumps with side actuation became available. When actuated, the pumps should deliver the whole dose as fine mist within a fraction of a second. Actuation forces should be in the range of 30-80 N (~3-8 kg). As a rule of thumb, higher viscosity will require higher actuation force. Of course there is also a great impact coming from the device side. Geometry of the metering chamber, design of the valve mechanisms, internal friction, spring forces, dimensions of the swirling chamber have a great influence on this parameter. Some of them can be adapted by the manufacturer to a certain degree to meet the requirements of a particular formulation. Beside this, it is always recommended to test several pump types with a new formulation to find the best match and to meet requirements of the target group (children, elderly).

Picture 3. Standard nasal spray pump on the left: This pump is actuated by pressing the actuator in a longitudinal movement towards the bottle. On the right side a nasal spray pump (Latitude®) designed for lateral actuation with the thumb which is preferred by some people.

3.4.4 Shot weight and delivered dose

Methods for the measurement of shot weight or delivered dose and acceptance criteria are well described in the appropriate guidelines. It should be recognized, that there are some differences between European and US guidelines which should be considered during development.

The volume of the metering chamber will define the delivered dose for a primed pump. This works normally fine for water or saline when the right actuation parameters (force, stroke acceleration) are used. Depending on surface tension and viscosity of the product, air may be trapped in the dosing chamber influencing priming and dose accuracy. Similar problems may occur when air bubbles come into the dip tube. Also too low actuation forces may lead to partial metering. To overcome the problem of partial metering, so called "user-independent" spray pumps were developed but are not widely used by the industry yet.

4. Bottles

Bottles or containers are an integral part of drug delivery devices and will also influence the general appearance of the final product. Special shapes may be used to differentiate a product from competitors. Glass bottles are less prone to cause interactions and will give good protection to the formulation even during long storage intervals. Sometimes the glass can influence the stability of the formulation (change in pH, release of trace metals). This depends of course on the quality of the glass which is described by its hydrolytic class (hydrolytic class I-III is normally used for pharmaceutical products). The disadvantages which glass bottles may have are the higher weight and the risk to break when dropped.

Bottles made of plastic material (e.g. polyethylene, polypropylene, PET) are sometimes used for nasal sprays but are mandatory for ophthalmic droppers because squeezing the bottles is needed to dispense the product. Pump supplier will most likely not manufacture these bottles themselves because a complete different technology is used. Parts for spray pumps or droppers are quite exclusively made by injection molding which gives high precision. Plastic bottle manufacturers use a process called blow-molding. The general principle is to make a hollow raw part and then blowing up the material to the final dimensions. The most important disadvantage for all bottles made of plastic material is evaporation/weight loss. Plastic materials are not a perfect barrier for gas or water evaporation. This problem can be tackled using laminated materials but these are more expensive. Another potential risk has to be considered: inks and adhesives from labels may migrate through the bottle wall and leach into the formulation.

Pure mechanics but critical for all types of bottles: the bottle opening must fit the delivery device exactly. It needs to be tested and dimensions need to be controlled because variations may cause leakages or destroy the closure during final assembly. To avoid troubles, consultation of the delivery system supplier is highly recommended as these companies are experienced in managing this interface. The pump supplier should be able to recommend a range of suited bottles from suppliers which provide reliable quality. Before switching to another bottle or bottle supplier, the compatibility with the device should be checked in advance.

5. Use of preservatives in multi-dose products

Multi-dose dispensers are widely used for the administration of liquid nasal, ophthalmic or dermal drugs because they are convenient and cost effective. To prevent degradation of the

product and to avoid the spread of potentially harmful microbes the content has to be kept sterile, both during storage and while in use by the patient or consumer. This requirement is in most cases met by a closely controlled manufacturing and filling procedure and by placing a suitable preservative or a combination of multiple preservatives in the formulations. Benzalkonium chloride (BAC) is by far the most widely used preservative, but thiomersal, chlorhexidine, chlorobutanol and phenylethanol, and parabens can also be found in topical medications (Freeman and Kahook, 2009).

Two general issues are linked to the use of these preservatives, one of which is the choice of materials. This is certainly of primary importance for the manufacturer only. Traditional glass containers do not interact with preservatives, but more and more used plastic containers and dispensing devices pose problems such as permeation of preservatives through the container or interaction with the plastic materials used in these. Rubber also reacts with preservatives but it is still used for closures, which in such events have to be pre-treated with the preservatives to minimize subsequent uptake during storage.

The issue of significance for the patient and consumer, however, is the high incidence of local side effects attributed to preservatives. The discussion is controversial, and published preclinical and clinical studies are not always consistent. It seems to be clear that short-term use of preparations containing preservatives at low concentrations is well tolerated, but preservatives can cause serious inflammatory effects with long-term use. The responses may include chemical irritation, hyperreactivity and true allergies (Hong and Bilory, 2009). The German Authorities (BfArM) addressed the use of BAC for nasal sprays in 2003 (BfArM notice, 2003) which pushed the preservative free systems for this administration route. Only recently, European authorities encourage the use of unpreserved multi-dose dispensers even for ophthalmic use (EMEA public statement, 2009).

Systems allowing multi-dose use of unpreserved formulations have the potential to solve some of the problems faced by pharmaceutical manufacturers (e.g. incompatibilities), as well as offer marked benefits for patients in need of chronic treatment. The technology starts to get widely known as PFMD devices (preservative free multi dose).

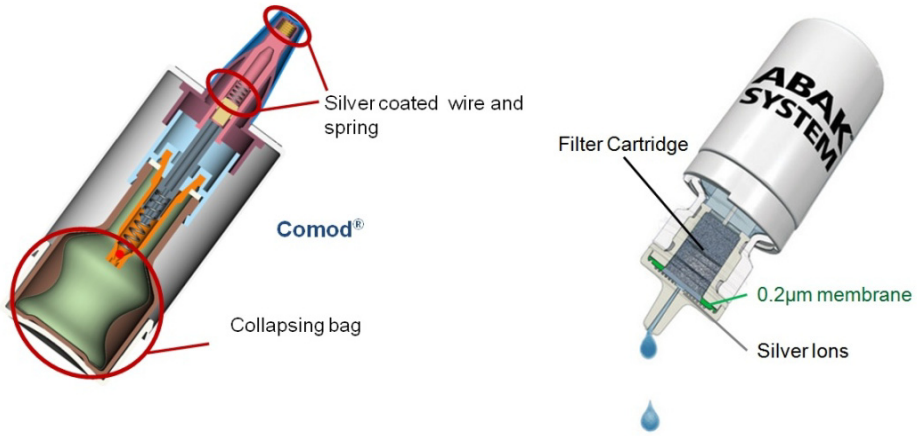
When using a preserved or unpreserved multi-dose product, there are three ways for microorganisms to enter the system:

- via the orifice and
- via the venting air which is replacing the dispensed liquid or
- insufficient container/dispenser fit.

In preserved formulations (conventional system), the added preservative controls microbial growth and no additional measures need to be taken to prevent microbial occupation via the orifice or venting air. If the formulation does not contain preservatives, the device must be able to keep microorganisms out off the system.

Today a range of technical solutions is available to overcome this issue. The highest risk of contamination obviously comes from the orifice, because it may come in contact with skin and mucosa as well as with infected body fluids. Some marketed systems use the oligodynamic activity of a silver wire in the tip of the actuator, a silver coated spring and ball (Groß, 2000). These components release silver ions into the formulation, which is a time dependent process. The system is able to keep microorganisms down between long dosing intervals, even when the tip is immersed into bacterial contaminated fluid (Bagel and Wiedemann, 2004). Silver ions are widely used for their antiseptic properties and even when used for wound dressings, it is safe and no adverse effects are attributed to this treatment. One general limitation of course must be considered: the silver ions may react with the

formulation, e.g. chloride ions and form micro-precipitations. This effect may be overlooked because it is most relevant for spans of 6-12 hours between individual actuations which are not routinely tested during development.



Picture 4. Examples for ophthalmic devices using the oligodynamic effects of silver in combination with other measures like collapsing bags or sterile filtration of the venting air.

Consequently, the most recent preservative free systems follow a purely mechanical approach to minimize interactions between parts of the device and the formulation. One technical solution to prevent contamination via the orifice is named "tip seal technology" which may be utilized for both nasal spray pumps and ophthalmic droppers. A spring loaded valve is located directly below the opening of the tip orifice and does not allow any microbes to migrate from any surfaces or contacted liquids into the system, the orifice is "sealed" under resting conditions. The tip seal keeps the system closed until a defined pressure (for nasal sprays it is more than 3 bar) is reached by actuating the system. Then the system will open and the formulation is forced through the orifice with a higher pressure than needed to open the valve. When the pressure drops at the end of the actuation the tip seal will immediately close the orifice with an outward movement. So no backflow of potentially contaminated medication or other liquid is possible. Depending on the pump system, the fluid path may even be "metal-free", which means the springs needed for the device operation do not come in contact with the formulation.

To avoid contamination of the formulation via venting air different technical solutions are used. The simplest way is sterile filtration of the venting air using separate filters or filter gaskets. For oxygen-sensitive formulations, so called collapsing bags or depressed systems are used. The formulation is filled in a special, microbial tight bag which is protected by a surrounding bottle. When dispensing the product, the bag collapses with the content not coming in contact with the ambient air. Some pumps are constructed in such a way, that the whole system is air-thigh and during use a certain vacuum (up to -300 mbar) is generated within the bottle. Those systems allow even a purging with inert gases to reduce oxygen content in the container head space.

These approaches to avoid the use of preservatives for multi-dose devices sound complicated but are well established and mature technologies. Preservative-free single dose

containers, most often presented as blow-fill-seal (BFS) containers can be an alternative for short term treatments. Disadvantages of these systems are linked to the quite complicated filling technology, the need to overfill and amount of material needed for each dose. Any efforts to provide reliable preservative free multi-dose systems are certainly appreciated by the growing number of patients who experienced discomfort with preserved formulations.

Prevent contamination via orifice	
Oligodynamic components in the actuator or inside the pump	Open orifice, but silver-ions are released into the formulation which kills germs but may interact with the formulation
"tip-seal" technology	Mechanical barrier system to prevent bacterial contamination
Prevent contamination via venting air	
Sterile filter incorporated into delivery system	The venting air in pressure balanced systems are forced though sterile filters with pore sizes less than 0.2 µm, the filter membranes are normally hydrophobic which prevents leakage from liquids out of the container via the venting system
Collapsing primary container	Best suited for oxygen-sensitive formulations, because the venting air does not come in contact with it, this technology also enables spraying at different angles
Unvented container/pump system	Tight fit of bottle and pump design prevent venting of the bottle, vacuum up to -300 mbar are reached

Table 4. Measures/technical solutions for preservative free multi-dose devices

5.1 Preservative free ophthalmic devices

With aging populations, conditions which require chronic treatment like dry eye syndrome or glaucoma are on the rise (WHO fact sheet, 2009) and preservative free multi-dose devices come into play. Chronic use of preserved formulation is not always tolerated by some patients. That is why the EMA encourages the development of such devices (EMEA public statement, 2009). This is not an easy task and poses some risks for the manufacturer. The standards for ophthalmic medications are set high to make sure that no patient or consumer 'risks an eye'. Agencies enforce guidelines on sterility of the product, absence of particles and microbial stability. Typically, the observation of microbial growth in a product leads to rejection of the entire batch, which will impact the manufacturer significantly.

Production and filling of ophthalmic medications are highly sophisticated, so pharmaceutical manufacturers are reluctant to alter established processes or to invest substantially in new filling technologies that obviously require further process qualification.

Because of these difficulties, only a very few multi-dose devices are available on the market yet, most of them relying on the oligodynamic effects of silver ions.

A patient-friendly, multi-dose system for unpreserved liquids is what patients and consumers are looking for, regardless whether one considers "simple" products like artificial tears or more complex pharmaceutical products such as prostaglandins or the like for treatment of serious diseases such as glaucoma.

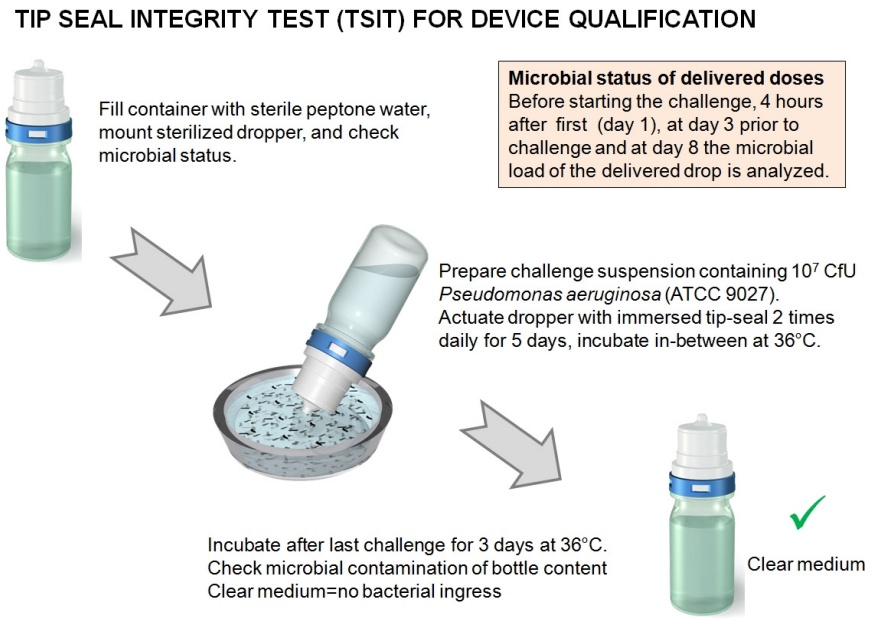
Picture 5. On the left: Rendered picture of the Ophthalmic Squeeze Dispenser (OSD) showing the tip seal and filter for the venting air in blue. Right: the complete system after filling and with removed protection cap.

A break through approach may be the Ophthalmic Squeeze Dispenser (OSD) which uses the mechanical tip-seal technology combined with sterile filtration of the venting air known from preservative free nasal spray pumps. The OSD is simply actuated by squeezing a LDPE (low density polyethylene) bottle and will deliver a drop in the range of 35 µl. The device can easily be adapted for a certain range of viscosity to keep forces within a rational limit and to deliver a consistent drop. Not only is the dropper highly sophisticated but the bottle too. The thickness of the bottle wall must be a compromise between minimizing water vapor loss and providing acceptable actuation forces. Of course the fit between bottle and dropper must be perfect to avoid contamination via this way. As some ophthalmic formulations have compatibility issues with metal, the OSD features a metal-free fluid path.

6. Microbial device integrity tests

To introduce a preservative-free multi-dose device successfully on the market, the microbial integrity must be clearly demonstrated to customers and authorities. Although there are guidelines in place for preserved multi-dose and preservative-free single dose containers, there is no guideline on microbial testing of preservative-free multi-dose devices. So the industry has to develop convincing test methods on their own. A well known procedure is the so called Wiedemann test, which was developed to characterize the 3K/Comod-system (Bagel and Wiedemann, 2004). This test for nasal sprays focuses on the microbial load of the next sprayed dose and was developed according to the needs of the system which releases silver ions into the formulation. The acceptance criteria are less than 100 CfU/ml delivered dose (according the EU Pharmacopeia) and no contamination of the bottle content. Similar tests were developed using a very agile germ (*Pseudomonas aeruginosa*) for the tip seal test and the tiny and robust spores from *Baccilus subtilis* for the whole package integrity test. These tests may be used for the characterization of preservative-free multi-dose nasal spray

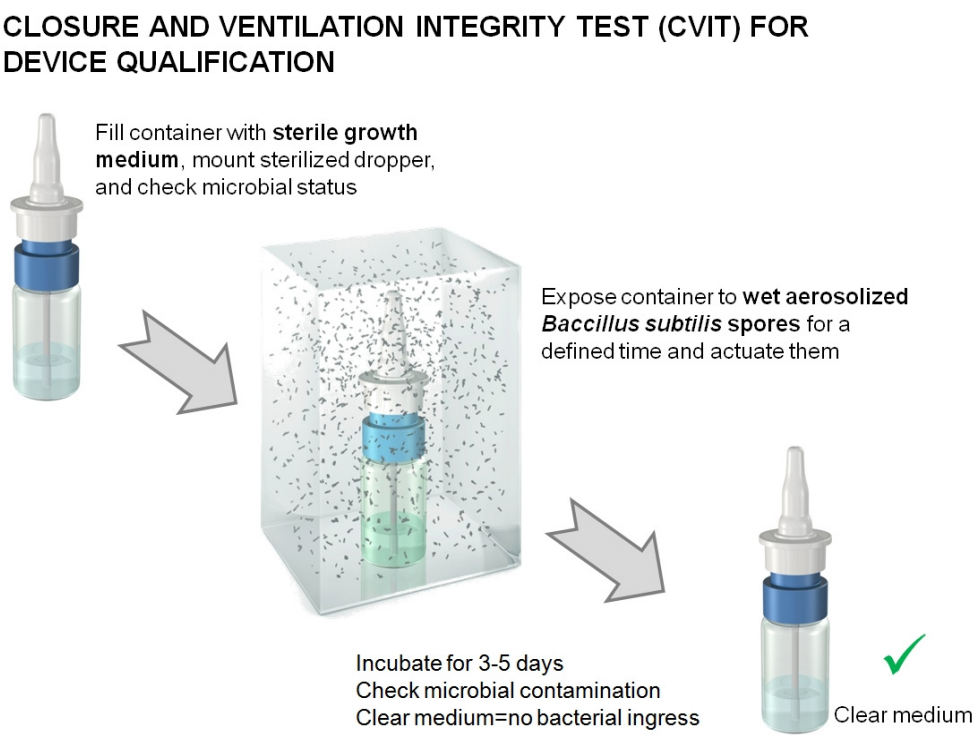
devices (Bommer et al., 2004) as well as for ophthalmic devices (Marx et al., 2010). For such device qualification tests most often the devices are filled with broth medium provide optimum conditions for the germs to growth in the case of any contamination. The final product will promote bacterial growth much less which provides more confidence.



Picture 6. Microbial test to challenge the tip seal function and contamination of delivered doses with a preservative-free ophthalmic dropper.

7. Summary

The intranasal route can be used for a wide range of applications and administration procedure is safe and easy. On the other side, development of a nasal formulation and to identify the right device is a complex process. Cooperation with an experienced pump manufacturer and specialized test laboratories on the selection of the delivery system and setting the right specifications can save resources and increase probability of success. Selecting the wrong spray pump or bottle can jeopardize the entire project so a range of pumps should be tested early in the development process. In this chapter we have focused on multi-dose devices for liquids because they clearly dominate the market. In the future we will see more of so called unit-dose devices which deliver only one dose for one or both nostrils and are discarded then. Such devices can be used for the administration of controlled substances (like fentanyl) to reduce the risk of misuse or for vaccinations (Marx et al., 2011). There are also some propellant-driven devices on the market (e.g. for nasal steroids) but discussion about the impact on the environment may limit their success. The future will also see powders for intranasal administration but all known projects are still in various stages of clinical trials. Last not least electronic add-ons, such as counters or locking features, may bring advantages and opportunities in nasal drug delivery, but will obviously need to comply with cost challenges.



Picture 7. Microbial test to challenge the venting system and closure tightness for preservative free multi-dose systems. During the aerosol-exposure the systems are actuated to generate an under pressure within the system.

Non-invasive administration of eye care products is still by far the most used way to reach therapeutic targets, let it be in diseases such as glaucoma or in self-medicated conditions such as dry-eye syndrome. Alternative methods such as implants or intravitreal injections are getting more attention lately, but given the implications associated with these methods it is fair to conclude that the multidose eye dropper still has a promising future. Avoiding or at least reducing the use of preserving agents in ophthalmic formulations is one way to maintain or extend life cycles of existing products and to ease acceptance of new entities. However, in particular in eye care habits of patients and consumers need to be taken into account carefully.

To answer the question from the beginning: will drug formulations for multiple use, presented in multi-dose containers for nasal or ophthalmic drugs, become preservative free? The answer is not as easy to give as it seems, because cultural aspects are no less important than regulatory aspects, safety concerns or clinical findings. However, as seen today in Europe, a trend begins to establish itself towards preservative-free multi-dose formulations and, consequently, delivery systems, in order to meet patient's expectations. Any development of novel drugs or research on life cycle management should take this into serious consideration.

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Microneedle-Assisted Transdermal Delivery of Opioid Antagonists for the Treatment of Alcoholism

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1. Introduction

It has been estimated that nearly 19 million Americans or 8% of the U.S. population need treatment for an alcohol problem. Alcohol dependence accounts for approximately 100,000 deaths each year. Alcoholism abuse and dependence costs the United States $185 billion dollars in direct and indirect social costs per year with more than 70% of the cost attributed to lost productivity.

Current treatments for alcohol dependence include acamprosate calcium, disulfiram, and naltrexone (NTX). NTX is a potent competitive opioid antagonist with high affinity for the mu-opioid receptor. NTX is currently available in an oral 50 mg tablet (ReVia® and generics) as well as a 380 mg depot injection for the treatment of opiate and alcohol addiction (Vivitrol®; PDR, 1996). The dosage forms have had limited success due to poor oral bioavailability (5-40%), intense side effects such as nausea and stomach pain, as well as inconsistent release from the depot form (Vivitrol®; PDR, 1996). An alternative approach to circumvent drawbacks from oral therapy and painful depot injections to treat alcoholism is transdermal delivery. By initially limiting the input of the drug dose directly to the systemic circulation, extensive metabolism in the liver is bypassed, thereby increasing efficacy and decreasing the chance of deleterious side effects. Transdermal drug delivery systems are trouble-free outpatient products, and monthly reoccurring healthcare visits to receive a painful injection can be avoided.

Opioid antagonists such as NTX, naloxone and nalmefene, a nonselective opioid antagonist, have been studied for delivery by the transdermal route for the treatment of alcohol dependence. However, most of the opioid antagonists do not have suitable physicochemical properties for required therapeutic skin permeation rates from passive-delivery. Previous attempts to deliver a controlled therapeutic dose of NTX through the skin have been unsuccessful in achieving desirable permeation rates in the high end of the therapeutic

range (Hammell et al., 2004; Pillai et al., 2004; Vaddi et al., 2005). NTX's primary metabolite, 6-β-naltrexol (NTXOL), is formed by reduction at the 6-keto group. NTXOL, which may provide a longer duration of action than NTX, also does not have optimal physicochemical properties for transdermal delivery (King et al., 1997; Verebey et al., 1976). NTXOL's maximum flux values have been reported to be 6-fold less than that of the parent NTX (Paudel et al., 2005). Approaches ranging from prodrug synthesis to codrug formation have been applied to enhance permeation of NTX and NTXOL through the skin with limited successes (Kiptoo et al., 2006; Valiveti et al., 2005).

The most recent approach to deliver therapeutically relevant levels of NTX is with the application of microneedle (MN) arrays to the skin (Wermeling et al., 2008). NTXOL has also been evaluated for MN skin permeation enhancement in hairless guinea pigs (GP) (Banks et al., 2010). Microneedles, composed from stainless steel, biodegradable polymers, and silicon, provide an aqueous conduit for drug to bypass the stratum corneum and enter the systemic circulation (Prausnitz, 2004; Prausnitz et al., 2005). MN study, while in its infancy, has focused on a broad range of compounds such as insulin, desmopressin, oligonucleotides, and vaccines for permeation enhancement (Cormier et al., 2004; Coulman et al., 2006; Gardeniers et al., 2003; Martanto et al., 2004; Mikszta et al., 2002; Prausnitz, 2004). MNs have been mostly used either as a pretreatment to permeabilize skin before applying a patch or with drug coated onto the MN array for rapid dissolution and release in the skin (Cormier et al., 2004; Martanto et al., 2004; Prausnitz, 2004).

Previous studies have shown that the transdermal flux of NTX and NTXOL can be enhanced by MN and can be optimized by using MN in combination with charged (protonated) drugs that have increased solubility in an aqueous patch reservoir and increased permeability through aqueous pathways created by MN in the skin (Banks et al., 2008). Another study determined the lifetime of MN-created aqueous pore pathways (Banks et al., 2010). MN pore lifetime was estimated by pharmacokinetic evaluation, transepidermal water loss (TEWL) and visualization of MN-treated skin pore diameters using light microscopy. A 3.6-fold enhancement in steady-state plasma concentration was observed *in vivo* with MN treated skin with the hydrochloride (HCl) salt of NTXOL as compared to NTXOL base. TEWL measurements and microscopic evaluation of stained MN-treated GP skin indicated the presence of pores, suggesting a feasible nonlipid bilayer pathway for enhanced transdermal delivery. Overall, MN-assisted transdermal delivery appeared viable for at least 48 h after MN-application.

It has been shown that formulation, specifically the viscosity of the formulation, influences the MN-enhanced transdermal transport of NTX·HCl (Milewski & Stinchcomb., 2011). Another study has combined microneedle skin pretreatment with the use of a highly water-soluble PEGylated NTX prodrug for further improvement in the percutaneous flux of NTX (Milewski et al., 2010). The main drawback of MN-assisted delivery, however, is that the micropores begin to close between 48-72 hour (Banks et al., 2010) and hence it is not possible to achieve sustained delivery of the drug for a prolonged period. A recent study has shown that by using diclofenac, a nonspecific COX inhibitor, daily with NTX enables the delivery of the drug over a 7 day period in hairless GP (Banks et al., 2011).

An *in vitro/in vivo* correlation (IVIVC) is an integral relationship in pharmaceutical dosage form development. Defined by the FDA, an IVIVC is a predictive model describing the relationship between an *in vitro* property of a dosage form and relevant *in vivo* response (FDA). In the case of transdermal delivery, the *in vitro* property is the rate of permeation or release through the skin while the *in vivo* response is the plasma drug concentration. An

IVIVC is an important aspect of transdermal delivery, because it gives validation to *in vitro* diffusion studies. Building confidence into the *in vitro* model is a cost and time saving factor that affords screening of multiple compounds and only testing the most successful *in vivo*. Previous studies looking at transdermal delivery of NTX prodrugs and a highly lipophilc drug, delta 8-THC, showed good correlation with the hairless GP pharmacokinetic model and *in vitro* flow through diffusion cell data (Valiveti et al., 2004a). The approach to deliver NTX at therapeutically relevant levels that has recently been studied is to use the water soluble form of NTX aided by MN facilitated permeation of the skin (Banks et al., 2008). In this present study, we describe IVIVC of NTX through MN treated skin. *In vitro* skin permeation studies were carried out across MN treated GP and human skin with a gel comprised of the hydrophilic HCl salt form of NTX. Then, *in vivo* studies were carried out in GP utilizing a MN gel patch delivery system to establish an IVIVC in GPs. Finally, the *in vitro* human skin data was compared with data from a previous healthy human volunteer study (Wermeling et al., 2008) to establish the human IVIVC.

2. Materials and methods

2.1 Materials

NTX·HCl was purchased from Mallinckrodt Inc. (St. Louis, MO, USA). Hanks' balanced salts modified powder and propylene glycol was purchased from Sigma Aldrich (St. Louis, MO, USA). Ammonium citrate was purchased from Alfa Aesar (Ward Hill, MA, USA). Sodium bicarbonate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), gentamicin sulfate, ammonium acetate, ethyl acetate, acetonitrile (ACN), triethylamine (TEA) and trifluoroacetic acid (TFA) were obtained from Fisher Scientific (Fairlawn, NJ, USA). A Barnstead nanopure Diamond Ultrapure water system was used for all aqueous solutions (Barnstead International, Dubuque, IA, USA). 1-Octanesulfonate was purchased from Regis Technologies, Inc (Morton Grove, IL, USA). Natrosol® (Hydroxyethylcellulose250HHX PHARM) was a gift from Hercules, Inc. (Wilmington, DE, USA). Benzyl Alcohol was purchased from Spectrum Chemical MFG. Corp. (Gardena, CA, USA).

2.2 Microneedle fabrication

In-plane microneedle rows with five microneedles were cut from stainless steel sheets (Trinity Brand Industries, SS 304, 75 mm thick; McMaster-Carr, Atlanta, GA, USA) using an infrared laser (Resonetics Maestro, Nashua, NH, USA) using methods previously described (Martanto et al., 2004) in the laboratory of Dr. Mark Prausnitz at the Georgia Institute of Technology. Briefly, the microneedle row was first drafted in AutoCAD software (Autodesk, Cupertino, CA, USA). Using this design, the infrared laser cut microneedles into the stainless steel sheet. The microneedle rows were then cleaned with detergent (Alconox, White Plains, NY, USA) to de-grease the surface and remove part of the slag and oxides deposited during laser-cutting. To completely clean the slag and debris and to sharpen microneedle tips, microneedle rows were electropolished in a solution containing glycerin, ortho-phosphoric acid (85%) and water in a ratio of 6:3:1 by volume (all chemicals, Fisher Scientific, Fair Lawn, NJ, USA). Electropolishing was performed in a 300 ml glass beaker at 70°C, a stirring rate of 150 rpm, with a current density of 1.8 mA/mm^2 applied for 15 min. A copper plate was used as the cathode (negative), while microneedles acted as the anode (positive). The electropolished microneedle rows were then cleaned by alternatively dipping in 25 % nitric acid (Fisher Scientific) and deionized water with a total of three repetitions. A

final rinse was performed under running deionized water before drying under pressurized air. Dry microneedle rows were stored in air-tight containers until later use. MN arrays for human studies were fabricated to produce patches containing 50MNs arranged in 5x10 arrays of MNs. Each MN measured 620 µm in length, 160 µm in width at the base, and 1 µm in radius of curvature at the tip. For *in vitro* studies and *in vivo* GP studies, each MN measured 750 µm in length, had a base width of 180 µm, and <1 µm radius in curvature at the tip. The distance between individual MNs was approximately 1 mm.

2.3 16% NTX·HCl gel formulation

The NTX gel formulation used for *in vitro* and *in vivo* studies consisted of NTX·HCl, (16.0%), propylene glycol (60.75%), sterile water for injection (20.25%), benzyl alcohol (1.0%) and hydroxyethylcellulose (2.0%). For human studies, the same formulation composition was used except that the gel was formulated, prepared, and tested according to current good manufacturing practices as outlined by the FDA and was carried out in collaboration with Coldstream Laboratories, Inc., formerly named The Center for Pharmaceutical Science and Technology (Wermeling et al., 2008).

2.4 *In vitro* studies

2.4.1 *In vitro* diffusion studies across full thickness hairless GP and human skin

Full thickness hairless GP skin was harvested from euthanatized animals. Animal studies were approved by the University of Kentucky IACUC. Human skin harvested during abdominoplasty was used for the diffusion studies. Human tissue use was approved by the University of Kentucky Institutional Review Board. Skin sections were obtained by removing the subcutaneous fatty tissue by scalpel dissection, and were stored at -20°C. A PermeGear flow-through (In-Line, Riegelsville, PA, USA) diffusion cell system was used for the skin permeation studies. Skin used for microneedle treatment was placed on a wafer of polydimethylsiloxane polymer, which mimicked the underlying mechanical support of tissue because of its comparable structural flexibility and elasticity. The human skin was pierced 20 times with an array containing 5 MN (Figure 1) (i.e., to make a total of 100 individual and non-over lapping piercings) before mounting the skin in the diffusion cell. Diffusion studies to determine flux values for GP skin were performed in such a way that only 3 insertions were made to obtain 15 individual and non-overlapping MN insertions. The insertion of MN into skin was carried out manually by applying gentle finger pressure followed by instantaneous removal. MN's were distributed evenly within the 0.95 cm^2 area of skin and could easily be visualized after each 5 MN insertion, as to prevent reapplication in the same area. Single MN sections of 5 were used simply out of ease for the experimental procedure. If any damage to an MN section was observed the section was replaced. Cells containing MN treated skin showed no presence of receiver solution back flow into the donor compartment. Untreated skin samples were simply placed in the diffusion cells. Diffusion cells were kept at 32°C using a circulating water bath. Data were collected using skin from a single guinea pig or human.

donor with three cells for untreated formulations and 3-4 cells for the MN treated formulations. The physiological receiver solution was HEPES-buffered Hanks' balanced salts with gentamicin at pH 7.4, and the flow rate was adjusted to 1.1 ml/h. Each cell was charged with 0.25 ml of the gel spread over the skin in the donor compartment of the chamber. The diffusion cells were covered with a stopper to prevent evaporation. Samples

were collected from the receiver compartment in six-hour increments over 48 h. All samples were stored at 4°C until analyzed by HPLC.

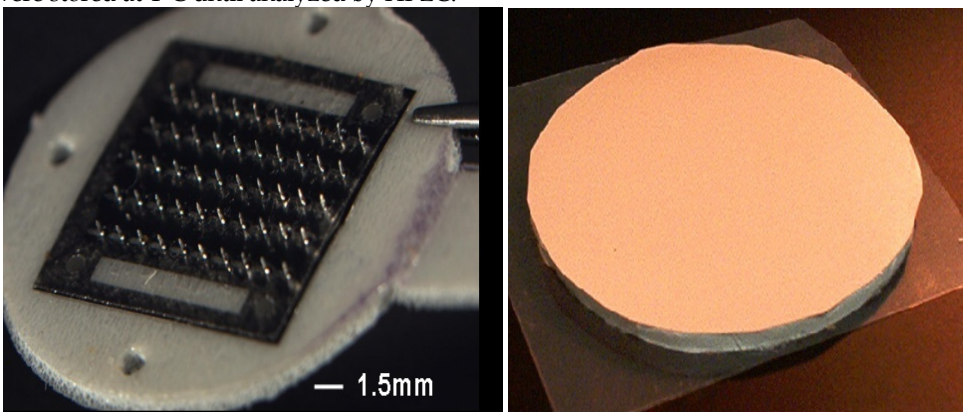

Fig. 1. Left depicts a 50 MN array used in human and GP studies and right shows a protective gel patch covering.

2.4.2 HPLC analysis

Quantitative analysis of NTX concentrations in receiver samples was carried out using a modification of the HPLC assay described by Hussain et al. (Hussain et al., 1987; Paudel et al., 2005). This HPLC analysis was performed as reported by Vaddi et al. (Vaddi et al., 2005). The receiver samples were diluted 10 fold with acetonitrile and injected directly into the HPLC. The HPLC system consisted of a Waters 717plus autosampler, a Waters 1525 Binary HPLC pump, and a Waters 2487 dual wavelength absorbance detector with Waters Breeze™ software (Milford, Massachusetts, USA). A Brownlee (Wellesley, Massachusetts, USA) C-18 reversed-phase Spheri-5 µm column (220 x 4.6 mm) with a C-18 reversed phase 7 µm guard column (15 x 3.2 mm) by Perkin Elmer® was used with the UV detector set at a wavelength of 215 nm. The mobile phase for NTX was 70:30 ACN: 0.1% TFA with 0.065% 1-octane sulfonic acid sodium salt, adjusted to pH 3.0 with TEA, and samples were run at a flow rate of 1.5 ml/min with a run time of 5 min.

2.4.3 *In vitro* data analysis

The cumulative quantity of drug collected in the receiver compartment was plotted as a function of time. The flux value for a given experiment was obtained from the slope of the steady state portion of the cumulative amount of drug permeated plotted over time. Apparent permeability coefficient values were calculated from Fick's First Law of diffusion:

$$\frac{1}{A}\left(\frac{dM}{dt}\right) = J_s = K_p \Delta C \tag{1}$$

where J_s is the flux at steady state (nmol/cm²/h), M is the cumulative amount of drug permeating through the skin, t is time, A is the area of the skin (0.95 cm²), K_p is the effective permeability coefficient in cm/h, and ΔC is the difference in concentrations of NTX in the donor and receiver components. Sink conditions were maintained in the receiver solution

for the duration of the experiment; thus ΔC was approximated by the initial drug concentration in the donor compartment.

2.5 *In vivo* hairless GP and human studies

2.5.1 Transdermal protective covering patches and dosing regimen

The transdermal covering was prepared as described by Wermeling et al. (Wermeling et al., 2008). Briefly, the transdermal occlusive protective covering patches of NTX·HCl (6.7 cm^2) were fabricated by sandwiching a rubber ringed barrier to create a reservoir between a drug-impermeable backing membrane (Scotchpak™ #1109 SPAK 1.34 MIL Heat Sealable Polyester Film, 3M Drug Delivery Systems, St. Paul, MN, USA) and an ARcare® 7396 adhesive around the edge of the nitrile spacer (Adhesive Research, Inc., Glen Rock, PA, USA). The impermeable backing laminate was adhered to the FDA approved medical grade nitrile retaining ringed barrier from Ilene Industries (Shelbyville, TN, USA) with ARcare® 7396. Finally, ARcare® 7396 was placed on the bottom of the rubber ringed barrier to maintain intimate contact with the skin and prevent evaporation of the 500 μL gel formulations. The protective patch was placed on a release liner composed of Scotchpak™ 9742 (3M Drug Delivery Systems, St. Paul, MN, USA). Human subjects were dosed with a total of 400 MN insertions and 2 g of gel. That is, the dose per patch was 0.5 mg gel and 100 MN insertions (Wermeling et al., 2008). MN arrays were applied manually to humans by only one clinician, after training to reproducibly apply with the same force. The same procedure was followed for the control subjects without the application of microneedle arrays. The gel patch systems were left in place on the skin for 72 hours, during which plasma samples were collected for LC-MS analysis of NTX and its active metabolite, NTXOL. The above described system was utilized on hairless GP (n=4) with only 15 MN insertions and 1 gel patch assembly system for 72 hours. For these studies, the insertion of MN was manual by applying gentle finger pressure followed by instantaneous removal. As in the human studies, only one researcher applied MN arrays to GP manually to reproducibly apply with the same force. The gel patch system was applied on the dorsal region of the hairless GP. Bio-occlusive tape was applied over the patches followed by a protective stocking. Samples were collected for LC-MS analysis. All plasma samples were stored at -70°C until analyzed.

2.5.2 Plasma sample extraction procedure

Samples were prepared and analyzed as described by a modified method as described by Paudel *et al*. (Paudel et al., 2005). One-hundred μL of plasma was extracted with 500 μL of ethyl acetate. The mixture was vortexed for 30 s and centrifuged at 10,000 x g for 20 min. The pellet and supernatant were placed in a -20°C freezer for 15 minutes to freeze the aqueous pellet. The supernatant was pipetted into a 3 ml glass test tube and evaporated under nitrogen at 37°C. The residue was reconstituted with 100 μL of ACN and sonicated for 15 min. The samples were transferred into autosampler vials containing low volume inserts, and 20 μL was injected onto the HPLC column.

2.5.3 Liquid chromatography

Chromatography was performed on a Waters Symmetry® C_{18} (2.1 x 150mm, 5 μm) column at 35°C with a mobile phase consisting of ammonium acetate (2 mM) containing 0.01 mM of ammonium citrate:ACN (65:35 v/v) at a flow-rate of 0.25 ml/min.

2.5.4 Mass spectrometry

The system consisted of HPLC with mass spectrometry detection (LC-MS) equipped with a Waters Alliance 2695 pump, Alliance 2695 autosampler, and a Micromass ZQ detector (Milford, MA) using electrospray ionization (ESI) for ion production. Selected ion monitoring (SIM) was performed in positive mode for NTX, m/z 324 $[M+H]^+$ and NTXOL, m/z 344 $[M+H]^+$. Capillary voltage was 4.5 kV and cone voltage was 30 V. The source block and desolvation temperatures were 120°C and 250°C, respectively. Nitrogen was used as a nebulization and drying gas at flow rates of 50 and 450 L/h, respectively.

2.5.5 *In vivo* data analysis

The pharmacokinetic analysis of NTX plasma concentration versus time profiles after MN treatment and gel patch application was carried out by fitting the data to a non-compartmental model with extravascular input (WinNonlin Professional, version 4.0, Pharsight Corporation, Mountain View, CA). The data generated were analyzed to determine peak concentration (C_{max}), steady state concentration (C_{ss}), lag time to steady state concentration (t_{lag}), and area under the plasma concentration time course from 0 to 72 h (AUC_{0-72}). The steady state plasma concentration of NTX after the application of patches was calculated by using the equation:

$$C_{ss} = AUC_{0-t}/\text{time} \tag{2}$$

2.5.6 *In vitro*/*In vivo* correlation

The predicted steady state plasma concentrations of NTX in the GP following the application of the TTS patch was calculated from the *in vitro* steady state flux by using the following equation:

$$C_{SS} = \frac{J_{SS}A}{CL} \tag{3}$$

where 'Css' is the predicted steady state plasma concentration (ng/ml); 'J_{ss}' is the steady state flux across human or GP skin; 'A' is area of the applied patch (26.8 or 6.7 cm^2 in humans or GP, respectively); 'CL' is the total body clearance in humans or GP.

Statistical analysis of the *in vivo* data obtained after the transdermal application of the patches was performed by one-way ANOVA using SigmaStat.

3. Results

3.1 16.0% NTX·HCl gel characterization

All of the release testing and stability testing of the 16.0% NTX·HCl gel was performed in Coldstream Laboratories, Inc. in accordance to cGMP regulations set forth by the FDA and the standard operating procedures of the facility. All curves generated for HPLC standard analysis of the gel had an average correlation value (r^2) of 0.999 ± 0.001 and precision for the assay had a % RSD of 0.67%. The solubility determined prior to formulation scale-up of NTX·HCl was 171.9 mg/ml. Thus, a 16% gel would be approximately 93.7% of the saturation limit, providing a satisfactory driving force according to Fick's first law of diffusion. The percent label claim of the gel at the product release testing, 1.5 months, and 5 months at 25°C/60% relative humidity was 97.0 ± 0.7%, 105.6 ± 3.5%, and 97.8 ± 1.7%,

respectively. All of the HPLC results were within the 90.0 – 110.0% of the specifications for label claim. The gel was clear, colorless and transparent, and over time a yellowish tint was observed. The pH was measured at 4.96 ± 0.03 and the viscosity was 16,859 ± 437 cP. Overall, the formulation was within percent label claim, had a pharmaceutically elegant appearance, excellent consistency to maintain intimate contact with the skin, and a skin compatible pH.

3.2 *In vitro* diffusion studies

3.2.1 HPLC validation

Calibration plots were prepared using NTX standards with the final concentrations over a range of 0.1-25 μg/ml. The correlation coefficient (r^2) obtained was 0.9997 for standard curves. The lower limit of quantification (LLOQ) was 0.1 μg/ml and the limit of detection (LOD) was 0.05 μg/ml.

3.2.2 *In vitro* diffusion studies across full thickness hairless GP skin

From cumulative permeation profiles the flux could be ascertained from the linear portion (apparent steady state). Extrapolation of this linear curve to the x-axis gives the lag-time to steady state. Figure 2 shows a cumulative NTX profile from 16% NTX·HCl gel on full thickness GP skin with 15 MN insertions and control intact GP skin that had no MN treatment. Table 1 shows the fluxes observed with 15 MN insertions as well as fluxes obtained from intact GP skin. Permeability coefficients and solubility are also reported. As observed in Table 1, a significant difference was observed ($p < 0.05$) in both flux and permeability parameters.

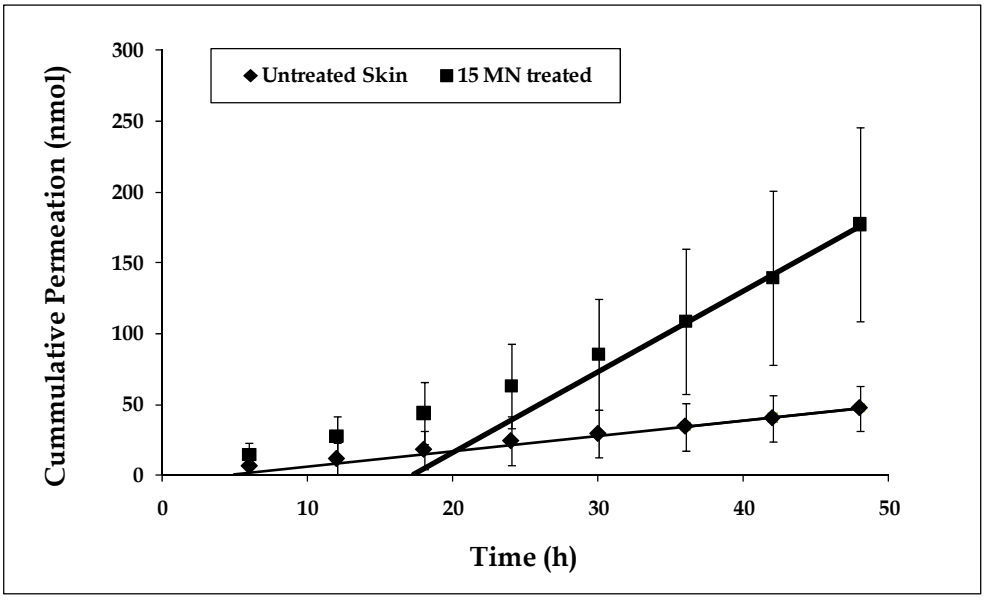

Fig. 2. Cumulative amount of NTX·HCl permeated through 15 MN treated and intact GP skin.

In vitro Study	MN treatment	Flux (nmol/cm²/h)	Kp (x 10⁵cm/h)
GP Skin	No MN	1.1 ± 0.9	0.26
	† MN treated	6.0 ± 1.5	1.4
Human Skin	No MN	0.13 ± 0.01	0.03
	†† MN treated	39.0 ± 13.1	9.2

† GP skin treated with 15 MN insertions for *in vitro* study.
†† Human skin treated with a total of 100 MN insertions for *in vitro* study

Table 1. *In vitro* permeation of a 16.0% NTX·HCl gel through human and GP skin

3.2.3 *In vitro* diffusion studies across full thickness human skin

Flux values and permeation profiles of NTX in human skin can be observed in table 1 and figure 3, respectively. Again, as shown in GP skin, a significant increase in flux ($p < 0.05$) was observed when comparing MN treated skin (39.0 ± 13.1 nmol/cm²/h) to intact full thickness skin (0.13 ± 0.01 nmol/cm²/h). Flux and permeability coefficients from MN treated skin were enhanced 300-fold when compared to intact full thickness human skin. As seen in figure 3, there is also a reduction in lag time when the skin is exposed to MN.

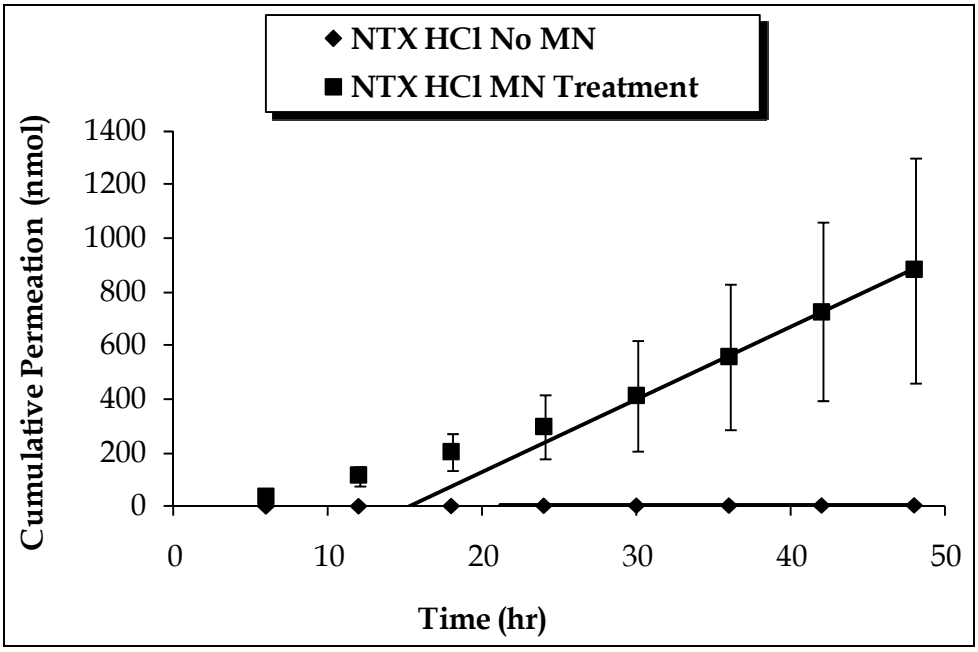

Fig. 3. Permeation profile of cumulative amount of NTX·HCl permeation through 100 MN treated and intact full thick human abdominal skin.

3.3 *In vivo* MN assisted transdermal studies

3.3.1 LC-MS Validation

In the current method, modification of the assay reported by Valiveti et al (Valiveti et al., 2004b) was validated to ensure proper sample analysis. Both NTX and NTXOL standards were first prepared in individual acetonitrile stock solutions. A standard curve of 0.5, 1, 2.5, 5, 7.5, 10, 15, and 25 ng/ml was generated after dilution of the stock solution for analysis of NTX and NTXOL containing 40 ng/ml naloxone as an internal standard. The retention times for NTX, NTXOL and naloxone were 4.3 ± 0.02 minutes, 3.1 ± 0.03 minutes, and 5.4 ± 0.2 minutes, respectively. The LOD and LOQ determined for NTX was 0.5 ng/ml in acetonitrile, however the LOQ was determined to be 1.0 ng/ml in plasma, as plasma interference made the blank plasma and 0.5 ng/ml plasma standards indistinguishable by LC-MS. The standard curve correlation coefficient for NTX in acetonitrile was 0.995 as compared to 0.979 for NTX extracted from plasma. However, both of these standard curves had values greater than $r^2 = 0.95$, the minimum value needed. The LOD and LOQ determined for NTXOL was 0.5 ng/ml in acetonitrile. The LOQ was determined to be 0.5 ng/ml as well in the plasma as plasma interference from the blank did not have an overlapping effect. The standard curve correlation coefficient for NTXOL in acetonitrile was 0.996, as compared to 0.987 for NTXOL extracted from plasma. The average extraction efficiency of NTX from plasma as compared to NTX in acetonitrile was 86.0 ± 6.8 %. Plasma standards (5 and 10 ng/ml) were placed in a -70°C freezer and allowed to freeze. The samples were then warmed to room temperature, and this freeze-thaw was repeated a total of three times over a 3 hour period. The samples were then extracted and analyzed. The three 5 ng/ml freeze-thaw standards had an average standard to internal standard ratio of 0.08 ± 0.002 with a %RSD of 2.7%. The average ratio of freeze-thaw samples was compared to the ratio of 0.08 ± 0.006 from 6 freshly extracted plasma standards (5 ng/ml) suggesting no degradation was occurring. The three 10 ng/ml freeze-thaw standards had an average standard to internal standard ratio of 0.13 ± 0.01 with a %RSD of 11.1 %. The average ratio of freeze-thaw samples was compared to the ratio of 0.14 ± 0.008 from 6 freshly extracted plasma standards (10 ng/ml) suggesting no degradation was occurring. Six replicate plasma samples of 5 and 10 ng/ml each were extracted as described above and injected in duplicate. The average ratio of standard to internal standard for the 5 ng/ml was 0.08 ± 0.006 with a %RSD of 7.8%. The reproducibility of extraction was in good confidence with a low %RSD. The 10 ng/ml ratio of NTX standard to internal standard was 0.14 ± 0.008 with an even lower %RSD of 5.6%. The average internal standard area for NTXOL throughout the day was 40,966 ± 3,462 with a %RSD of 8.5%.

3.3.2 *In vivo* hairless GP studies

GP pharmacokinetic data from 15 MN insertions from a section of 5 MNs (Figure 1) and one 6.7 cm^2 occlusive protective patch containing 0.5 g 16.0% NTX.HCL gel can be observed in table 2 and plasma profiles of NTX are shown in figure 4.

The plasma concentration rapidly reached a steady state level and maintained an average plasma level of about 2.6 ng/ml for 48 hours. Conversely, without the aid of MN insertions no detectable levels of NTX were observed after placement of one 6.7 cm^2 patch containing 16.0 % NTX·HCl gel.

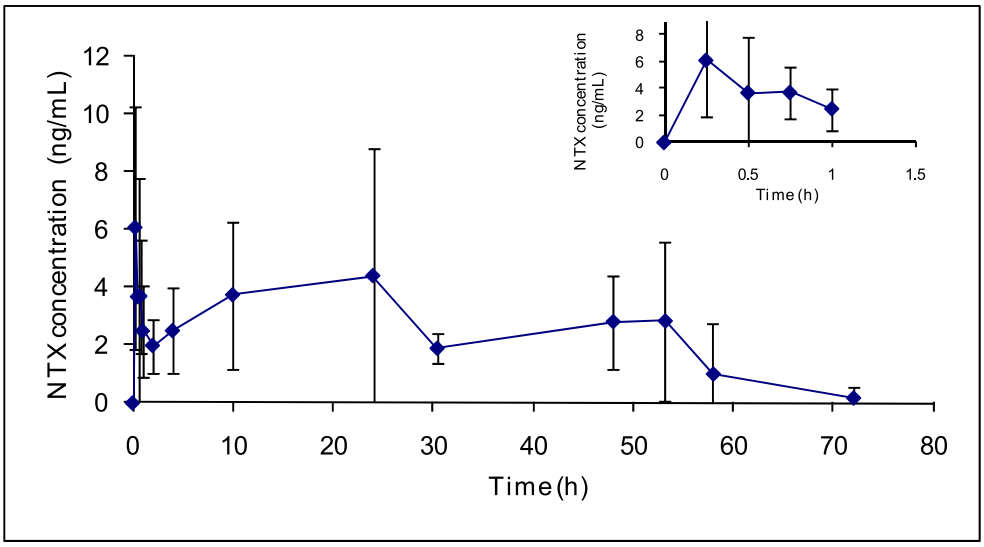

Fig. 4. Plasma NTX concentration profile from 16% NTX·HCl gel with 15 MN insertions in hairless GP.

	Healthy Human Subjects(*)		GP	
PK parameters	**NTX MN treatment**	**NTXOL MN treatment**	**NTX MN treatment**	**NTX No MN treatment**
C_{ss} (ng/ml)	**2.5 ± 1.0**	**0.6 ± 0.5**	**2.6 ± 0.6**	**ND**
T_{lag} (h)	**1.8± 1.1**	**1.4 ± 1.4**	**4.0 ± 0.0**	**ND**
AUC (ng/ml*h)	**142.9 ± 43.9**	**39.7 ± 25.9**	**158.2 ± 63.5**	**ND**
C_{max} (ng/ml)	**4.5 ± 2.4**	**1.9 ± 1.3**	**8.0 ± 1.8**	**ND**
T_{max} (h)	**8.8 ± 7.6**	**37.5 ± 31.3**	**0.3 ± 0.0**	**ND**
C_{last} (ng/ml)	**1.8 ± 1.0**	**0.4 ± 0.6**	**0.0 ± 0.0**	**ND**

(ND) NTX was not detected in the control (no MN) animals therefore PK parameters were not established.
(*) Human pharmacokinetic parameters are values obtained from earlier human study (Wermeling et al., 2008).

Table 2. Pharmacokinetic analysis of 6 healthy subjects and hairless GP treated with MN arrays and a 16.0% NTX·HCl gel patch.

3.3.3 *In vivo* human studies

We recently completed a human study with MN assisted transdermal delivery of NTX (Wermeling et al., 2008). Volunteers in the study wore four gel patch systems and were treated with a total of 400 MN insertions with an MN array as described by Wermeling et al.(Wermeling et al., 2008). Subjects reported no pain, only a sensation of pressure upon insertion of the MN arrays into each subjects upper arm region. As shown in table 2 the data exhibit a rapid increase in plasma NTX concentration above 2.5 ng/ml (therapeutic goal) within 2 hours and this level appears to remain for at least 48 hours. The average steady state concentration observed was 2.5 ± 1.0 ng/ml with 4 patches and a total of 100 MN insertions per patch (Wermeling et al., 2008).

3.3.4 *In vitro/In vivo* correlation

The average steady state plasma concentrations in GP and humans were estimated from equation 3 and were compared with the observed concentrations *in vivo* (Table 3). The average predicted steady state concentration in GP was 2.7 ± 0.7 ng/ml and the actual observed steady state concentration was 2.6 ± 0.6 ng/ml, using a clearance value of 5.65 L/h and a patch area of 6.7 cm^2 (Paudel et al., 2005). These two values were within 96% agreement with each other and there was no significant difference between the estimated and observed C_{ss} values ($p > 0.05$). Similarly, a good correlation was seen upon comparing human skin and healthy human individuals *in vivo*. The predicted and observed steady state concentrations in human skin and humans was 1.9 ± 0.7 ng/ml and 2.5 ± 1.0 ng/ml (Wermeling et al., 2008), respectively, using a clearance of 3.5 L/min and a patch area of 26.8 cm^2 (Vivitrol®).

Healthy Human Volunteers			Hairless GP	
(ng/ml)	**No MN**	**MN**	**No MN**	**MN**
C_{ss} Observed	**ND***	**2.5 ± 1.1***	**ND**	**2.6 ± 0.6**
C_{ss} Predicted	**0.01 ± 0.0**	**1.9 ± 0.7**	**0.5 ± 0.4**	**2.7 ± 0.7**
(nmol/cm^2/h)				
J_{ss} Observed	**0.13 ± 0.01**	**39.0 ± 13.1**	**1.1 ± 0.9**	**6.0 ± 1.5**
J_{ss} Predicted	**ND**	**51.9 ± 20.2**	**ND**	**5.8 ± 1.3**
% Correlation	**NC**	**76.0**	**NC**	**96.7**

(ND) No NTX detected in plasma
(NC) No correlation due to absence of NTX in plasma
(*) Results obtained from previous human study (Wermeling et al., 2008).

Table 3. *In vitro/in vivo* observed and predicted steady state fluxes and plasma concentrations of NTX in humans and hairless GP.

4. Discussion

4.1 *In vitro* diffusion studies

4.1.1 *In vitro* diffusion studies across full thickness hairless GP skin

Treatment of the GP skin with as little as 15 MN insertions caused an increase in flux and permeability compared to MN untreated skin. Permeability increased as the resistance from intact to MN treated skin decreased. Even with a minimal number of insertions, permeation was enhanced to a level that would be predicted to be observable in the hairless GP *in vivo*. Flux enhancement was 5.4 times higher with 15 MN insertions as compared to that of intact skin. Based on results from these experiments and previous other trials reported, the hairless GP model is a good choice for *in vitro* screening of transdermal candidates (Valiveti et al., 2004a; 2005).

4.1.2 *In vitro* diffusion studies across full thickness human skin

As observed with GP skin, an increase in both flux and permeability coefficient was observed in MN treated human skin compared to intact full thickness skin. Earlier work with 100 MN insertions in human skin gave a flux enhancement ratio of 8.3 in contrast to our 300 fold enhancement in flux and permeability coefficient (Banks et al., 2008). Differences between the formulations and concentration (10% vs. 16%), excess solid, and viscosity can definitely influence the NTX flux in the presence of MN micropores. A reduction in lag time when the skin is exposed to MNs is a strong indication that there has been a change in the diffusivity and/or permeation pathway. Thus, not only can flux indicate a change in the route of diffusion and resistance of the skin, but also the lag time is a good indicator of the drug re-routing.

It is well established that a 2.5 ng/ml NTX plasma concentration in humans provides approximately an 85% narcotic blockade of a 25 mg IV injection of heroin (Verebey et al., 1976). It was predicted that an area of 26.8 cm^2 would be required to obtain a therapeutic NTX plasma level, based on the flux information presented in table 1 and figure 3, and the gel formulation used. Thus, a four patch 16% NTX.HCl gel system with 100 MN insertions per patch was proposed to determine the feasibility of MN assisted delivery in humans (Wermeling et al., 2008).

4.2 *In vivo* MN assisted transdermal studies

4.2.1 *In vivo* hairless GP studies

Of importance in GP studies was a rapid increase in NTX plasma concentration to steady state and that C_{ss} remained reasonably constant for at least 48 hours after 15 MN treatment. Steady state concentrations remaining constant for 48 hours compares with earlier work, where NTXOL plasma concentrations, transepidermal water loss, and microscopic staining and visualization of pores over time were also consistent for 48 hours (Banks et al., 2010). No detectable levels of NTX were observed in GP without MN insertions. As expected, one would not likely see quantifiable amounts *in vivo* due to the lack of a significant amount of pore pathways, such as sweat glands or follicles, through which hydrophilic NTX could permeate. However, even with as little as 15 MN insertions to the skin a rapid increase in flux is observed and the MN created aqueous pores remain open. The steady-state profile of NTX concentrations in the plasma prior to 48 h when the pores begin to close suggests that

once treated with an MN array a constant flow is created for a substantial period. These micropores could theoretically create a more precise and controlled release of drug delivery, as compared to passive delivery systems that rely on inter-individual variations in stratum corneum lipid bilayer resistance. As well, the initial "burst" observed in the plasma concentrations could be caused from a rapid influx of the drug followed by the attainment of the equilibrium steady-state flow through the aqueous channels and constant permeation rates. The "burst" effect in the plasma concentration profile circumvents one of the major complaints about transdermal delivery, the typically long lag times. Drug therapies like transdermal fentanyl could benefit from a "burst" effect as a bolus dose, followed by maintenance therapy for at least 48 hours. Thus, the MN gel patch assembly appears to be a successful candidate for the transdermal delivery of NTX in the GP model.

4.2.2 *In vivo* human studies

As previously described, there are limited successful treatments for opiate and alcohol abuse with NTX (Vivitrol®; PDR, 1996). In a recent study, a therapeutic approach to MN transdermal delivery of NTX in healthy human volunteers was completed (Wermeling et al., 2008). As seen in the GP experiment, there was a rapid initial increase in the plasma drug concentration followed by controlled release of NTX in these human volunteers. For opiate and alcohol abusers, a fast acting form of NTX is desirable and achieving a steady-state permeation of NTX and maintaining that rate for an extended time will benefit addiction therapy. C_{max}, an important PK parameter, was rapidly achieved in all six subjects (4.5 ± 2.4 ng/ml), and this onset was observed within a relatively short time (8.8 ± 7.6 h) (Wermeling et al., 2008). No plasma levels were observed in human non-MN controls. NTXOL plasma levels were lower than NTX, but the same overall permeation profile shape was observed as compared to NTX. *In vivo* human data including C_{ss} and other PK parameters in table 2 had very little subject to subject variability (Wermeling et al., 2008). Conversely, after oral administration, there appears to be a much higher degree of variability in humans, as much as eight-fold (Meyer et al., 1984; Rukstalis et al., 2000). Not only did the study prove that a water soluble molecule could be delivered through an MN-created pore, but delivery rates were in a therapeutic range for a poorly bioavailable NTX (Wermeling et al., 2008).

4.2.3 *In vitro/in vivo* correlation

Using equation 3 to predict the steady state concentration *in vivo* or the steady state flux *in vitro*, a correlation can be made to determine the likeness of the *in vitro* model to actual *in vivo* pharmacokinetic evaluation of a compound. During this research both the *in vitro* and *in vivo* studies proved to be highly successful. There was no significant difference between the estimated and observed C_{ss} values in GP. The GP *in vitro* skin model, therefore, is an excellent *in vitro* based set up to screen potential candidates for MN enhanced transdermal delivery. Previous studies from our group has shown GP as a good model for NTX prodrugs and other highly lipophilic compounds for passive delivery (Valiveti et al., 2004a; 2005). However, this is the first time a study has shown good *in vitro* and *in vivo* correlation in GPs for MN assisted transdermal delivery. The percent correlation between human and human skin was 76%, which showed an under estimation for the *in vitro* studies. This could be due in fact to the location of the patches worn by volunteers compared to the area of skin used for *in vitro* studies. The patches were placed on the upper region of the arm as

compared to the full thickness abdominal skin used for *in vitro* permeation studies. Another reason may be the enzymatic activity differences in human skin *in vitro and in vivo*. It may also be possible that polar NTX also has significant diffusion through follicular routes in the skin *in vivo*. Overall, however, a good correlation between the *in vitro* and *in vivo* studies was observed in both GP skin and GP as well as in human skin and healthy human volunteers. Either skin type could be utilized with confidence when screening compounds for MN enhanced permeation studies *in vitro*.

5. Conclusion

In the present study, the aim was to evaluate the transdermal delivery efficiency of NTX·HCl salt via MN enhancement of skin in both *in vitro* and *in vivo* studies. In human and GP skin, excellent MN enhancement was observed as compared to intact skin. The same trend was observed *in vivo* and it was shown that a therapeutic plasma level could be achieved and maintained for at least 48h in human subjects, whereas no plasma levels were observed in either human or GP non-MN treated controls. Overall, an excellent IVIVC was observed between humans and human skin, as well as GP and GP skin. Thus, both human and GP *in vitro* models are excellent cost saving and predictive models for MN enhanced transdermal candidate screening. In regards to future applications, the work lies in developing a patch system that is patient friendly and cost effective for the treatment of alcoholism. Such work is ongoing in the lab of Dr. Audra Stinchcomb to develop a microneedle and patch system that can be effectively transitioned to the market for the treatment of alcohol abuse.

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Part 4

Drug Development Strategies

22

Innovative Proposals for Incentivizing Drug Development

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1. Introduction

The confluence of rising drug costs, more effective innovative medicines, and increasing public outrage have heightened criticism of the pharmaceutical industry and intensified the debate over pharmaceutical prices and access to medicines. As a consequence, policymakers and scholars from a variety of fields have argued for a reexamination of the patent system as an efficient mechanism for encouraging pharmaceutical innovation and drug development. The pharmaceutical industry is uniquely characterized by its production of a social good featuring high fixed costs, substantial informational and regulatory costs, and relatively low marginal costs of production. As such, pharmaceutical innovation is well suited to alternative incentive mechanisms to foster drug development. A variety of alternative mechanisms have been proposed, focusing on welfare improvements ranging from lower drug prices to increased consumer surplus, enhanced access to medicines and greater innovation.

This chapter describes the pharmaceutical industry's existing operating model as well as the subtleties of the current patent system and how it impacts drug development. Second, the chapter explores the characteristics that would make for a socially preferable, welfare-enhancing mechanism. Next, the chapter reviews a number of proposed mechanisms and evaluates the advantages and disadvantages of each. The chapter then examines the overwhelming prevalence of "pull" mechanisms, those aimed at incentivizing successful innovation with prizes or other rewards, relative to "push" mechanisms which reduce the cost of research and development through funding awards. Following this, the chapter presents an analysis and comparison of the policy proposals, the similarities in their strengths and limitations, and the characteristics which would enhance the political feasibility of implementation. Finally, the chapter concludes with an appeal for the reexamination of the patent system and further exploration of the promise of alternative mechanisms to enhance access to medicines and incentivize additional research on neglected diseases.

2. Current environment and challenges to drug development

The minimum standards for pharmaceutical patent protection are defined by the Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS), as set forth by the World Trade Organization (WTO). The TRIPS Agreement was signed in 1995 at the end of the

Uruguay Round negotiations and requires that all WTO member states provide 20 years of market exclusivity for patented drugs. As a result, the generic products that characterized the pharmaceutical markets of many developing countries prior to TRIPS disappeared as stronger patent protection was adopted. Under TRIPS, generic producers must wait for patent expiry to bring generic versions to market.

The apparatus embodied in the TRIPS Agreement encourage additional pharmaceutical research and development (R&D) by guaranteeing innovators a period of exclusivity during which they are able to recover their R&D investments. Without the protection provided by the patent system, innovators would have little incentive to invest in new technologies that could easily be copied and sold by their competitors. Such free riding on the initial investment constitutes a market failure that would stymie most innovation. The market exclusivity incentivizes firms to invest in the difficult and expensive research and development necessary for pharmaceutical advances. This incentive system is the heart of the static/dynamic tradeoff that characterizes the existing patent system. In exchange for temporary (20 years under the existing patent system) monopoly power, a static loss, new knowledge is forever brought into the public domain, a dynamic gain. While the existing patent system has addressed the market failure in one way, there are clearly problems with the current mechanism.

Public health advocates argue that the patent protection afforded by the TRIPS Agreement is a significant determinant in establishing pharmaceutical prices and availability. Specifically, prior to the TRIPS Agreement, developing countries tended to have weaker patent protection (many had no patent protection for pharmaceuticals) which enabled generic drug producers to manufacture generic versions of drugs, even those still under patent protection in industrialized nations. Arguably, this created price competition and lowered drug prices. Critics point to the market exclusivity provided by TRIPS as raising drug prices and reducing availability in the developing world.

Clearly this creates a barrier to access for many of the most vulnerable. In the context of access, "extreme poverty is universally the most important determinant. This is true both for individuals and for countries too poor to fund control efforts fully, still less to undertake needed research." (Musgrove & Hotez, p.1694)

While poverty and the existing patent system prevent some individuals who would benefit from medical innovations from buying them, a demand side problem, the existing patent system also suffers from problems on the supply side. Glennerster and Kremer (2000) adeptly identify four supply side issues that contribute to the insufficient incentives for innovation: potential purchasers are unwilling (or unable) to pay monopoly prices; some of the benefit from the innovation, in the form of consumer surplus, accrues to consumers; some of the benefit from the innovation is captured by consumers who purchase generic versions after patent expiry; and lastly, some of the benefit goes to other researchers who draw on the original innovation. The authors note that many studies estimate that innovators realize less than half their returns to their inventions. In a similar vein, Kremer (1998) estimates that the social value of a pharmaceutical innovation will be 2.5 times the private value. In essence, for developing countries, the existence of a market is constrained by the poverty of the patients as well as the inability of private researchers to capture the benefits of potential treatments.

The problems embedded in the existing patent system are perhaps more significant in the realm of pharmaceuticals because of the lives that may be lost and the tremendous costs

associated with untreated diseases. "Some eighteen million human beings die prematurely each year from medical conditions we can cure – this is equivalent to fifty thousand avoidable deaths per day, or one-third of all human deaths." (Pogge, p.182) Musgrove and Hotez (2009) provides an excellent description of the disease burden (death and disability) and economic costs (damage to children's cognitive development and adults' physical productivity) from neglected tropical diseases.

Nevertheless, given the incentives created by the existing patent system, very few resources are devoted to the diseases of the poor. Pharmaceutical research is motivated by potential profits and the diseases endemic to developing nations and the limited purchasing power of these consumers hold little appeal. As described by the World Bank Group (2009), global health research spending is characterized by a 10/90 gap, i.e., less than 10 percent of global health research expenditures are devoted to conditions which account for more than 90 percent of preventable mortality. This imbalance is perhaps best exemplified by the numbers on new drug approvals. Of the "1,556 new drugs approved between 1975 and 2004, only 21 (1.3%) were specifically developed for tropical diseases and tuberculosis, even though these diseases account for 11.4% of the global disease burden." (DNDi, p.1) Notably, of these 21 drugs, five emerged from veterinary research. (Poggee, p.190) The absence of a profitable market for neglected tropical disease medicines directs research efforts and resources to other endeavors.

Given the challenges surrounding pharmaceutical pricing, access to medicines, and research on neglected diseases, scholars, public health advocates and policymakers are working to overcome these challenges and design a better mechanism for incentivizing pharmaceutical innovation. In pursuit of a better mechanism, it is important to identify the points in the research and development process where the failures occur. Failures may occur at several stages of the drug development process: insufficient funding for research on neglected diseases, insufficient funding to take potentially promising research forward to the clinical development stage, and distribution problems that prevent existing treatments from reaching patients.

Alternative mechanisms address these failures in one of three ways: those that work within the existing system (compulsory licensing, bulk buying, differential pricing), push mechanisms (research grants, government funding), and pull mechanisms (advance market commitment, prize funds, priority review vouchers). Push mechanisms incentivize pharmaceutical innovation by reducing the cost of research and development. Pull mechanisms remunerate welfare-enhancing innovations with prizes, supplemental profits or other rewards. All mechanisms seek to incentivize pharmaceutical research and development while overcoming particular flaws within the existing system. Greatest emphasis is placed on reducing the price of pharmaceutical innovations and incentivizing work on neglected diseases. Each mechanism is distinguished by its own advantages and limitations, providing a distinct set of benefits and disadvantages to compare to the existing system.

3. Characteristics of a welfare-enhancing mechanism

The alternative mechanisms considered below aim to improve upon the existing system, generating additional societal welfare and overcoming some of inefficiencies that plague the current patent regime. Ideally, a new mechanism would improve access to medicines, presumably through lower drug prices, and incentivize additional drug development for

the treatment of diseases endemic to developing nations. At the same time, a new mechanism would avoid the pitfalls that frequently accompany pharmaceutical innovation. This section describes the welfare-enhancing characteristics that would characterize a preferable mechanism as well as the inefficiencies that should be eliminated. The alternative mechanisms reviewed in the next section are then evaluated in light of the capacity to enhance social welfare and avoid these inefficiencies.

Marginal cost pricing The global patent system and international pharmaceutical market currently engender differential pricing across countries. Through Ramsey Pricing pharmaceutical prices reflect the price elasticity of demand of consumers. The result is higher prices in industrialized nations and lower pharmaceutical prices in the developing world. Access to medicines could be enhanced through more affordable drug prices which would also reduce the deadweight losses associated with monopoly pricing. Most efficient would be marginal cost pricing, prices set equal to the cost of the additional inputs needed to produce one additional unit of output. Marginal cost pricing guarantees that anyone willing to pay the cost of production is able to purchase the good, increasing access and maximizing consumer welfare.

Reduction of pharmaceutical counterfeiting The market exclusivity granted by the existing patent system may result in a substantial mark-up over marginal cost. This mark-up and the associated profitability of the pharmaceutical market create an attractive target for fraudulent production and pharmaceutical counterfeiting. Global health and the security of the international pharmaceutical supply chain would be enhanced if the incentive to counterfeit could be reduced.

Increased R&D on treatments for neglected diseases with high social value As repeatedly noted above, the existing system fails to incentivize research on diseases of the poor due to the absence of a profit motive. Overcoming this obstacle to drug development for neglected diseases would enhance global social welfare and provide the potential for therapeutic relief to many of the world's most vulnerable.

Mechanisms for rewarding incremental innovation Although incremental innovations will contribute less to social welfare than innovations that are both first-in-class and best-in-class, it remains important to encourage follow-on innovation. An effective mechanism will reward innovations in proportion to their therapeutic value and will also allow original innovators to capture a share of the returns from all follow-on innovations spurred by the initial advance.

Continued commitments to safety and efficacy Any alternative mechanism should preserve the existing system's commitment to safety and efficacy, ensuring the health of the patient above all. While more rapid review times increase firm profitability, these incentives should not come at the cost of assured safety.

Reduction of excessive marketing Critics frequently point to excessive marketing expenditures within the pharmaceutical industry as a significant contributor to high drug prices. An effective alternative mechanism would incentivize firms to reduce this spending, in essence de-linking profitability and marketing efforts. Elimination of these costs would reduce drug costs and should help to lower prices.

Preservation of the incentives to innovate Alternative mechanisms should preserve the incentives that are in place that encourage innovative activity and foster creativity. Although flawed, the existing patent system effectively does this and has successfully rewarded inventors with returns to their innovations, encouraging a continued stream of innovations and advances.

Reduced launch times In its current form, the international pharmaceutical market is characterized by differential prices such that drugs are sold at a premium in some countries over others. Given this, firms have an incentive to launch new products in the most profitable nations first, those characterized by higher prices and stronger patent protection. The result may be a significant delay in launch dates for less profitable, frequently developing nation, markets. Reduced launch times, as facilitated by an alternative mechanism, improve global welfare as innovative treatments reach patients more quickly.
Low information costs Alternative mechanisms will ideally require limited, easily attainable information for adoption. Significant informational requirements or difficult (impossible) to obtain information will drive up the cost of implementation.
Reduced duplicative research and development of "me too" drugs The existing patent system and the resultant monopoly prices incentivizes innovators to dedicate R&D efforts to drugs that are unique enough for a patent award but may provide little or no therapeutic advance. "According to the US Food and Drug Administration, over 77% of the drugs approved from 1990-2004 were duplicative rather than breakthrough drugs." (Ravvin, p.112 citing a 2005 report by the US Food and Drug Administration) While providing the innovating firm with a return, such duplicative research efforts are an inefficient use of scarce research talent and financial resources. An improved mechanism would provide greater incentives for breakthrough therapies and reduce the incentives for "me too" drugs.

4. Review of the alternatives to the existing patent system

As envisioned, the existing patent system seeks to balance the static inefficiency of the temporary monopoly power guaranteed by a patent grant and the dynamic benefits of knowledge spillovers resulting from the sharing of the patented knowledge. While providing incentives to innovate, many argue that the current system has failed the developing world. High drug prices and a dearth of treatments for many neglected and tropical diseases constitute barriers to access to medicines for many patients in the developing world. Frustration with the status quo has driven the call for a reexamination of the patent system and resulted in the development of several alternative mechanisms. The challenges of reforming the patent system and balancing the pharmaceutical industry's incentive to innovate with widespread access to medicines have been approached by numerous scholars in a variety of ways. This section considers nine proposals that have addressed this challenge. Each proposal is described and evaluated in turn. The mechanisms proposed utilize different means to reward pharmaceutical innovation and vary in their ability to enhance access through lower pharmaceutical prices while simultaneously providing a reward to the innovator that would be proportional to the social value of the innovation.
The proposals analyzed here all advocate changes to the existing patent system, through a variety of creative mechanisms advocated from a variety of perspectives. These works date back as far as 1998, and each is characterized by its own set of advantages and challenges, though there is significant overlap in both areas. Each of the proposals is described and examined below.

4.1 Abramowicz (2003)

Description: The Abramowicz proposal provides an evaluation of a prize system from the perspective of public administration. This mechanism relies on a retrospective reward

system to complement the existing patent system. As described, the innovators have the option to elect the prize system, but the decision to participate is irrevocable. Given that the administrative agency has the ability to tailor the procedure for valuing the innovation to the particular patent, the mechanism is flexible which is very attractive. This mechanism provides for the government to reward firms that surrender their patents. The proposal is designed such that the prize is proportional to the patent's value relative to the total value of prize submissions.

Advantages: The Abramowicz mechanism places patents in the public domain, which reduces the static inefficiency associated with monopoly power and the associated deadweight losses. Since the technology is in the public domain, the incentives for "me too" drugs and inventing around existing patents is reduced, removing an additional inefficiency inherent in the existing system. Moreover, projects that would produce high social value that may not be pursued by private firms are incentivized for development through this mechanism.

Limitations: The administrative agency operationalizing this mechanism will require significant amounts of information in order to estimate the value of the innovation. This information will be both difficult and expensive to collect, when it is available. In addition, the innovating firm must also have a great deal of information to accurately determine the quantity of resources to devote to research and development. Specifically, firms require information in order to accurately estimate the social value of every project for which the prize is sought in order to calculate potential returns and the efficient level of investment. Finally, the Abramowicz mechanism is administrated by a government body and is therefore subject to the bureaucratic weaknesses and inefficiencies which characterize government control.

4.2 Civan (2009)

Description: Civan proposes extending the patent length beyond 20 years for treatments for diseases which disproportionally affect developing nations. The additional years of patent protection are argued to increase the incentives to research treatments for neglected diseases. Citing the work of Diwan and Rodrik (1991), Civan suggests that patent protection in developing countries could be as much as one and one half times that of patent protection in industrialized countries (20 years under the World Trade Organization).

Advantages: Increased patent length would extend the period of time that pharmaceutical innovators would have to recoup their R&D investment, enhancing the incentive to research treatments for neglected diseases. This mechanism would be very easy and inexpensive to implement, a law change in participating developing nations would be sufficient.

Limitations: The Civan proposal takes its recommendations from early papers, specifically from static models that may fail to capture important dynamics of the global pharmaceutical market. While enhancing the incentives for research on neglected and tropical diseases, this mechanism would increase the patent length and result in monopoly prices for drugs for a longer period of time in the developing world. Granting that pharmaceutical companies price discriminate, this would still lead to higher prices for the most vulnerable consumers. Given the tremendous opposition to any patent protection for pharmaceuticals in the developing world, one can imagine tremendous opposition to this mechanism by poor nations.

4.3 Grabowski, Ridley & Moe (2006, 2009)

Description: The essence of this policy proposal is the award of a priority review voucher (PRV) to any innovator that gains US FDA (or European Agency for the Evaluation of

Medicinal Products) approval for a new pharmaceutical or biological treatment for a neglected tropical disease that is shown to be clinically superior to existing treatments. The value of the voucher is that it entitles the bearer to a priority review for another drug submitted for US FDA approval. In contrast to the 15 months required for a standard review, the median review time for a priority review is approximately seven months. The authors find that getting a new drug to market more quickly, even measured in mere months, can be worth hundreds of millions of dollars.

Advantages: The US Congress enacted the Food and Drug Administration Amendments Act of 2007 in September of that year, based on this policy proposal. In September 2009 the PRV program went into effect and the first voucher was awarded to Novartis following the US FDA approval of their anti-malarial drug, Coartem. The priority review voucher is transferable and may either be used or sold by the innovating company. As demonstrated by the experience of the US FDA, the mechanism is straightforward to adopt and operationalize. Regulatory standards for safety and efficacy are preserved, though the priority review does require additional resources at the US FDA to quickly analyze the additional data. The authors claim that the priority review will increase costs to the regulatory agency by approximately $1 million per review, though this cost is paid by the innovative firm and amounts to only a small fraction of the value of the voucher. The incentives provided by the priority review voucher are also highly efficient because the innovating firm is able to determine which treatments should be pursued based on internal information and evaluation, increasing the likelihood of success. The incentives provided by the voucher will allow for faster access to blockbuster drugs and increased research on neglected tropical diseases. Based on a study of historical data, Grabowski and Kyle (2007) estimates that the priority review voucher may increase the effective patent life for drugs facing generic entry. In addition, the value of the priority review voucher creates a market mechanism for identifying those drugs for which expedited review is most efficient. Finally, this policy proposal is complementary to other push and pull mechanisms that may be adopted.

Limitations: The proposal requires the US FDA to employ additional resources in order to quickly evaluate the additional regulatory data. The mechanism does not provide for how the treatment will reach patients in developing countries, implementation challenges remain for enhancing access to the medicines. In like manner, while this proposal enhances the incentives for R&D, it fails to address high drug prices as a barrier to access since the mechanism does not specify any conditions on the affordability of the price of the medicinal intervention.

4.4 Hollis (2004, 2005)

Description: The Hollis proposal aims to overcome two of the most significant shortcomings of the existing patent system: the high drug prices which prevent widespread access, and the lack of incentives for research and development on diseases endemic in developing countries. As envisioned, patent rights are relinquished in exchange for a reward calculated to be proportional to the incremental therapeutic benefit of the technology. As an optional reward mechanism, the proposal suggests that this system would operate in conjunction with the current patent system. Key to the Hollis mechanism is the development of an International Pharmaceutical Innovation Fund which would be the source of payments to innovators surrendering their patent rights. Participants would agree to freely license all patents related to the drug when used for developing country markets in exchange for payments for a period of perhaps twelve years.

Advantages: This mechanism serves as a complement to the existing patent system. The optional reward mechanism would incentivize the development of innovations that provide high social value but are distinguished by low appropriability, precisely the characteristics needed to generate research into neglected diseases. Beyond incentivizing the research, this mechanism also enhances access and reduces deadweight losses by avoiding monopoly pricing. As described, the drugs would be competitively produced and sold at the average cost of production, increasing access for the poor and vulnerable in the developing world. As an additional benefit, the reward is calculated to be proportional to the innovation's social value as determined by a *quality-adjusted life year* (*QALY*) measure of disease burden, which includes both the quality and the quantity of life lived. In principle, the use of a QALY measure will provide more information than a market-price valuation, and avoid some of the agency and informational problems that currently characterize pharmaceutical markets.
Limitations: In order to operationalize the Hollis proposal, both the administrative agency and the innovating firm must have a great deal of information about the value and cost of the innovation. Determination of the reward requires calculations of both pharmaceutical sales and QALYs for covered innovations, which may be difficult and costly to attain. For the innovator, R&D investment decisions are based on information about the expected social value of every successful drug in order to calculate the innovator's relative share of the total reward. The mechanisms may induce bureaucratic abuses including regulatory capture and corruption, as well as attempts at gaming the system. Finally, the proposal lacks details on the funding and management of the International Pharmaceutical Innovation Fund.

4.5 Hubbard & Love (2004)

Description: The Hubbard and Love proposal is based on the establishment of a national R&D contribution with a suggested value of 0.1% of national Gross Domestic Product (GDP). In line with the existing patent system and current market mechanisms, this contribution could be achieved through the purchase of patent protected drugs (under the assumption that national expenditure on pharmaceuticals is approximately 1% of GDP and a tenth of the revenue from drug sales is ploughed back into research and development). Alternatively, nations that financed their R&D contribution in other ways (for example through direct funding of drug development) could place drug patents into the public domain, allowing for competitive production.
Advantages: This mechanism would operate in conjunction with the existing patent system. For those countries electing alternative means of making their R&D contribution, weaker patent protection would provide for competitive production, lower prices and enhanced access to medicines. In addition, the proposal would eliminate the need for excessive drug marketing and the associated deadweight losses. In principle, funding for pharmaceutical research and development is sustained without the welfare losses generated by monopoly pricing. The authors also claim that R&D resources will be drawn into the areas of greatest need, though the means by which this will happen is not clear or described.
Limitations: This proposal primarily suffers from informational and measurement challenges. The Hubbard and Love proposal lacks any information on the specifics necessary to operationalize the mechanism: how to calculate and establish the contribution norm, means of global coordination, establishment of an administrative body, distribution of the contribution funds, and design of acceptable alternative mechanisms. Finally, this mechanism requires significant government oversight and control.

4.6 Kremer (1998)

Description: This paper proposes employing a government auction to estimate the private value of patents, then providing an opportunity to buy out the patent at a multiple of this value. In an effort to overcome the insufficient incentives for such research, the multiple would approximate the social value of the innovation. Innovators could opt to sell the patent or retain their rights to the technology. Given a government purchase, patents would be placed in the public domain. In order to avoid distortions and to induce accurate valuations, a fraction of patents would be sold to the highest bidder according to government randomization.

Advantages: The principle advantage of this mechanism is the elimination of monopoly pricing through the placement of patents into the public domain. At the same time, the private incentives to innovate are enhanced to levels closer to the social value since the government aims to establish the buyout at this value. Kremer's proposal provides a market mechanism, specifically auctions, to value innovations and provides for substituting and complementary patents. Patent holders would have the ability to request a second auction in those cases. An additional advantage is the reduced incentive for duplicative research on "me too drugs" through the auction mechanism. Since the patent-generated distortions are severe in the pharmaceutical industry, this mechanism is especially well-suited to this technology. In addition, the U.S. Food and Drug Administration approval process will automatically generate a great deal of the information necessary to value the innovation.

Limitations: A significant criticism of the Kremer mechanism is that transferring the patent into the public domain would reduce the incentive for follow-on research and further development. That is, in the pharmaceutical industry additional clinical development frequently occurs only after a predetermined sales threshold is reached. As such, this proposal may be best suited to the development of vaccines and other specialty products that are narrowly focused with specific, well-defined clinical objectives. In addition, the Kremer mechanism relies upon the assumption that auction participants honestly reveal their valuation of the innovation. Although the government's randomized auction (government purchase of the innovation or sale to the highest bidder) should induce honest valuations, the proposal remains vulnerable to collusion among bidders or bribery from the patent owner.

4.7 Lanjouw (2002)

Description: This proposal is predicated on the existence of two types of nations, rich and poor, and two separable drug markets: one for global diseases, such as cancer, and one for diseases endemic to developing nations, such as malaria. In the case of global diseases, Lanjouw's mechanism would require innovating firms to elect to exercise their patent rights in either rich countries or poor countries. This would be done as part of the US Patent and Trademark Office (USPTO) foreign application process, through a declaration attached to the request for a foreign filing license. Nations generating perhaps two percent or less of total global pharmaceutical profit would be defined as poor. For diseases endemic to developing countries, the incentives derived from the patent system would remain in place to encourage innovation. For global diseases with a high incidence in all nations, patent rights exclusive to rich nations would result in competing firms in developing countries where patent protection was not elected, ultimately bringing prices down for developing country residents. As nations developed and markets evolved, the license declaration would be updated to reflect these changes.

Advantages: The Lanjouw proposal targets patent protection by disease and by country. The mechanism draws on the information advantages possessed by the firm regarding the links between patents, products and diseases, as well as information on the relative size of national markets. The proposal would increase access to medicines in the developing world, for global diseases, while maintaining existing incentives for research on neglected diseases. Finally, the mechanism would be easy and inexpensive to implement, both of which are significant advantages.
Limitations: As envisioned, the Lanjouw proposal requires separation of markets to permit price discrimination. This will be made more difficult by increased globalization, international arbitrage and internet sales. Although the poor nation markets are quite small (two percent), the loss of market power in these countries will (marginally) reduce the incentives to innovate. This mechanism requires information about market size and global pharmaceutical sales that may be difficult to collect. In addition, operationalizing this alternative will require consensus in measurement and in the establishment of the profit share benchmark. Moreover, the policy change describe in this proposal is already available to interested firms on a voluntary basis. One imagines that, however small, the loss of sales revenue from surrendering patent protection is not profitable and therefore the elect not to. Presumably this also indicates that innovative firms would resist the adoption of the changes presented by this mechanism. Finally, the Lanjouw proposal is subject to the lack of stability and consistency in governments' decision-making which may be corrupt, irrational and unpredictable.

4.8 Lybecker & Freeman (2006, 2007)

Description: The Lybecker & Freeman proposal rewards innovative firms based on the social value of their drug. The pharmaceutical innovator would receive direct tax credits in exchange for marginal cost pricing, based on production and distribution. The calculation of social value (the sum of consumer and producer surplus) will be based on one year of benchmark sales at market prices, reflecting private value of the innovation. A multiple of this figure will then be utilized to calculate compensation for the patent holder. Participation by innovative firms is voluntary and participation requires marginal cost pricing or licensing the patent for generic production. National participation is also voluntary. Participating nations buy in by providing a share of the financing which is a function of the social value, national disease burden and per capita GDP.
Advantages: While government involvement is inherent in the Lybecker & Freeman mechanism, the valuation of the innovation continues to be established by market forces. If the innovation offers a therapeutic benefit, a market will emerge in the benchmark year. The efficiency of tax credits is established in earlier work which has shown that one dollar in tax credits for R&D is associated with one dollar increase in R&D expenditure at the margin. Duplicative innovations will capture smaller market share and garner smaller direct tax credits. In addition, this mechanism transfers the burden of R&D financing from those afflicted with a particular disease to all taxpayers, and with global adoption halts the US subsidization of pharmaceutical research and development. Given that firms are expected to freely license the technology, generic competition should emerge to reduce prices to competitive levels in participating nations. Moreover, the mechanism should improve global product dissemination and reduce the launch delays that currently characterize developing country markets.

Limitations: Implementation will require significant international cooperation on pricing, estimation of social value, financing shares (proportional disease burden and per capital GDP), and reimbursement policies. These calculations will be particularly difficult to make for the least developed countries. In addition, there will be an incentive to manipulate the figures to lower the share of compensation. Since peak sales are reached three to five years post-launch and this proposal uses one benchmark year, this mechanism may underestimate the social value of the innovation. Moreover, given that a single benchmark year is utilized to calculate market valuation, marketing practices may intensify pre-launch and in the benchmark year. Marginal cost pricing is admittedly difficult to estimate and will be a challenging aspect of implementing this proposal. Internet sales and transhipment to non-participating nations may make enforcement difficult. Finally, while the Lybecker and Freeman proposal should lower drug prices and increase access to medicines to developing countries, it does not provide additional incentives for research into treatments for neglected diseases.

4.9 Pogge (2005)

Description: The Pogge proposal would provide a mechanism to operate in tandem with the existing patent system. Innovative firms would have the option to surrender their conventional patent rights in exchange for an alternative patent which would reward them with a guaranteed payment stream in proportion to the health impact of their medical intervention during its initial 10-12 years of availability. The patent hold would have two options for implementation: grant of an open license to generic producers, or maintaining exclusive production of the drug under the condition that reward payments would be reduced by the amount of sales revenues.

Advantages: Innovative firms would have incentives to develop cost-effective technologies with the maximum health impact. Generic production as well as marginal cost (or less) pricing would be incentivized to maximize access and health improvements among all populations, including the global poor. The mechanism would also discourage duplicative research efforts and excessive marketing. The costs of research and development would be borne globally, by all taxpayers, rather than by those afflicted with a particular disease or condition.

Limitations: Significant challenges are presented by the necessity for measuring an appropriate reward, quantifying the global disease burden and assessing the health impact of new medical interventions, a particularly difficult charge in the context of combination therapies (the so called AIDS "triple cocktail"). In addition, the mechanism requires the coordination of governments and the pharmaceutical industry. The necessary consensus on essential and nonessential drugs may be contentious and subject to political battles and gamesmanship. Moreover, implementation is contingent on raising the resources needed for the Health Impact Fund, an estimated US $45-$90 billion annually, a formidable challenge. Pogge problematically asserts that pharmaceutical companies may be willing to bear the additional costs of widespread accessibility, such as improved infrastructure and distribution systems. As argued by Sonderholm (2010), due to the free rider problem, an individual firm has little incentive to invest in the expensive efforts that would then benefit all firms. Finally, there must be agreement on the allocation of the costs across nations, a negotiation process that promises to be difficult and contentious.

Table 1 below presents a snapshot of the characteristics of each of these proposals, briefly summarizing the authors' perspective, the essence of the proposed mechanism, the proposal's goal, as well as the primary criticisms and advantages of each.

	Perspective	**Mechanism**	**Goal**	**Criticism**	**Advantages**
Abramowicz	Lawyer	Retrospective Reward based on social value	Reduce DWL, Access	Government control, No specifics, Informational Requirements	Patents in public domain, Complements patent system
Civan	Economist	Longer patent length in developing nations	Therapeutic Need	Coordination between nations, Requires lots of participating countries, Bad for global diseases	Market mechanism, Easy to implement
Grabowski, Ridley & Moe	Economists	Priority Review Voucher	Therapeutic Need, Lower Prices	Smaller prize value than APC, Potential for limited clinical benefits, Safety risks of Priority Reviewed Drugs	Operationalized and in place Market mechanism at work, Low cost to taxpayers, faster access
Hollis	Economist	Reward system via innovation fund, QALY Measurement	Therapeutic Need, Access	Government control, Difficult measurement	Complements patent system, Reward linked to therapeutic benefit
Hubbard & Love	Consumer Advocate	National contribution norm via alternative R&D funding models	Therapeutic Need, Lower Prices	No Specifics, Government Control	Continued R&D fund via mandated contribution
Kremer	Economist	Patent Buyout via Auction mechanism	Reduce DWL, Pay Social Value	Collusion, Further development discouraged	Market Mechanism Public domain patents
Lanjouw	Economist	Patents tailored to two different drug markets	Therapeutic Need, Access	Enforcing separate markets, Industry cooperation	Low cost, Easy to Implement
Lybecker & Freeman	Economists	Direct Tax Credits to fund innovation	Therapeutic Need, Access, Lower Prices	Difficult measurement, consensus, global cooperation	Marginal Cost pricing, faster launch times, reduced duplicative research
Pogge	Political Scientist	Reward system based on health impact	Therapeutic Need, Lower Prices	Publicly funded, challenging measurement, global cooperation necessary	Incentive to maximize health benefit, incentives for cost-effective drugs

Table 1. Summary of Proposals

The selection of proposals examined here represents a variety of perspectives, from economist to lawyer to political scientist, and utilize a variety of mechanisms to incentivize drug development, from auction to tax policy to license declaration. The objectives of most of the proposals focus on improved access to medicines for the poor, especially patients in developing nations, enhanced research on neglected diseases and reduced deadweight loss. Unfortunately there is also great commonality in the limitations of the mechanisms: large (sometimes impossible) informational requirements, tremendous government involvement, dearth of details, and coordination challenges.

5. Comparison and analysis

This section seeks to compare and contrast the nine proposals presented above. Specifically, given the alternative policy proposals considered here, the following questions must be answered: How do they differ and how are they most similar? Which are the most politically feasible? What are the greatest advantages? What are the most significant limitations?

As described earlier, alternatives to the existing patent system fall into three categories: those that work within the existing system, push mechanisms and pull mechanisms. Push mechanisms reduce the cost of research and development while pull mechanisms reward successful innovation with prizes, supplemental profits, or some other reward. In essence, push mechanisms reward effort while pull mechanisms reward results. Of the policy proposals considered here, eight of the nine would be described as pull mechanisms. The sole push mechanism is the one advocated by Hubbard and Love (2004) which proposes a national R&D contribution nom to be met by any means, which could then expand direct funding for drug development.

The remaining eight proposals are all forms of pull mechanisms ranging from priority review vouchers to longer patent lives in developing nations, to direct tax credits. Given that so many of these proposals focus on pull mechanisms, one must ask whether this is a superior approach to the challenges surrounding neglected diseases and barriers to access. In an excellent discussion of the comparative advantages of push vs. pull mechanisms Grace and Kyle (2009) examine the strengths and weaknesses of each. In the context of pull mechanisms, there are several important advantages. Grace and Kyle note that economic principal-agent models suggest that pull mechanisms are superior when agents are not capital constrained, when it is straightforward to specify the desired innovation and its specific characteristics, and when the principal is risk-averse. Perhaps most advantageous is the fact that pull mechanisms only reward successful outcomes. This correctly aligns the firms' incentives: only those whose chances of success are great enough will be willing to invest the resources necessary for research and development. The market mechanisms work to ensure those best able to succeed are those most likely to participate. Pull mechanisms allow for the contributions of a large, varied and disperse group of experts and researchers. While this may increase the speed of innovation, it may also risk duplicative research efforts. On the other hand, Grace and Kyle argue that push mechanisms may be considered superior when effort is easy to monitor and measure and when the principal has a greater tolerance for risk. An important advantage of push funding is the elimination of research and development risk since the funding is assured regardless of results. This is particularly beneficial for encouraging basic research, though it is arguably inefficient since many funded research efforts are unsuccessful and de-linking risk and reward may incentivize less fruitful efforts.

The pull characteristic is not the only similarity across the proposals considered in this chapter. Importantly, the majority of mechanisms are focused on incentivizing research on those diseases for which there is greatest therapeutic need and enhancing access to medicines through lower drug prices. Though the means to the end are very different across the proposals, the goals are virtually identical.

The proposals are also characterized by similar advantages and limitations. Perhaps the most common advantage seen across the alternative mechanisms is the reliance on a market mechanism to incentivize invention and determine the valuation of the innovation. This both ensures that resources are invested prudently (risks are balanced against potential returns) and that research efforts are pursued by the most efficient firms. In the case of the limitations, those most frequently cited are informational requirements and coordination/cooperation challenges. The uncertainty surrounding both of these elements poses a noteworthy challenge to implementation in each case.

Finally, it is important to consider which of the nine policy proposals are the most politically feasible. In this spirit, it is valuable to reflect on the Priority Review Voucher (Grabowski, Ridley and Moe) which went into effect in the United States in September of 2009. Several aspects of this mechanism are politically attractive and important to the likelihood of adoption. First, this mechanism is optional for the firm. This ensures greater buy-in by the industry, which will be favourably received by policymakers as well. It is also voluntary at the national level. While the United States is now experimenting with the Priority Review Voucher, other nations have the option of instituting a similar mechanism to enhance their patent systems as well. In addition, mechanisms that complement the existing system will be easier and less costly to adopt than those that require a new institutional architecture for adoption. Again, such mechanisms would be implementable at the discretion of national authorities. Lastly, the most politically feasible mechanisms will utilize the market forces which preserve the incentives to innovate through potential profits. Market forces are highly efficient and ensure that resources are invested only when risk levels and expected returns warrant that investment.

Overall it is apparent that the majority of creative thinking in the realm of alternatives patent mechanisms utilize pull mechanisms to both incentivize research on neglected diseases as well as lower drug prices to enhance access to medicines. While the policy proposals vary greatly in the design of their mechanisms, there are tremendous similarities in their strengths and limitations. Ultimately the value of these proposals will lie in the change they engender: through their implementation and/or the creative thinking they inspire. While only one of these proposals has been operationalized, it is clear that there is a set of identifiable characteristics making adoption more politically feasible and therefore likely.

6. Conclusions

The current policy debate over pharmaceutical patents and drug development is largely focused on the high prices which create a barrier to access for many consumers in the developing world. An obvious tension exists between public health policy and shareholder expectations. Emotionally charged discussions frequently ignore the importance of research and development funding to a productive pipeline of therapeutic compounds and new pharmaceutical compounds. The growing public outrage has inspired scholars and policymakers to re-examine the patent system and search for alterative mechanisms capable

of incentivizing pharmaceutical innovation which simultaneously ensuring greater access to medicines.

This chapter presents a review of nine alternative policy proposals seeking to reward innovation while improving social welfare. As an industry which produces social goods characterized by high fixed and/or sunk costs and relatively low marginal costs of production, the pharmaceutical industry is well-suited to an alternative mechanism which may greatly improve global social welfare. The challenges of improving access to medicines and incentivizing research on neglected diseases demand creative thinking to overcome the tremendous loss of life and quality of life. Although the advantages to these proposals are significant, each is also characterized by costs and drawbacks that limit the potential for implementation. In spite of this, growing frustration with the existing system and increasing criticism of the pharmaceutical industry point to the need for consideration of alternatives and indicate these policy proposals are worth exploring.

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Drug Experimentation in Healthy Volunteers

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1. Introduction

Drug development is a complex process that includes drug discovery/product development, pre-clinical research (*in vitro/in vivo)* and clinical trials. The new chemical entities, which show promising pharmacodynamic activity in *in vitro* experiments on particular biological targets, thought to play critical pathophysiological roles in specific diseases, emerge from the process of drug discovery and are candidates to undergo safety and toxicity tests, as well as pharmacokinetic and metabolism evaluations in *in vivo* pre-clinical models. Moreover, pre-clinical investigations are focused on determining the dose and administration schedule to be used in the first human clinical trial (first-in-man or first human dosing).

Clinical drug development is currently arranged into four phases, with phase I traditionally representing the very early stage of drug development in humans. Phase I is conducted to establish safety and tolerability, to evaluate pharmacokinetics and to obtain preliminary data on pharmacodynamics. Phase I begins with the first administration of a new compound in humans (Pocock, 1983).

Based on differences in the experimental design, various types of phase I trials can be distinguished: (1) Single ascending dose studies, in which small groups of subjects receive a single dose of the test drug, afterward they are observed and examined for a given period of time. If subjects do not experience any remarkable adverse effect, and pharmacokinetic data are roughly consistent with pre-specified safety values, the dose is escalated up, and a new group of subjects is then given a higher dose (Buoen et al., 2005). This stepping-up dose is continued until the pre-calculated pharmacokinetic safety levels are achieved, or intolerable side effects occur, indicating the point at which the drug appears to have reached the maximum tolerated dose (Friedman et al., 1996). The first dose to be tested in phase I is estimated as a fraction of the so called "no adverse effect dose", which is the highest dose found not to harm animals under appropriate toxicity/safety testing. (2) Multiple ascending dose studies, characterized by an experimental design similar to single ascending dose studies, with the exception that, at each step, a small group of subjects undergoes repeated administration of the same dose of the test drug. Such studies can be conducted to better understand the pharmacokinetics and pharmacodynamics of the new drug at the steady state. (3) Short trials, designed to investigate variations in the absorption of the new drug following its oral administration in the presence of food.

The increasing cost of novel drug development, in conjunction with ethical considerations about the safety of first-in-man trials, has fostered the implementation of novel procedures with the purpose of optimizing, rationalizing, and enhancing the ability of eliminating redundancies in early phase clinical trials without compromising safety. Accordingly, a new procedure, designated as "phase 0", has been introduced into the very early stage of clinical drug development in order to gain insight into the clinical suitability of novel compounds before starting conventional phase I trials (EMEA, 2004; Pasqualetti et al., 2010).
Additional clinical studies, which are conducted in healthy volunteers, include bioequivalence pharmacokinetic tests. To assess bioequivalence between two medicinal products containing the same active ingredient, such as a commercially available brand product and a generic formulation under clinical development, cross-over pharmacokinetic studies in healthy volunteers are currently regarded as the most suitable experimental approach (Del Tacca et al., 2009). Furthermore, bioequivalence studies are performed in healthy volunteers during pre-registrative development phases, in order to assess different formulations of the new active ingredient.
The present chapter focuses on issues concerning the enrollment of healthy volunteers in the early clinical phases of drug development from different points of view, including regulatory, methodological, normative, ethical, and logistical perspectives.

2. Healthy volunteers in early clinical studies

Defining a healthy volunteer is not an easy task, since different criteria, underlying the concept of wellness, can be implied in this condition. The Royal College of Physicians has defined the healthy volunteer as an "individual who is not known to suffer of any significant illness relevant to the proposed study, who should be within the ordinary range of body measurements, such as weight, and whose mental state is such that he is able to understand and give valid consent to the study" (Royal College of Physicians, 1986). Moreover, in the Association of the British Pharmaceutical Industry guidelines for medical experiments in human volunteers, it is highlighted that the individual cannot be expected to receive therapeutic benefit from the proposed study (Association of British Pharmaceuticals Industries, 1988). The EMEA guideline proposes also a general definition of healthy volunteer for studies aimed at assessing pharmacokinetics: "healthy, adult volunteers, in well-defined and controlled conditions" (EMEA, 1998). On this basis, the selection of healthy volunteers is conducted by enrolling subjects without relevant pathologies and with organ functions, such as heart, liver and kidney, in the normal range. However, the general definitions of healthy volunteer, as those proposed by current guidelines, allow wide margins of discretion. For example, might an asymptomatic subject with allergic rhinitis or affected by knee swelling be considered eligible for a phase I clinical experimentation of a new antidepressant drug? Furthermore, might women under treatment with oestrogen derivatives be eligible for early phases of drug experimentation? These simple examples support the notion that we can use different definitions of healthy subjects and that a critical judgment is required (Pasqualetti et al., 2010).

3. Enrollment procedures

From the industry standpoint, recruitment problems translate into potential revenue losses resulting from delays in bringing a new drug into the market (Harris et al., 2005).

Accordingly, clinical research units must ensure a fast recruitment of healthy volunteers into phase I studies, since an inadequate enrollment may increase the study costs, delay the time to completion, and possibly invalidate the trial outcome due to insufficient study power. Recruiting volunteers is an unavoidable, often time-consuming, and difficult task in clinical research (Bramstedt, 2007). Clinical pharmacological units may overcome recruiting problems by facilitating the access of healthy volunteers to information about clinical experimentation. Internet advertising of clinical research studies can be accomplished in various ways, including websites dedicated to specific studies, clinical trial databases, which store basic information about studies, and direct e-mail solicitation to target populations. The U.S. Food and Drug Administration (FDA) requires that institutional review boards (IRB) examine and approve the advertising materials and methods used to recruit human subjects. Additionally, FDA has issued a guidance regarding internet advertising. In particular, FDA does not require IRB to review internet listings of clinical trials as long as the listings provide only "basic trial information" (e.g., study title, study summary, study location, contact information). Internet databases, which are designed with formal system limits (e.g., font size, font style, entry of only basic study information), satisfy this guidance, and thus, do not require IRB approval for each study listing. Bramstedt (2007) analyzed the incidence and nature of ethically inappropriate recruiting advertisements on internet, and provided a descriptive guidance to clinical investigators for responsible internet recruiting. The majority of advertisements satisfied the FDA guidance. However, 18% of them were ethically questionable with regard to font size, font style, and/or verbiage. This author concluded that inappropriate recruiting advertisements can be coercive and misleading.

4. Pharmacokinetic and pharmacodynamic investigations

Clinical pharmacokinetic studies are performed to examine absorption, distribution, metabolism, and excretion patterns of investigational or approved drugs in healthy volunteers and, where appropriate, in patients. Data obtained from early pharmacokinetic testing are useful for designing and conducting extensive clinical trials. Pharmacokinetic studies are also necessary in the post-marketing setting for bioequivalence assessments (Del Tacca et al., 2009). Moreover, clinical pharmacokinetic studies are also relevant for determining the appropriate use of medicines in particular populations, mainly in patients with impaired renal or liver function, for predicting the outcome of pharmacokinetic drug interactions and for assessing genetic variants in drug metabolism. Data on drug concentration-time profiles, obtained from clinical trials, can also provide information for therapeutic drug monitoring in clinical practice.

In a single-dose study, the concentrations of an investigational drug and its metabolites are measured in blood samples following a single administration to healthy volunteers and/or patients. Furthermore, the levels of the investigational drug and its metabolites in blood, urine and, when necessary, faeces are measured to evaluate the elimination pathways. Drug binding to plasma proteins, time-concentration profiles, and the effects of meals on its bioavailability should also be investigated in single-dose studies. In order to evaluate the relationship between the dose and the respective pharmacokinetic profiles, several doses should be tested. Some pharmacokinetic studies, both in healthy volunteers and patients, aiming at evaluating not only the relationship between dose and blood concentrations, but also that between pharmacological effects and blood levels, may provide valuable information for future clinical development.

As far as pharmacodynamic investigations during a clinical trial are concerned, drug effects can be studied both with hard endpoints, like the patient survival, or surrogate endpoints. A surrogate endpoint (or marker) is an effect measure of a certain treatment that may correlate with a real clinical outcome, but doesn't necessarily have a firm relationship with it. The US National Institutes of Health define a surrogate endpoint as "a biomarker intended to substitute a clinical endpoint" (Cohn, 2004).

Several authors have suggested the use of biomarkers as a method for obtaining early indications of drug effectiveness and safety, both for research and regulatory approval purposes, thereby reducing costs and development time (Frank & Hargreaves, 2003; FDA, 2004). Indeed, it has been estimated that small improvements in clinical trial outcomes and decision-making translate into hundreds of million dollars of development cost-savings and a faster time-to-market (DiMasi, 2002). Therefore, the use of biomarkers is encouraged to such an extent that they are routinely examined as a part of many new drug trials.

The traditional gold standard for evaluating drug safety and efficacy relies on prospective, randomized, well controlled, double-blind clinical trials (Merrill, 1996). Although this paradigm has served well the public health in ensuring that new drugs are thoroughly and scientifically evaluated before reaching the consumer, some investigators have argued that reliance on morbidity and mortality data – the so-called 'true endpoints' – could have had the unintended consequence of contributing to the high cost of pharmaceutical innovations. In an attempt of avoiding such cost increments, with the enactment of the FDA Modernization Act of 1997, the FDA was given explicit authority to approve drugs for the "treatment of a serious or life-threatening condition…upon a determination that a product has an effect on a clinical endpoint or on a surrogate endpoint that is reasonably likely to predict clinical benefit".

Traditional biomarkers, such as analytes assayed in serum, have been employed for decades in the clinical practice and drug development. However, a variety of new biomarkers have recently fostered great interest. In particular, the use of imaging biomarkers for the assessment of drug therapies – a field designated as "pharmaco-imaging" – has become very common in recent years. Not surprisingly, the rise in use of pharmaco-imaging methodologies has coincided with the impressive technical advances occurred in medical imaging, with particular regard for noninvasive *in vivo* imaging methodologies such as computerized tomography (CT), magnetic resonance imaging (MRI) (Rudin et al., 1999; Beckmann et al., 2004) and positron emission tomography (PET) (Fischman et al., 2002; Gambhir, 2002).

Several characteristics of imaging biomarkers differentiate them from traditional biomarkers. First, non-invasive imaging has been applied routinely to diagnosis and disease management for several decades, and the ability to identify a wide spectrum of pathophysiological conditions by means of imaging techniques is well established. Second, imaging biomarkers tend to be more closely associated with disease phenotypes, thus enabling a direct association between therapy and its effect. Third, imaging allows a marked versatility in providing continuous, structural and functional assessments of therapy, thus offering snapshots of drug bioactivity over time (Pien et al., 2005).

5. Efficacy-related surrogate endpoints

Several techniques can be used to measure indirectly the effect of drugs in healthy volunteers (Passchier et al., 2002). Currently, drug dosing regimens for patients are based

mainly on the outcome of preclinical and phase I–II human studies, such as in vitro autoradiography, dose–effect relationships, plasma concentration, tolerability, electroencephalography (EEG), and functional MRI (fMRI) or PET. Although these measurements have their own merits, they cannot provide direct insight into the relationship between the amount of drug administered and the occupancy of its target, thus not allowing a correlation of the results with the effectiveness of the drug in treating the disease. For example, a drug that is assumed to exert its action on the central nervous system (CNS) may instead induce a systemic release of endogenous factors, such as cortisol or noradrenaline, thus leading to changes in EEG patterns or changes in cerebral blood flow, which can be misinterpreted as the drug having a direct effect on CNS. Likewise, measuring drug plasma levels following single or repeated administration, and assuming that target occupancy is linearly related to the dose or plasma concentration, may be incorrect.

Two cases can be easily identified in which plasma concentration does not reflect the actual target occupancy. First, if brain uptake is high and the drug off-rate from its target is slow, a significant fraction of the target receptor will remain occupied for prolonged periods, even if the clearance of the drug from plasma is rapid. Second, a drug can be effective on CNS, even if it shows a very little brain uptake and, consequently, a very low target occupancy. (Sawada et al., 1991; Gibson et al., 2000).

Two techniques are currently available to provide a direct measurement of drug-related receptor occupancy. PET and single-photon emission computed tomography (SPECT) both use radiolabelled ligands specifically designed to bind the desired target with high selectivity and specificity. Although SPECT has certain advantages, such as a relatively long half-life of radionuclides, it displays also major drawbacks, including low sensitivity, limited temporal resolution, impossibility of labelling the native drug since its conjugation with ^{99m}Tc, ^{131}I, and ^{111}In may result in changes in its chemical properties (Sawada et al., 1991; Gibson et al., 2000).

Although various issues, concerning the study design and data interpretation, must be taken into account, PET can play a relevant role in the evaluation of novel drugs. Imaging studies may demonstrate whether the drug reaches its target, if there is a linear relationship between dose and target occupancy, if the occupancy reflects plasma drug levels, and how long the drug remains bound to its target. PET data from healthy volunteers can thus provide very useful information for the design of early clinical trials (Passchier et al., 2002).

5.1 Use of biomarkers in neuroscience

Treatment of neuro-degeneration or CNS diseases has been a daunting task, and therefore, substantial efforts have been made to identify and validate biomarkers to support the implementation of effective therapies (Bakhtiar, 2008). Notably, understanding the molecular bases of Alzheimer's and Parkinson's disease, and identifying novel brain molecular targets has been subject to intense research and high investment by industry, government, and academia. Examples of methodologies employed to identify biomarkers in CNS include brain imaging techniques such as PET, CT, MRI and SPECT. In addition, facial expression recognition task, Visual Analogue Mood Scale, anxiety tests, genetic markers, catecholamine concentrations, psycho-immunological markers, and neuroendocrine markers are among other complementary approaches (Bieck & Potter, 2005). Since drugs that directly target CNS receptors must show good permeability across the blood–brain barrier (BBB) and sufficient brain exposure, an approach is needed to determine the extent of receptor occupancy, which is defined as the percent of receptor population that is

occupied by the drug at a specific dose or concentration in plasma. The data obtained from such imaging biomarkers are then correlated with the pharmacokinetic data to generate a pharmacokinetic/pharmacodynamic (PK/PD) model, understand the mechanism of action, select the most promising compounds candidate to clinical development, guide dose selection, and/or develop predictive tools at early stages of clinical development (de Boer & Gaillard, 2007). There are two common approaches for obtaining information on BBB penetration: to apply an imaging technique (non-invasive); to sample cerebrospinal fluid directly from the central compartment (invasive). Recent advances in medical imaging have made it possible to employ PET in order to detect picomolar levels of radiolabelled drugs in both preclinical models and humans (Frank & Hargreaves, 2003; Lee & Farde, 2006).

5.1.1 Magnetic resonance imaging and pharmaco-MRI

In contrast with the use of ionizing radiations in CT, MRI employs radio-frequency pulses and magnetic fields to obtain signals from changes in nuclear magnetic moments (Pien et al., 2005). In particular, as the alignment and relaxation of protons occur in response to pulsed radio-frequencies, characteristic relaxation times can be measured, most notably T1 (longitudinal relaxation time) and T2 (transverse relaxation time) (McRobbie et al., 2002). While CT images result from a single parameter, namely the X-ray attenuation by the tissue along the propagation path, MRI is generated by multiple parameters, including proton density, T1, T2, flow, diffusion and susceptibility. MRI is useful for several applications, including central and peripheral nervous system function and visualization, both under basal conditions and after environmental stimuli.

Although MRI displays lower resolution and requires a longer time for data acquisition than CT, the former offers higher soft tissue contrast, thus making MRI the technique of choice in the brain, besides specific applications in musculoskeletal and gastrointestinal systems. MRI, with or without the aid of contrast agents, is also employed for a number of functional assessments, including tissue perfusion, tumour permeability, and blood oxygenation level-dependent (BOLD) studies (Le Bihan, 1995; Sorensen & Reamer, 2000).

Pharmaco-MRI allows in vivo visualization of human brain activity and enables non-invasive assessments of drug-related changes in this activity (Windischberger et al., 2010). Several studies have investigated the effects of antidepressant drugs, such as selective serotonin reuptake inhibitors (SSRIs) on neural activation (Anderson et al., 2008; Arce et al., 2008), indicating area-specific and dose-dependent effects on the BOLD response in both healthy subjects (Loubinoux et al., 2002; Del-Ben et al., 2005) and patients suffering from major depression and obsessive–compulsive disorder (Hoehn-Saric et al., 2004). These effects were particularly pronounced in the amygdala, the key brain region in processing and consolidating aversive emotional cues. For instance, a study on acute citalopram administration in twelve healthy male volunteers showed an increased BOLD signal in those brain areas typically involved in depression (McKie et al., 2005).

5.1.2 Positron emission tomography and single-photon emission computed tomography

The basis of radionuclide imaging is the use of bi-functional compounds containing a radiolabelled moiety, which confers detectability, and a chemical and/or pharmaceutical moiety, which determines uptake and distribution throughout the body. In the case of positron-emitting radioisotopes, the emitted positron passes through tissues and is

ultimately annihilated upon combination with an electron, resulting in two photons emitted in opposite directions. Detectors are arranged in a ring around the tissue of interest, and only triggering photonic stimuli arriving near simultaneously at diametrically opposite detectors can be recorded. Tomographic methods are then used to construct the resulting PET images. Several radioisotopes are used for nuclear imaging. These tracer isotopes can be substituted directly into drug compounds to mimic naturally occurring compounds, or can be conjugated with other molecules to form new compounds referred to radiopharmaceuticals. 2-^{18}Fluoro-2-deoxy-D-glucose (FDG), for example, is an analogue of glucose labelled with a positron-emitting form of fluorine, and it is used in PET imaging of metabolic activities that involve glucose uptake (Gambhir, 2002).
In the case of investigations concerning the dopaminergic system, other iodine radiolabelled compounds can be employed (e.g., [^{123}I] FP-CIT: N-ω-fluoropropyl-2β-carbomethoxy-3β(4-iodophenyl)tropane) (Figure 1).

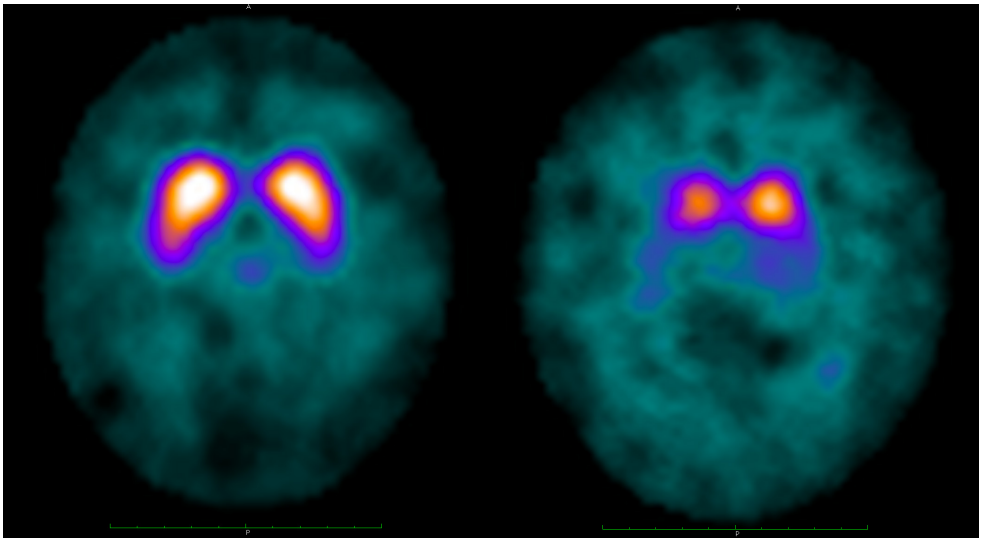

Fig. 1. Transversal sections of [^{123}I]FP-CIT SPECT from brain of a normal subject (left) and a patient affected by PParkinson's disease (right). Radiolabelled compound pattern shows a reduced uptake, both in putamen and caudatum, associated with the degeneration of nigrostriatal nerve fibers (by courtesy of Prof. G. Mariani, Nuclear Medicine Unit, University of Pisa)

It is this diversity in imaging capabilities that allows imaging biomarkers to address a spectrum of drug development issues (Pien, 2005).
The utility of PET studies in healthy volunteers could be represented by drug effect evaluation in specific cerebral areas, in terms of receptor occupancy. In the study by Smith and colleagues (2007), the receptor occupancy produced by treatment with mirtazapine differed significantly among brain regions, showing that higher receptor occupancies were achieved by 7.5 and 15 mg of mirtazapine in high-binding regions (e.g., cortex, amydgala and hippocampus) than in regions with less binding properties (e.g., thalamus and putamen). Thus, PET studies of regional receptor occupancy using [^{11}C]mirtazapine may

provide a reliable mean for testing hypotheses concerning the role of central alpha2-adrenergic dysfunctions in psychiatric disorders (Smith et al., 2007).

5.1.3 Pharmaco-EEG studies of psychotropic drugs

EEG can detect with high sensitivity functional alterations of human brain. Specific EEG patterns related to various psychotropic drugs, such as antipsychotics, antidepressants, anxiolytics, psychostimulants and nootropics, have been reported (Mucci et al., 2006).

By a combination of computer-assisted quantitative analyses of EEG with statistical procedures (quantitative pharmaco-EEG) and mapping techniques (pharmaco-EEG mapping), Saletu and colleagues (2005) classified psychotropic substances and indirectly evaluated their bioavailability in the human brain. In particular, by means of pharmaco-EEG techniques it is possible to determine, at an early stage of drug development, whether a drug is effective on CNS as compared with placebo, what clinical efficacy will be likely achieved, at which dosage it acts, when it acts and equipotent dosages of different galenic formulations. Pharmaco-EEG patterns and maps of neuroleptics, antidepressants, anxiolytics, hypnotics, psychostimulants and nootropics/cognition-enhancing drugs differ, and these differences could reflect the influence of several factors, such as acute or chronic drug administration or differences between normal subjects and patients. Pharmaco-EEG evaluations of these drug classes could anticipate their effects on CNS, their PK/PD profiles and their therapeutic efficacy.

Saletu et al. (2002) investigated the relationship between alterations induced by mental disorders and psychotropic drugs by means of an EEG source analysis, designated as low-resolution brain electromagnetic tomography. Through this approach, they found that some neuroleptics, antidepressants, anxiolytics, hypnotics, psychostimulants and nootropics/cognition-enhancing drugs exerted EEG effects which appeared to counteract the EEG changes associated with mental disorders (i.e., schizophrenia and generalized anxiety disorder). Such a phenomenon has been termed "key–lock principle". In line with this principle, Yoshimura et al. (2007), who performed a placebo-controlled pharmaco-EEG study on two conventional antipsychotics (chlorpromazine and haloperidol) and four atypical antipsychotics (olanzapine, perospirone, quetiapine and risperidone) in healthy volunteers, observed that, under perospirone and haloperidol, the EEG pattern was opposite as compared with the pattern previously reported in schizophrenic patients, thus suggesting a key–lock mechanism.

A compatibility or incompatibility with the key–lock hypothesis does not necessarily have implications for the effectiveness of the drug, but rather for the potential mechanisms of action. Indeed, a key–lock pattern of drug response suggests that the drug acts upon bioelectrical processes that are present both in patients and healthy controls, although in patients such processes are quantitatively different, thus suggesting functional alterations (Yoshimura et al., 2007).

5.1.4 Facial expression recognition task

The area-specific and stimulation-dependent changes in human brain activation by SSRIs are important issues for improving our understanding of the mechanisms evoked by pharmacological treatments. For instance, dysfunctions of the emotion processing circuitry are associated with depression and anxiety disorders (Akimova et al., 2009), and SSRI-induced changes in reactivity within this circuitry can be taken as indicators of treatment response and efficacy (Anderson et al., 2008; Cipriani et al., 2009). In particular, it has been

suggested that, under SSRI treatment, the decrease in clinical symptoms can depend on changes in the processing of emotional stimuli (i.e., enhanced positive emotion processing in concomitance with an attenuated negative emotion processing) (Nathan et al., 2003).
Several studies have shown that changes in serotonin neurotransmission can modulate the identification of emotional faces, particularly fearful faces. Alves-Neto and colleagues (2010) performed their studies on healthy volunteers by using faces from the Pictures of Facial Affect Series, portraying six basic emotions (anger, disgust, fear, happiness, sadness, and surprise), which had been morphed to range from neutral (0%) to a standard emotion (100%), in 10% steps of emotion intensity. For each emotion, pictures of 2 males and 2 females were presented at each intensity level, thus comprising 40 stimuli for each emotion. The faces were displayed in the computer screen for 0.5 s, with an interval (blank screen) of 4.5 s among the various stimuli. Volunteers were requested to select the response that best described the emotion shown in the picture and to record their responses as soon as possible by pressing one of the labelled keys on the keyboard. A single oral dose of escitalopram (10 mg) or placebo was administered to healthy male volunteers 3 hours before testing the Facial Affect Series. Escitalopram facilitated the recognition of sadness and inhibited the recognition of happiness in male, but not female, faces. These results confirm that serotonin modulates the recognition of emotional faces, and suggest that the gender of the face subject can play a role in this modulation (Alves-Neto et al., 2010). Based on these findings, the facial expression recognition test represents an innovative model suitable for studying old and new antidepressant drugs in healthy volunteers.

5.1.5 Biomarkers for antipsychotic drugs

Studies of novel antipsychotics in healthy volunteers deal traditionally with pharmacokinetics and tolerability, but useful information can be also obtained from biomarkers of clinical endpoints. A reliable biomarker should meet the following requirements: consistent response across studies of different antipsychotics; clear response of the biomarker to a therapeutic dose; dose-response relationship; plausible relationship among the biomarkers, pharmacology and pathogenesis (Figure 2).
A review by de Visser and co-workers (2001) has identified 65 studies investigating the effects of 23 neuroleptics by means of 101 different neuropsychological tests, which could be clustered into seven neuropsychological domains. Subjective and objective measures of alertness, as well as those of visual-visuomotor-auditory and motor skills were most sensitive to antipsychotics, although over half of studies failed to show significant differences from placebo (de Visser et al., 2001). With regard for the subjective assessments, most individual analogue scales, in order to assess alertness, mood and calmness, have been proposed and applied to the evaluation of psychotropic drugs. Other scales can be used to examine anxiety, subjective psychotropic drug effects and extrapyramidal side effects (Norris, 1971; Bond et al., 1974).
In addition to EEG, as discussed above, other valid tools can be used for psychological and neurological assessment of novel drugs in healthy volunteers. Smooth pursuit and saccadic eye movements have been extensively validated to assess the side effects of psychotropic drugs. These effects are not specific for a class of drugs (van Steveninck, 1993), but they rather allow to quantify sleep/wake transitions. For instance, schizophrenic patients display abnormalities in stimulation-related potentials, which are postulated to reflect characteristic changes in the stimulus discriminability and decision making. Typically, these changes

consist of a reduction in the amplitude and a prolongation in the latency of the P300 component of evoked potentials.

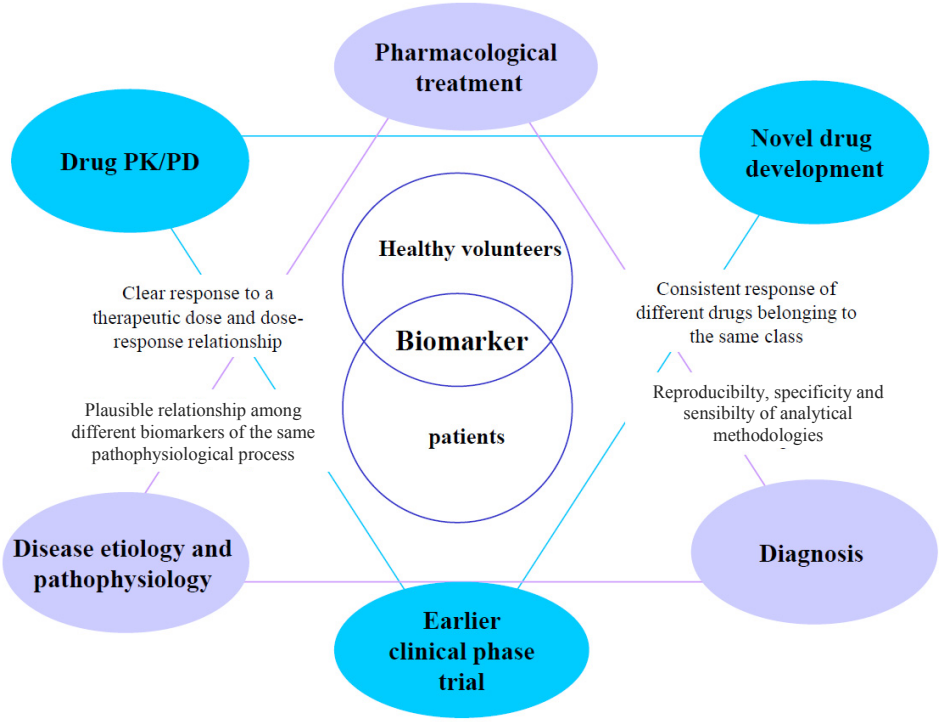

Fig. 2. Features and usefulness of reliable biomarkers and their investigational and clinical application targets. PK/PD: pharmacokinetic/pharmacodynamic relationship

Increments of serum prolactin response in healthy volunteers reflect the "therapeutic" antidopaminergic effect of antipsychotic drugs, while changes in growth hormone and cortisol secretion are studied as biomarkers for antipshycotic activity of drugs on the serotoninergic system. The most consistent effects have been associated with prolactin increase, where 96% of all studies reviewed by deVisser et al. (2001) showed statistically significant effects.

5.2 Biomarkers for anti-inflammatory drugs

Endotoxin or lipopolysaccharide (LPS) (Thorn, 2001; Kharitonov & Sjöbring, 2007) is a component of the outer cell wall of Gram-negative bacteria. LPS is a highly potent pro-inflammatory substance, which, upon inhalation, causes fever, chills and bronchoconstriction in a dose-dependent fashion. These symptoms are accompanied by a pro-inflammatory response, detectable in sputum and bronchoalveolar lavage fluid as elevation of neutrophils, macrophages and certain cytokines/chemokines. Such a response can be partly modified by specific drugs.

There is increasing evidence that diseases caused by organic dusts are mainly inflammatory in nature. Among the several agents found in organic dusts, LPS is a major candidate for the

pathogenesis of inflammatory reactions. Consistently with this view, the inhalation of LPS increases neutrophils, lymphocytes and fibronectin levels in the bronchoalveolar lavage fluid from healthy volunteers (Sandström et al., 1992). Changes in mediators of inflammation, such as eosinophilic cationic protein, myeloperoxidase, interleukin-8 (IL-8), IL-1beta, tumor necrosis factor alpha and C-reactive protein have also been found into the airways and/or blood. In particular, the inhalation of LPS elicits a neutrophilic inflammation with IL-8 elevation in both normal and asthmatic subjects (Kharitonov & Sjöbring, 2007).

Future studies with LPS inhalation need to be focused on relevant diagnostic tools for detecting the inflammatory reaction in subjects exposed to LPS-containing organic dusts, and evaluating whether the large inter-individual variations observed in the response to organic dusts or LPS could depend on differences in the molecular mechanisms underlying the toxicity of the injuring agent.

Models of provoked asthma are very valuable tools for understanding the pathobiology of asthma, aiding the diagnosis, helping to clarify the mechanisms of actions of effective drugs and supporting the development of new drugs. Some provoked models are useful in the clinical setting, particularly those that measure direct airway hyper-responsiveness (for instance, bronchoconstriction elicited by inhaled methacholine), while others, particularly those based on allergen challenge, can be used both in animal models and humans to study the mechanisms of allergen-induced airway inflammation and related pathophysiological changes, as well as in the development of new drugs for asthma (O'Byrne et al., 2009). In particular, investigations on novel bronchoactive drugs in healthy volunteers can be accomplished by testing their effects on basal airway calibre and induced bronchoconstriction. In this context, gaining further knowledge on the molecular and pathophysiological bases of the inflammatory response associated with LPS-induced bronchoconstriction is expected to foster the use of this model for investigating new antiasthmatic drugs.

5.3 Biomarkers for immune-allergology drugs

Allergic rhinitis is a chronic inflammatory disorder of upper airways which evokes characteristic signs and symptoms in sensitized individuals exposed to relevant allergens. Allergic airway inflammation is characterized by immunoglobulin E (Ig E)-triggered mast cells and activated eosinophils, which release pro-inflammatory mediators, such as histamine and leukotrienes. Furthermore, allergic rhinitis is associated with elevated serum IgE levels and positive skin prick test (SPT) to corresponding allergens. In addition to their diagnostic value, serum specific IgE levels and SPT tests may serve as biomarkers to monitor the disease activity in response to anti-allergic therapy (Boot et al., 2008).

Nasal challenge with allergens is a validated, reproducible clinical model for investigating the pathophysiology of allergic rhinitis, which also allows the evaluation of kinetics of nasal inflammatory responses (Doyle et al., 1995). As such, it may serve as a tool to study the effects of anti-allergic interventions, targeting specific inflammatory mechanisms related to the upper airway response (de Graaf-in't Veld et al., 1995). Although nasal biopsies are the golden standard for investigating the cellular inflammatory response, there are some limitations to this invasive technique (Godthelp et al., 1996): a) it can only be performed by an experienced ear-nose-throat physician; b) it provides information just on a limited part of upper airways; c) it does not allow repeated sampling within short time intervals (Jacobson et al., 1999). By contrast, nasal brushing is a less invasive method, which has emerged as a

possible viable alternative for interventional trials requiring repeated sampling. In patients with allergic rhinitis, Jacobson et al. (1999) showed that seasonal changes in the number of mast cells and eosinophils in nasal brush samples correlated well with those found in nasal biopsies. Likewise, intranasal fluticasone produced a similar degree of reduction in these inflammatory cells in both brush and biopsy samples. Nasal lavage is another relatively non-invasive sampling technique which allows serial assessments of the effects of anti-inflammatory drugs on soluble factors released by upper airway inflammation (de Graaf-in't Veld et al., 1997).
In the study performed by Boot and colleagues (2008), SPT, serum specific IgE levels and inflammatory biomarkers in nasal lavage and material obtained by nasal brushing were analyzed in 20 subjects with mild allergic rhinitis, randomly assigned to undergo an intranasal challenge with a relevant allergen (n=10) or diluent (n=10), in order to simultaneously assess the kinetics of several biomarkers of allergic airway inflammation. The authors concluded that serum specific IgE assay and SPT displayed good reproducibility in patients with clinically stable allergic rhinitis, and that nasal allergen challenge, when used in combination with nasal lavage and brush sampling, is a suitable research tool for early drug development.

5.4 Biomarkers for anticancer drugs

In oncology, besides toxicity, alternative endpoints have been proposed for early phase trials evaluating novel drugs targeted against relevant molecular factors, including the assessment of target inhibition in tumours or surrogate tissues, and/or the evaluation of biomarker pharmacokinetics (Kelloff et al., 2004; Goulart et al., 2007; Arrondeau et al., 2010). In this respect, phase 0 trials can provide critical human PK/PD data to support the design of future studies (Kummar et al., 2009). Healthy volunteers, as indicated by international guidelines (EMEA, 2004), can be enrolled to perform phase 0 and early phase I trials to study new anticancer drugs, evaluating specific surrogate biomarkers of antitumor activity and preliminary pharmacokinetics.
For instance, sunitinib, a thyrosin kinase inhibitor, endowed with antiproliferative and antiangiogenic effects, was tested in 12 healthy volunteers at the oral dose of 50 mg for 3–5 consecutive days in order to perform a preliminary evaluation of its PK/PD profile. The parameters assessed in this trial were blood pressure, plasma concentration-time-course of sunitinib, its major metabolite SU12662, vascular endothelial growth factors VEGF-A and VEGF-C, as well as soluble VEGF receptor-2 (sVEGFR-2). The authors found that the time-course of blood pressure under treatment with sunitinib was highly consistent with published data in patients, while changes in circulating biomarkers were greater in patients, as compared with simulations suggested for healthy subjects. Overall, the tumour-independent pharmacological response to sunitinib in healthy volunteers can be described by PK/PD models, thereby facilitating model-based investigations of novel antitumor antiangiogenic drugs, using blood pressure and circulating proteins as biomarkers (Lindauer et al., 2010).
Another interesting example of early phase trial on healthy volunteers in oncology is represented by the study carried out by Reid et al. (2011). In order to accelerate the clinical development of SR13668, an orally active Akt pathway inhibitor, which has demonstrated cancer chemopreventive potential in preclinical studies, these authors designed and conducted a phase 0 trial for evaluating and comparing the effects of food and pharmaceutical formulation on bioavailability of the novel drug. Healthy adult volunteers

were randomly assigned to receive a single 38-mg oral dose of SR13668 in one of five different formulations, with or without food. Blood samples were obtained pre- and post-drug administration for pharmacokinetic analyses and the area under the plasma concentration-time curve (AUC) was defined as the primary endpoint. Overall, the authors found that the AUC values of SR13668 were higher in the fed state and different across the formulations. Moreover, they identified a lead formulation of SR13668 for further clinical research, supporting the use of phase 0 trials to accelerate the development of new anticancer drugs (Reid et al., 2011).

6. Safety of experimental drugs

One of the main goals of phase I trials is the gathering of preliminary data on the safety profile of new compounds in humans, and also to ensure the safety of enrolled subjects. Participation in phase I trials can involve significant risks, and there is no expectance of medical benefits, particularly for healthy subjects. Preclinical data must support an acceptable level of risk for a new drug given to humans for the first time. On this basis, to optimize safety in first-in-man trials, a comprehensive understanding of the new molecule, its target, and its expected pharmacokinetics in both normal and pathological tissues is required. This concept is particularly important for biopharmaceuticals with slow elimination, such as monoclonal antibodies, where the potential for persistent target modulation and alteration of downstream cellular processes requires careful assessment (Tibbitts et al., 2010). However preclinical toxicology, as testified by several toxic reactions observed in early phase trials (Kenter & Cohen, 2006), may not provide suitable information on drug safety to predict which potential adverse effects might occur in humans. For this reason, adverse reactions must be intensively monitored throughout all early phase studies, and first-in-man administration must be conducted in an appropriate pharmacological unit, which can rapidly provide intensive cares. Guidelines issued by both American and European agencies (EMEA 2007a, 2007b, FDA, 2005, 2009) have been implemented to improve the safety of first-in-man trials. In particular, they provide important information to investigators and organizations involved in the design and interpretation of preclinical programs supporting first-in-man trials. Selecting the first dose to be administered in man represents a crucial step for preserving the safety of participants to phase I studies and it requires the integration of data provided by multidisciplinary approaches (Tibbitts et al., 2010). Once sufficient preclinical data are available, the first dose in humans is selected on the basis of both relevant toxicological and toxikinetic endpoints, such as the dose not eliciting any adverse effect in animals (no observed adverse effect level, NOAEL) and the evidence obtained in PK/PD animal models. Moreover, the first dose is calculated by application of a safety factor, which takes into consideration the overall robustness and quality of preclinical data, as well as the potential for adverse effects in the target population (Tibbitts et al., 2010) (Figure 3).

In March 2006, TGN 1412, a new monoclonal antibody directed against a human lymphocytic antigen, which was studied in a first-in-man clinical trial at the Northwick Park Hospital of London, caused a catastrophic systemic organ failure in six healthy subjects exposed to the new drug (leading to hospitalization of all six volunteers in intensive care units), despite being administered at a supposed sub-clinical dose of 0.1 mg per kg, which was about 500 times lower than that estimated as safe in animals (Kenter & Cohen, 2006).

After the London tragedy, in an attempt of mitigating the risk associated with phase I clinical trials, the regulatory agencies, including EMEA, issued new guidelines to aid sponsors in the transition from preclinical to early clinical development, including the "Guideline on requirements for first-in-man clinical trials for potential high-risk medicinal products" (EMEA, 2007a) and "Strategies to identify and mitigate risks for first-in-human clinical trials with investigational medicinal products" (EMEA, 2007b). The first guideline provides criteria to classify new investigational drugs as potential high-risk medicinal products. The second one is intended to assist sponsors in the transition from preclinical to early clinical development. These efforts have been collectively gathered under the slogan "New Safe Medicines Faster" in Europe and "The Critical Path" in the U.S. (Buoen et al., 2005). For these reasons, a new procedure, designated as "phase 0," has been introduced into the very early stage of clinical drug development in order to gain early insights into the clinical suitability of novel compounds to shorten the duration of phase I studies.

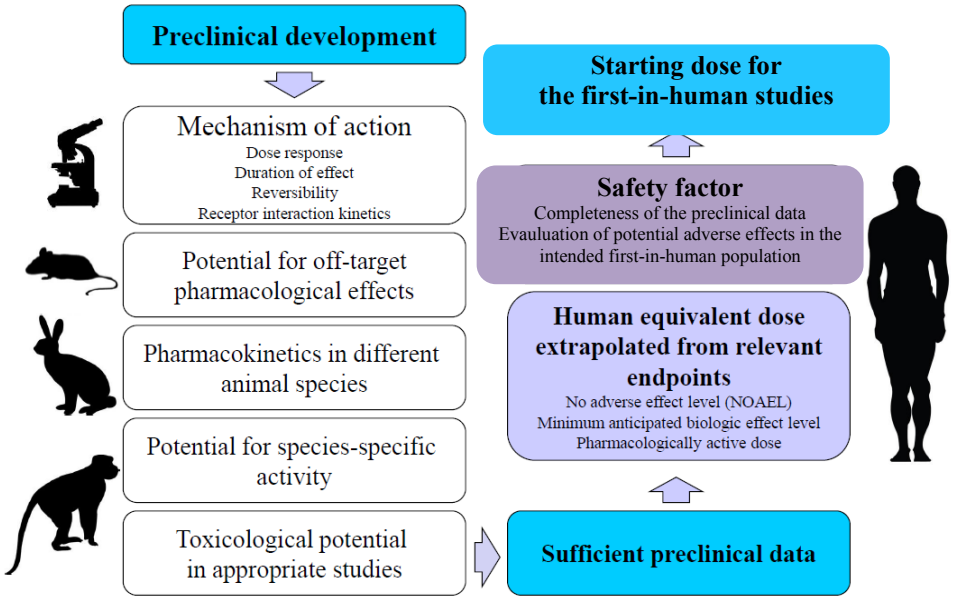

Fig. 3. Flow chart for determining starting dose in the first-in-human studies. The acquisition of sufficient preclinical data and a suitable safety analysis can allow to obtain the human equivalent dose and selecting the starting dose for the first-in-human study (Modified from Tibbitts et al., 2010)

As anticipated above, phase I clinical experimentation encompasses an array of studies, which can be performed on healthy volunteers or patients and deal with the determination of experimental drug tolerability and its PK/PD profile. In the setting of patients affected by a particular disease, these studies can include the evaluation of pharmacodynamic activity, mainly for those drugs with a balance between expected therapeutic effect and toxicological risk not justifying their administration to healthy subjects. In particular, studies evaluating high-risk drugs should be performed in patients when the potential therapeutic effects of

the test drug are expected to overcome its known toxicity (e.g., antiblastic, anti-HIV drugs) or when the expected risks are not acceptable for healthy volunteers. These concepts have been widely accepted until recently, when the new "phase 0" or "early phase I" studies were introduced, according to which the so-called high risk drugs (e.g., antiblastics, antipsychotics, antiepileptics, antiarrhythmics) can be administered to a small number of healthy subjects in subtherapeutic micro-dosing studies, with a consequent reduction of toxicity risk. Accordingly, phase 0 trials are not intended to replace the traditional dose escalation, safety, and tolerability studies, and they cannot indicate whether a candidate drug will have a positive impact on the target disease (Schellens, 2009). The scientific rationale underlying phase 0 trials includes determining as early as possible whether a new drug is capable of modulating the therapeutic target in humans and/or generating pharmacokinetic data, such as the biodistribution and metabolism. This early knowledge is critical in the process of drug development and it may avoid larger phase I and II trials for drugs shown to have unfavorable pharmacologic properties in phase 0 trials (Hill, 2007).

7. Reimbursement procedures

The main goal of early phase trials is to gain knowledge about the clinical suitability of novel compounds, without pursuing specific therapeutic or diagnostic purposes. As anticipated in the previous section, innovative drugs, investigated in these early phases, could be harmful to the health of volunteers. Therefore, it is reasonable to assume that altruism and idiosyncratic interests alone are unlikely to motivate a sufficient number of healthy subjects to act as volunteers in phase I trials. Without payments, recruitment can be slow, resulting in a thwarted and unfitting phase I. For these reasons, there is no doubt that an adequate remuneration must be offered to subjects recruited in early phase investigations. At the same time, coercion and excessive psychological influence should be avoided when obtaining consent (Dickert et al., 2002).

For many years, there has been an ongoing ethical-scientific debate on how to take decisions about fair payment of research subjects, viewed as an attempt of compensating them for their "lost wages" and "discomfort" due to their participation in clinical trials. As in the U.S. and other European countries, in Italy a detailed guideline or a specific law about research volunteer reimbursement is still lacking (Pasqualetti et al., 2010).

Dickert et al. (2002) reported that only 37.5% of clinical research organizations included in their analyses (academic research centers, pharmaceutical companies, contract research organizations, and independent institutional review boards) had written guidelines about the payment of healthy subjects. These organizations disclosed that investigators and IRBs make decisions about payments and that in some studies both healthy and ill subjects are paid for reimbursement of their time, inconvenience, and travel, as an incentive, or for incurring risk. Dickert et al. (2002) underlined also some methods adopted by different organizations to establish "how to pay" by specific formulas: payment for time by the hour, payment per inpatient day or outpatient visit, payment by a flat rate per day or visit, supplemental payment for the "inconvenience" associated with certain procedures. Overall, most organizations require that the estimated payment must be delivered to subjects as prorated (i.e., fractional payment based on the amount of time or procedures actually accomplished) rather than as a contingent payment at the completion of the study (Iltis, 2009).

Several authors support the opportunity of a reimbursement to healthy volunteers and propose different hypotheses to resolve this issue (Pasqualetti et al, 2010). Among the

proposed ways, those indicated by Iltis (2009) and Resnick (2008) appear to be quite interesting. Iltis (2009) underlines the conflict existing between the practice of providing appropriate payments, thus avoiding undue influence, and the requirements of justice when recruiting normal healthy volunteers for phase I clinical trials. By keeping payments low intentionally, in line with recommendations by IRBs, investigators might target or systematically recruit healthy subjects from lower socio-economic levels, thus not fulfilling criteria of social justice. On the other hand, higher reimbursements to volunteers might prompt more persons to enter clinical studies, while not discouraging the less well-off from enrollment. Although investigators would likely prefer to achieve the goal of having a sufficient number of subjects within a reasonable time-frame, the latter method might appear as a wrong influencing procedure for the recruitment of research volunteers.

An alternative way to decrease or increase payments homogeneously is to offer reimbursements based on various and personalized contributions, which may differ according to the specific procedures required by trial protocols (Iltis, 2009). Resnick (2008) describes five distinct models of payments to research subjects: (1) free market model: subjects are paid for providing services (such as completing surveys and undergoing tests and procedures), goods (such as blood, tissue or other biological samples), and to cover potential risks; (2) wage payment model: participants earn a wage equivalent to that of a typical unskilled labourer; (3) reimbursement model: in this setting, participation in clinical research is regarded as a public service (altruism) and volunteers can be paid for their travel expenses, lost wages, baby sitting expenses, etc.; (4) appreciation model: subjects are not compensated for the costs of participation, but they receive money or gifts, such as t-shirts, mugs, gift certificates, as a sign of the investigators' appreciation; (5) fair benefits model: subjects are neither paid labourers nor paid volunteers, but partners in research, sharing the benefits of research, including the economic ones.

The first two models represent a form of "compensated labour" and could motivate individuals to take part in a clinical trial merely to obtain consistent gains. By contrast, the remaining models reflect a "free and voluntary contribution" to community, but they can't embrace the actual motivations of healthy volunteers, who might be seeking free clinical tests or monetary gain (Resnick, 2008).

The lack of international and local guidelines about some crucial aspects related to the recruitment of healthy volunteers in early phase clinical trials, such as the definition of healthy status, payments, advertisement, and participation of the same subject in different experimentations, stimulated a proposal by the Centre for Clinical Drug Experimentation of Pisa University Hospital to implement specific operative procedures (Pasqualetti et al., 2010), attempting to properly address the following issues: (1) advertising to healthy volunteers for recruitment in early phase clinical trials after approval by the local ethics Committee; (2) evaluation of the clinical and psychological status of a potential healthy volunteer; (3) creation of a database containing information on selected healthy subjects to be contacted for possible enrollment in a clinical trial, and allowing a proper monitoring of their participation in different experiments; (4) calculation of adequate reimbursements to healthy volunteers participating to clinical trials. The latter operative procedure is based on criteria which, although not yet fully comprehensive, provide a useful frame to estimate the amount of fair payment to healthy volunteers. In particular, it encompasses a number of items for which the amount of specific reimbursement is indicated, including the number of blood samples, peripheral venous catheter or needle placement, number of clinical laboratory analyses performed during the screening and follow-up (blood and urine

sampling, ECG, psychopathological test administration); other biological fluid sampling; restrictions imposed by a specific study protocol (for example, smoking and alcohol ban, physical exercise, etc.); investigational drug administration route (invasive: intramuscular, intravenous, by naso-gastric tube; not invasive: oral); filling in questionnaires and diaries; time spent at the clinical pharmacology centre (per hour); discomfort or distress caused by study procedures (classified as minor, moderate and major).

8. Conclusion

Drug experimentation in healthy volunteers is aimed at investigating pharmacokinetics and pharmacodynamics as well as documenting safety and tolerability of new compounds. Therefore, strategies to evaluate risks versus benefits during phase I and phase II clinical studies need to be refined continuously to improve efficiency and quality. At the same time, there is a clear need to better predict inter-subject variability, off-target toxicity, clinical outcomes and disease pathophysiology (Cohen, 2006).

Since drug development is a complex, costly and risky process, evidence has now been accumulated concerning the promising role of imaging in highlighting risks and costs associated with clinical drug experimentation by validating targets, confirming mechanisms of action, obtaining early markers of pharmacological activity, assessing pharmacokinetic profiles and providing prognostic indicators. Despite these interesting promises, consideration must be given to the possibility of confounding factors, which could mislead surrogate endpoints, and the extent to which biomarkers are validated for their intended purposes.

The issue of validation of new imaging biomarkers needs to be addressed in a more systematic and rigorous way, including the search for correlations among imaging and molecular biomarkers, elucidating the relationships between particular imaging biomarkers and the purported pathophysiological pathways. With recent advances in genomic, proteomic imaging and computational sciences, pharmaco-imaging is gaining an important role in drug development (Bakthiar, 2007).

Other critical aspects of drug experimentation in healthy volunteers include both legislative and ethical issues, pending the lack of international guidelines suggesting specific procedures for the recruitment and reimbursement of healthy subjects enrolled in clinical studies.

In order to clarify the main unresolved questions, the international scientific community needs to address and resolve urgently the following points: a) international definition of healthy status based on standard physical, psychological and clinical parameters; b) appropriate advertisement addressed to potential participants to first-in-man clinical trials; c) international standard criteria for offering fair payments to healthy volunteers enrolled in phase I trials; d) need of a national register, following the examples of some Countries (Resnik & Koski, 2011), in order to monitor the participation of healthy subjects and avoid their simultaneous enrollment in early clinical trials at the same or other centre for drug experimentation.

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Changes in Research and Development of Medicinal Products since the Paediatric Regulation

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1. Introduction

The lack or incompleteness of evidence of the efficacy and safety of drugs used in children has been a growing concern in the recent past. The great majority of drugs prescribed to children are often given either on an unlicensed or an "off label" basis simply by extrapolating data for adults and without conducting any paediatric clinical, pharmacokinetics, dose finding, or formulation studies in the paediatric population. The paediatric pharmaceutical repertoire therefore comprised pills too large to swallow and extemporaneous formulations containing excipients unsafe or unpalatable to children.

Diseases in children, however, are often different from their adult equivalents, and the processes underlying growth and development might lead to a different effect or an adverse drug reaction unseen in adults.

The health and, therefore, quality of life of the children in Europe suffer from a lack of testing and authorisation of medicines for their use. It means that children are "orphans" of appropriate medicinal products and children continue to be exposed to risks, and at the same time miss out on therapeutic advances.

This is particularly ironic considering that our modern system of medicines regulation, that ensures the high standards of safety, quality and efficacy of medicinal products for use in adults, was developed primarily in response to therapeutic disasters, or "drug catastrophes", that occurred in children in the past (such as the numerous cases of icterus induced by sulphanilamide which occurred in 1937 and the well-known phocomelia caused by thalidomide in the 1960s).

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For these reasons, the development of regulations and the adoption of stringent criteria on quality, safety and efficacy medicinal products used in the paediatric population have been enforced and led in January 2007 to the introduction in the European Union (EU) of the "Paediatric Regulation" (European Parliament and the Council of the European Union, 2006a, 2006b) governing the development and authorisation of medicines for use in children aged 0 to less than 18 years. The Paediatric Regulation is the latest in a number of incremental regulatory steps to improve public health for children through increasing research, information and availability of medicines. It brings in many new tasks and responsibilities for the European Medicines Agency (EMA), chief of which is the creation and operation of an *ad hoc* Paediatric Committee (PDCO) within the EMA to provide objective scientific opinions on any development plan for medicines for use in children.
Since the entry into force of the Paediatric Regulation, the regulatory environment for paediatric medicines has dramatically changed, a marked "paediatric revolution" aimed at improving the health of children in the EU took place and pharmaceutical companies have been faced with a number of measures, obligations and incentives.

2. The Paediatric Regulation

2.1 Objectives

The objective of the Paediatric Regulation is to improve the health of children in Europe by increasing and facilitating the development and availability of medicines for children aged 0 to less than 18 years, that means changing the way in which medicines are developed. Increasing the development of medicines for children is to be reached by ensuring that those medicines are subject of high quality, ethically researched and authorised appropriately in the relevant population subsets including the neonates, to avoid, at the same time, unnecessary clinical trials in children and not delaying the authorization of medicines for the adult population.
The new key element of the Paediatric Regulation is the early involvement of a pharmaceutical company in the research and development programme of a medicinal product by the requirement to consider the needs of the paediatric population, also in terms of age-appropriate formulations, according to a Paediatric Investigation Plan (PIP) that describes the paediatric development (quality, non clinical and clinical aspects) and all adopted measures necessary to investigate the medicine in the paediatric population. The PIP has to be agreed with an *ad hoc* committee of experts, namely the Paediatric Committee. In case that a) the specific medicinal product or class of medicinal products is likely to be ineffective or unsafe in part or all of the paediatric population, b) the disease or condition for which the specific medicinal product or class is intended occurs only in adult populations, or c) the specific medicinal product does not represent a significant therapeutic benefit over existing treatments, it is possible to apply for a waiver. The Paediatric Regulation also foresees the opportunity to request a deferral thus avoiding the delay of the authorisation for other populations. A deferral allows the postponement of the initiation or completion of some or all of the measures set out in PIP, such as the completion of trials, but not the delay in the submission of the PIP itself.

2.2 Obligations and incentives

The Paediatric Regulation is the first piece of European legislation that requires the pharmaceutical industry to develop medicines for use in children through a system of obligations and incentives, depending on the type of medicinal product concerned.

For *medicinal products not yet authorised* in the EU, according to article 7 of the Regulation, there is an obligation to include the results of studies (conducted in compliance with an agreed PIP), unless a waiver or a deferral was granted by the PDCO, for the validation of a marketing authorisation application for adult and paediatric medicines.
For *authorised products protected by a supplementary protection certificate, or by a patent eligible for a supplementary protection certificate*, according to article 8 of the Regulation, there is an obligation to include either the results of studies (conducted in compliance with an agreed PIP), unless a waiver or a deferral was granted by the PDCO, in applications for variations or extensions (of an existing marketing authorisation) concerning a new indication, pharmaceutical form or route of administration.
The compliance with such obligations allows pharmaceutical companies to benefit incentives and rewards, aimed at encouraging the development of paediatric medicines. Once authorisation is obtained in all EU Member States and study results (whether negative or positive) are incorporated into the product information (SPC), the pharmaceutical company grants rewards and incentives.
In details when an agreed PIP is completed and all the information has been submitted to the regulatory authorities, the medicinal product, falling under article 7 or 8 of the Regulation, will be granted an extra 6 months patent protection (extension of the duration of its Supplementary Protection Certificate [SPC]). This extension will be granted whether or not the data support a paediatric indication.
For orphan medicinal products the incentive takes the form of an extra two years market exclusivity.
The Regulation also establishes a new type of marketing authorisation, called the paediatric use marketing authorisation (PUMA), intended to stimulate the development of off-patent products for use in the paediatric population. The PUMA is the specific instrument created by the Regulation aimed at stimulation the development of off patent drugs which represent a still unmet need in paediatrics, since most of these compounds widely used daily in children of all age groups, have not been adequately tested in the paediatric population. The Paediatric Regulation includes provisions for funding of research into off-patent medicines (Community Framework Programmes for Research, Technological Development and Demonstration Activities or any other Community initiatives for the funding of research). Public funding is necessary as off-patent medicines are of little commercial interest for pharmaceutical companies.

2.3 The Paediatric Investigation plan

As described above, the central key instrument of the Paediatric Regulation is the agreement of the PIP, defined as a research and development programme aimed at ensuring that the necessary data are generated to determine the conditions in which a medicinal product may be authorised to treat the paediatric population.
The PIP details the planned development in terms of efficacy, safety and quality (age-appropriate formulation) and timelines for children from birth to 18 years. In other words, the PIP contains a full proposal of all the studies necessary to support the paediatric use of an individual product, intended for diagnosis, prevention or treatment of a condition.
The paediatric population is in fact composed of a number of population subsets, thus the PIP has to specify which population subsets need to be studied, by what means and by when.

In addition, PIP must describe any measures to adapt the formulation of the medicinal product to make its use more acceptable, easier, safer or more effective for the different subsets of the paediatric population.

The introduction of the PIP in the legal framework of medicines has finally ensured, or at least is expected to ensure, the availability of paediatric data and results, to cover the needs of all age groups of paediatrics, from birth to adolescence. All the regulatory and scientific strategy for developing a product is changing and the development of medicinal products for potential use in the paediatric population becomes also an integral part of the development of medicines for adults.

The PIP has to be submitted to the Paediatric Committee early during the product development before marketing authorisation applications are submitted. Particularly applications for a PIP, including a deferral if relevant, and/or for a waiver should be submitted no later than the completion of the relevant human pharmacokinetic studies in adults, unless justified. This time point was chosen to ensure that the paediatric development of the product is considered at a very early stage of the overall product development rather than as an afterthought.

An *ad hoc* procedure is followed by the PDCO to evaluate and finally agree the PIP. In the specific the evaluation of a PIP is conducted over two periods of 60 days each—maximum. EMA scientific coordinators and two members of the PDCO contribute to the PIP evaluation in a written report (PDCO Summary Report), which is then discussed by the PDCO. In most cases, the Paediatric Committee requests modifications of the proposal at the end of the first 60 days period. The request for modification identifies all necessary changes to study non clinical and/or clinical protocols and/or formulations. At this point it is expected a clock stop at day 60 to allow companies to respond to requests for modification of the plan (approximately 3 months) and once evaluated, PDCO positive or a negative opinions on PIPs and waivers are transformed into binding EMA decisions.

Notably the PIP is legally binding for the pharmaceutical companies willing to seek marketing authorisation since the results generated by the agreed PIP are then assessed by the Competent Authority granting the marketing authorisation (the Committee for Medicinal Products for Human Use for the centralised procedure) or the variations.

2.4 The Paediatric Committee

The Paediatric Regulation brings the creation and operation of a new scientific committee of experts at the EMA, the Paediatric Committee, set up in July 2007 and primarily responsible for reviewing and agreeing applications for paediatric investigation plans including deferrals, and/or waivers.

This committee aims to ensure expertise and competence in paediatric medicines and to provide scientific opinions on any development plan for medicines for use in children.

The PDCO membership includes five members of the Committee on Human Medicinal Products (CHMP) plus their alternates. These five members will provide an important link between the two committees. Member States, that have no representatives as CHMP members in the Paediatric Committee, appoint a member and an alternate. In addition, the European Commission appoints three members plus alternates, to represent health professionals, and three members plus alternates to represent patient associations, following a public call for expressions of interest and after consulting the European Parliament. The EMA and the Commission are expected to cooperate to ensure that the final composition of the Committee, including members and alternates, covers those scientific areas relevant to

paediatric medicines. The members, alternates and experts must not have any financial or other interests in the pharmaceutical industry that could affect their impartiality.
The Paediatric Committee has a number of important responsibilities. Its most important task is to assess the content of PIPs (which may also include requests for waiver and/or deferrals) for medicinal products and to release opinions on them. The Committee may itself impose a waiver from the requirement to provide data from a PIP, if it considers that the product may be unsafe or ineffective in the paediatric population or that the product will not provide any significant therapeutic benefit. In fact, when assessing a Paediatric Investigation Plan, the Paediatric Committee must consider whether or not any proposed studies can be expected to be of significant therapeutic benefit to and/or fulfil a therapeutic need of the paediatric population.
Other specific tasks include establishing an inventory of specific needs for paediatric medicinal products (still under preparation) and giving scientific input in the development of any documents related to achieving the Regulation's objectives. The Committee should avoid requirements for studies in children causing any delay in the marketing authorisation of the medicine for other populations. This is to ensure that medicines are developed for children based on the therapeutic needs of children rather than just on the basis of when the paediatric market may be profitable or incentives might be financially attractive. The Committee also has an advisory role for the EMA and for the European Commission on any question relating to the paediatric use of medicines. This includes giving advice on communication issues relating to conducting research in the paediatric population, on the compliance of an application for marketing authorisation with an agreed PIP or on the safety, quality and efficacy of a medicinal product for paediatric use.

2.5 How to increase paediatric research

One of the above described objectives of the Paediatric Regulation is to increase paediatric research. Clinical trials in the paediatric population require specific expertise, in some cases specific methodology and specific facilities, and should be carried out by appropriately trained investigators. Thus in order to facilitate this aspect the Paediatric Regulation established the creation of an European Network of Paediatric Research and research funding.
The EMA is responsible for establishing a network of existing networks, centres and investigators of paediatric research, which was set up in 2011 and whose objectives are to coordinate studies relating to paediatric medicinal products, to build up the necessary scientific and administrative competences at European level, avoiding duplication of studies and testing in children. The EU network is expected to serve as a tool for industry to perform trials with children in keeping with the PIP. Both technical and/or administrative competences in the performance of paediatric clinical trials through effective collaboration are expected benefits, which will allow to avoid duplication of work and efforts, making the use of facilities more efficient and profitable, to develop common methods of working with special attention to quality assurance. Facilitation of recruitment of patients and avoiding unnecessary studies in children are expected.

2.6 Transparency and information

Transparency and information are other key words of the Paediatric Regulation. Through increased availability of information, the safe and effective use of medicinal products for

children can be increased so promoting public health, preventing the duplication of studies in children and avoiding unnecessary studies. One of the measures is making all paediatric trials included in the European database (EudraCT) accessible to the public both for protocol and results-related information. The increase in paediatric clinical trials transparency beginning from the planning and recruiting of patients to the on-going and finalised studies, is another target of the Regulation. All decisions of the EMA on PIPs, deferrals or waivers of the paediatric development are made public and routinely published on EMA website. Moreover where authorisation is granted, the results of all those paediatric studies, any waivers or deferral, are included in the Summary of Product Characteristic and, if appropriate, in the Patient Leaflet of the medicinal product, whether or not all the paediatric indications concerned were approved by the competent authority.

3. What is changing with the Paediatric Regulation

The Paediatric Regulation is a remarkable step forward, because for the first time in Europe it is a regulation that is provided by law and provides direct economic support for paediatric clinical trials and indirect support for pharmaceutical industries.
Since its establishment in July 2007 the PDCO has assessed a large numbers of procedures: by August 2011, 1087 validated PIPs/waivers have been submitted by pharmaceutical companies covering nearly 1516 indications., and of which 259 have been requests for a full waiver.
Of the 1087 validated applications:

- 74% referred to medicinal products not yet authorised in the EU at the time of the entry into force of the Regulation (so called "Article 7 applications").
- 24% referred to products already authorised, still under patent or supplementary protection certificate, in view to submitting a variation/extension for a new indication, pharmaceutical form or route of administration (so called "Article 8 applications").
- 4% referred to an off-patent product developed specifically for children with an age-appropriate formulation (so called "Article 30 applications").

In the first year of the implementation of the Regulation, most of the applications were "Article 8 applications". After about a year, the balance changed towards a higher proportion of "Article 7 applications". This change is confirmed in 2010 and 2011. For "Article 30 applications", the number of applications submitted is still very low.
In 2011, Olski et al. published the first analysis of the general impact of the Paediatric Regulation on the development of medicinal products in Europe. Three years after its implementation an increase in the availability of medicines with age-appropriate information in the next years is shown at least as reported by the high number of PIPs despite the still modest number of clinical trials performed (Olski TM et al., 2011; Davies et al., 2010; Rocchi et al., 2010).
Most of the paediatric developments will be performed in therapeutic areas, such as endocrinology, oncology, infectious diseases and cardiovascular diseases, which relates to the economical importance in the adult market, while other areas such as pain management still remains less studied.
A high number of full waiver requests are reported for more prevalent adult-only conditions, such as atherosclerosis.
On the light of these results and from a qualitative approach, the Paediatric Regulation appears to fulfil its core goals. The quality of the plans submitted has improved also due to the PDCO demanding better methodological approaches. The PDCO's intervention has also

increased the number of medicines that will be studied in neonates, the most neglected subset to date. A high proportion of agreed PIPs require a specific age-appropriate formulation, more than originally proposed, and all this should help meet the clinical needs of children and health professionals.
The agreed-upon PIPs will provide important short- and long-term safety data.
Some paediatric development plans have been also preliminary discussed during scientific advice procedure, a free access granted by the Paediatric Regulation for any request containing questions on the paediatric development. The advice is provided by the *ad hoc* SAW /Scientific Advice Working Party) of the Committee for Medicinal Products for Human Use (CHMP) and is adopted by the CHMP. For the paediatric requests, members of the PDCO are routinely involved in the procedure as experts. Notably, since the entry into force of the Paediatric Regulation, the number of scientific advice on questions related only to paediatric development has increased steadily, with a total of 32 procedures in 2010. A high number of so-called "mixed" scientific advice/protocol assistance requests, i.e. covering both adult and paediatric development, have also been submitted for which members of the PDCO are generally involved. Compared to 2009 where 35 procedures were submitted, the figure increased to 48 in 2010 (European Medicines Agency [EMA], 2011, thereafter EMA, 2011).
Indeed, there is still a need for additional paediatric information on off-patent medicines and to date, in June, 2011, only a PUMA has been granted for the medicinal product Buccolam (midazolam) intended for the treatment of prolonged, acute, convulsive seizures in paediatric patients from the age of 3 months to 18 years. The funds for the research of off patent drugs granted by the European Commission are currently in stand by and there is hope that next calls of the EC Framework programs will take into account the paediatric population.
One thing is for certain, the whole R&D process from pharmaceutical, non –clinical and clinical to post-marketing studies is continuously evolving and changing starting from *quality* (need for age appropriate formulation specifically addressed to paediatrics), to *non clinical* (need for studies on juvenile animals to evaluate the toxic potential before any administration to paediatrics), *clinical* (need for non conventional approaches in terms of study design and size of the population, choice of adequate endpoints), and *post-marketing* issues (need for *ad hoc* pharmacovigilance measures).
In the following sections, a short overview of how R&D process is improving in the light of new Paediatric Regulation's requirements is provided.

4. Quality: Need for age appropriate formulation specifically addressed to paediatrics

The development of ad hoc formulations intended for the paediatric population is a crucial issue and unfortunately, nowadays there is still limited scientific and regulatory experience.
Regulatory initiatives have been undertaken to provide guidance in developing ad hoc medicine. As mentioned above, the adoption of the Paediatric Regulation plus the consequent demand for paediatric studies have started to change the context by finally strengthening the focus on better formulations for children.
The current legislation establishes that pharmaceutical companies need to develop a specific age-appropriate paediatric formulation together with an adequate packaging, user instruction and where relevant dosing device and/or administration device prior to

performing clinical studies in the paediatric population. All these pharmaceutical development aspects may be fundamentally different to those of the existing adult product.
This new regulatory environment stimulates the rational development and testing of age-appropriate medicinal products intended for the paediatric population. Part of this legislation (Recital 9, 10 ; Art. 15) requires that applicants for medicinal products for use in the paediatric population should submit their plans for the development of such products in the form of a PIP for approval, or a waiver, or a deferral.
The spirit of the legislation was to i) to encourage companies to develop specific, 'age-appropriate' paediatric formulations and ii) to develop relevant and acceptable formulations with convenient and precise dosing characteristics, on an industrial scale suitable for marketing. (i.e. the spirit is not to place the preparation of paediatric medicines in a context which is ad hoc, extemporaneous, or magistral preparations).
It is now well accepted that children are not simply small adults and their treatment with pharmaceutical medicines poses problems which are not seen to the same extent in adults. For example, the lower age group subsets of the paediatric population are simply unable to swallow conventionally sized tablets; they may be particularly sensitive to certain excipients that are otherwise acceptable in adult formulations, or there may be compliance problems since they often need to be persuaded to take their medicines, and so on.
Few European Medicines Agency (EMA) documents have been released with the aim of providing guidance on the principles that should be taken into account in the development and the assessment of pharmaceutical aspects of medicinal products for paediatric use.
In the regulatory framework, the starting point was the "Concept paper on the development of a quality guideline on pharmaceutical development of medicines for paediatric use" (Committee for Medicinal Products for Human use [CHMP] et al., 2008, thereafter CHMP et al., 2008) which was the first step to a scientific and harmonised approach to the development of a guideline that provides adequate tools for responsible development of a medicinal product for use in the different subsets of the paediatric population. Information sharing with authorities from other regions (e.g. the FDA) would further support global development.
This guidance however, was preceded by other guidelines published by the EMA, such as:

- "Excipients in the Dossier for Application for Marketing Authorization of a Medicinal Product" (CHMP, 2007a): a key guideline on the quality of excipients, into effect on January 2008.
- "Excipients in the Label and Package leaflet of Medicinal Products for Human Use" (European Commission [EC], 2003, thereafter EC, 2003): one of the most relevant guidelines in the context of quality related to safety, even if established safety profiles and warning statements are based mostly on data in adults, and apply to the adult population. However, it acknowledges that some excipients are not entirely inert but may have side effects.

One of the issues to consider is that the benefits of a medicinal product for paediatric use should outweigh the potential risks associated with its use by the different subsets of the paediatric population as defined in the "International Conference on Harmonisation (ICH) Topic E 11 Note For Guidance On Clinical Investigation Of Medicinal Products in the Paediatric Population" (Committee for Proprietary Medicinal Products [CPMP], 2003, thereafter CPMP, 2003), namely:

- Preterm newborn infants.
- Term newborn infants (0-27 days).
- Infants and toddlers (1 month to 23 months).

- Children (2 – 11 years).
- Adolescents (12 – 16 or 18 years).

The pharmaceutical development aspects should be chosen with the care for each subset of the paediatric population. This classification does not match pharmacological stages. Often, the different subsets (from preterm newborn infants to adolescents) require different approaches. As a consequence, there might be a need to develop more than a single `formulation` which would be appropriate for all ages. On July 2006, the "Reflection paper on formulations of choice for the paediatric patient" (CHMP, 2006) was finalised by the EMA with the aim of providing a comprehensive summary of the physiological and pharmaceutical issues. These could be taken into account in the development of paediatric medicines, looking at the acceptability of different dosage forms, administration volumes, size of unit dosage, taste and the acceptability and safety of excipients in relation to the age and development status of the child.

Afterwards, on January 2010 the "Guideline on the investigation of Medicinal Product in the Term and Preterm Neonate" (CHMP & Paediatric Committee [PDCO], 2009, thereafter CHMP & PDCO, 2009) was effective and specifically addressed to the investigation of medicines intended for neonates.

An additional consolidated guidance on the pharmaceutical aspects of medicinal products for paediatric use was anticipated to be finalised before the end of 2009 in the concept paper. However, "the Draft of Guideline on Pharmaceutical Development of Medicines for Paediatric Use" (CHMP, 2011) has been released for consultation in May 2011. As the deadline for comments is December 2011, the guideline will likely to be published in 2012 as an outcome of a collaborative work between the CHMP, Safety Working Party (SWP), Paediatric Committee, and external experts. This guideline aims to provide additional tools for the rationale pharmaceutical development of medicines for children between birth and 18 years of age.

4.1 Points to consider in developing an age appropriate formulation

Paediatric formulations that permit accurate dosing and enhance adherence (i.e., simple dosing regimen, better palatability) are required for paediatric clinical pharmacology studies and an age-appropriate dosage form must be made available for children.

The goal should ideally be to develop relevant and acceptable safe formulations, which have convenient and precise dosing characteristics for the intended population, made on an industrial scale suitable for marketing.

Basically, the critical points of the paediatric formulations to be considered are related to:

- Routes of administration.
- Appropriate dosage forms: taking into account a formulation that the child can take (size, volume, taste, ease of administration, dosing regimen).
- Dosing accuracy (strength, dose criticality, administration device).
- Treatment duration and setting.
- Appropriate safety considering administration device, handling, excipients such as preservatives, antioxidants, colorants.
- Acceptable organoleptic properties including taste, after taste, smell, colour, texture.
- Minimal impact on life style, ease of administration, good acceptability by children and parents/carers.
- Cost-effectiveness.

4.2 Development of paediatric formulations for paediatric subsets

4.2.1 Preterms and neonates

As above cited, the guidance (CHMP & PDCO, 2009) was released by the EMA and entered into force on January 2010 on the investigation of medicinal products in the term and preterm neonates. This guideline addresses the considerations, requirements for the design and conduct of clinical trials in premature and term neonates. It includes background information on the maturation of organs and of body functions. Formulations and route of administration are also mentioned.

Age-appropriate formulations and strengths using appropriate excipients must be developed to avoid extemporaneous preparations, even more so for neonates. In neonatal practice medication errors are commonly due to use of inappropriate formulations or strengths which require complex calculations and measurement of very small volumes or multiple dilutions. Special care should be given to extremely low birth weight (birth weight <1000 g) and very low birth weight (birth weight <1500 g) newborns.

Notably, attention must be focused on the excipients which can be used on adults and older children, may be toxic in neonates because of their immature and rapidly changing metabolic & elimination system (e.g. less predictable absorption, different volume of distribution, immature clearance mechanism). The salt of the active ingredient and the chemical nature of the preparation must be carefully considered to avoid administration of excessive amounts of electrolytes.

The intravenous (IV) route is normally used in clinically unstable term and preterm neonates. The volume of IV infusions (blood products, total parenteral nutrition (TPN), other IV medication) contributes critically to daily fluid intake. Therefore careful thought must be given to the volume of injection not to exceed the daily fluid allowance. Osmolarity of solutions, pH and infusion rates must be carefully considered. A suitable strength of an IV formulation with an appropriate solvent should enable to administer appropriate volume.

Oral administration should be used when possible and appropriate in the neonatal population. Preparations for oral administration are most likely to be liquid dosage forms, keeping the volume to be administered as small as possible. However, as liquid preparations more often contains excipients like preservatives and antioxidants, special care should be taken regarding the choice of excipients as some may have toxic effects. Sterile and/or single use oral dosage forms may be considered in order to avoid the use of preservatives and to avoid or to reduce antioxidants. When preservatives are required, the concentration should be at the minimum level and a thorough justification for the choice of the preservative should be provided.

Neonates have special needs. If the product is likely to be administered via an enteral (e.g. nasogastric, nasojejunal) tube, issues such as viscosity of formulation (to permit flow of the product through small tubes [e.g. 6FR/8FR] and avoid blockage), size of particles, adsorption to commonly used enteral tubes and interaction with common formula/breast milk should be investigated.

For local or systemic effect a topical administration may be suitable, taking into account skin immaturity, especially in preterm neonates, and the large and more permeable and moisturised surface area.

On the contrary, rectal and intramuscular (IM) administration are not commonly used in this age group, due to erratic absorption and in case of IM also to painful injections that may cause tissue damage. Adverse effects such as muscle contraction and abscess development

can be seen after IM administration. In premature neonates, inefficient muscle contractions and vasomotor activity may alter pharmacokinetics of the drug. In neonates, decreased blood flow may cause variability in drug delivery and absorption.

4.2.2 Infants

The rapid maturation, immune system development and total body growth should be carefully taken into consideration in developing relevant and acceptable formulations for infants. The developmental pattern may occur at a variable level between individuals.

While the rate of oral absorption is slower in neonates than in older paediatric patients, absorption by IM route may be greater by the rich supply of capillaries. In early infancy, the low concentration of bile salts may decrease the absorption of lipid-soluble drugs. Above 1 year of age, infants metabolic activity increases, dose adjustments should be made accordingly. Infant skin is mature; their epidermis is hydrated to a greater extent than adults. During the first years of childhood, the surface area to body weight ratio in infants may be twice that of adults and subject to a change.

The choice of the excipients should be properly justified in PIP in relation to age, treatment duration, severity of disease, rational use, due to infants' juvenile metabolizing capacity. Risk-benefit analysis should support proposed excipient selection. Some excipients are not entirely inert, therefore may present safety problem. Even the so-called child-friendly excipients should be kept to a minimum. When only intended for aesthetic purposes, excipient use should be avoided. It would be a better approach to use marking or embossing instead of inclusion of colouring agent into formulation composition.

As a result of lack of cognitive maturity and coordination, infants are similar to neonates in terms of being fully dependent on caregivers. Appropriate dose delivery and administration devices have to be used. The physiological capability of the infant should be considered. For instance, dry powder inhalers are not appropriate for infants as they cannot generate sufficient air flow. Pressurized metered dose inhalers may be applied to infants from birth if in combination with a specific spacer system and face mask. Administration devices providing accurate dose delivery should be considered. Validated droppers are convenient for infants. Concomitant administration with TPN using the same IV access is discouraged, as TPN formulations are highly variable and may induce oxidation.

4.2.3 Children

This period is much more mature compared to newborn and infants. However, children constitute a subset of paediatric population, with a distinct age range situated at different stages of their physiological and cognitive development. The magnitude of doses varies throughout childhood. Hence, major difficulty would be to choose the optimum formulation applicable for all age range. In this respect, the points to be considered are as follows; younger children are still under psychomotor development, school age children are subject to skeletal growth, weight gain and switch from home life to school life, the onset of puberty can occur as early as 9 years of age. Therefore, stratification by age within this category may be needed for the decision on drug delivery system. Considering the child's cognitive ability, further subdivision of this age group into pre-school children (2-5 years) and school children (6-11 years) would be helpful. The formulation strategy should be chosen on a case by case depending on the drug's physicochemical characteristics, dose and patient's disease status and age. Accurate dosing must be ensured with an appropriate packaging and

administration device. For instance, if the incorrect dosing risk is high, it would be a sound approach to use unit dose, pre-filled oral syringes or cups for the single use . The criticality of the dose should be established to determine the choice of the drug delivery system.

Oral solid unit dosage forms which are intended to be ingested whole may be acceptable for older children. The compelling question is at what age children can safely swallow conventional tablets and capsules. Anecdotal evidence suggest that with support and training, children aged < 6 years can learn to take solid dosage forms but little information on acceptable size of tablets/capsules is available. Formulations providing dose flexibility, such as liquids and multiparticulates (e.g. sprinkles, minitablets, pellets, granules) can be used across different age groups of children. Opening the capsules and mixing with food/beverage facilitate the administration to young children. However, this process should not cause any inconvenience for the child and parent/caregiver. It must be ensured the bioavailability of the formulation would not be affected and handling would not cause any harm to parent/caregiver (e.g. exposure to irritating or cytotoxic drugs must be avoided). Minitablet is suitable for young children. Orodispersible formulations (e.g. orodispersible films and tablets) are encouraging due to their ease of administration, lack of necessity to use water and thereby, adaptability to different settings such as developing countries depending on the feasibility of pharmaceutical manufacturing. Liquid formulations would be alternative if the medication can be administered at an appropriate volume (< 5 ml for children under 5 years and < 10 ml for children of 5 years and older). Furthermore, formulation should taste pleasant to avoid noncompliance.

The usability of the device by the paediatric patient is essential. For instance, inhaling devices should not be too complicated for the pre-school children or children with lack of coordination. Educational training programmes would be helpful to increase adherence to inhalers. Children should benefit from advances in drug delivery. Innovative formulations such as sipping straw (Clarosip® Straw) and dispensing spoon (comprising preloaded drug that on exposure to water turns into a pudding like texture) (Parvulet®) are promising approaches facilitating the administration of dose in an appealing way to paediatric patient. Nevertheless, still few innovative formulations are for prescription drugs on the market. The main limitation of available innovative formulations is the delivery of a single dose. For older children who are able to swallow solid oral dosage forms, multiple dose delivery approaches exist. For example, the rectangular tablet with multiple fraction bars and deep score lines on both sides has been a smart design allowing flexible drug dosing in function of the body weight of paediatric HIV patients. Modified release formulations would be an easy option to omit administration of doses during school hours.

The place and pattern of treatment should be taken into account in paediatric formulation development. The administration setting affects the selection of dosage form. Application of different formulations would be feasible in hospital, while it is not in community. The practicality of using different drug delivery systems in different clinical settings, particularly in paediatric intensive care setting should be considered. The condition (acute/chronic) and severity of the disease plays a role on the selection of appropriate dosage form. During an acute illness, the ease of application becomes an critical parameter. Because the child is more fractious, uncooperative and the medication is relatively unfamiliar each time it is used. Formulation used for a chronic condition may cause repeated cumulative exposure to excipients. Therefore, the acceptable daily intake and safety limits of excipients for children must be checked.

Acceptability on entire age group should be considered. In the matrix given in "Reflection paper on formulations of choice for the paediatric patient "(CHMP, 2006), 'children' have been further divided into pre-school children (2-5 years) and school children (6-11 years) because of the significant changes in the ability to handle some dosage forms between 2-12 years of age. The pre-school period is particularly challenging: neither passive nor active compliance, can be expected. Patient should be kept at the forefront of any evaluation. Conducting research directly with children across various defined age subsets is necessary to gain insight, in particular, to understand acceptability and applicability of different dosage forms.

Palatability is critical to compliance. Many drugs taste bitter or irritate the oral cavity. Undesirable taste and aftertaste have to be improved to provide acceptability of the formulation . Taste masking may rely on sweeteners but often requires multiple approaches. The taste has been started to be seen as a major component of pharmaceutical quality aspects. Additionally, texture (grittiness), smell and ease of swallowing need to be evaluated. Different types of flavours may be more acceptable in one region than another, due to cultural differences.

Topical administration of drugs is also feasible. However, it should be considered that young children have a larger surface area to weight ratio than adults which may result in profound systemic effects.

As alternative promising drug delivery systems, transdermal formulations improve patient acceptability, ideally suit to needlephobes, provide controlled delivery to relevant skin layers and systemic circulation, reduce dose requirement and bring advantage of ease of application and parent drug management. Needle-free drug delivery systems (e.g. microneedles) can mitigate against the pain that can be associated with the parenteral route of administration.

4.2.4 Adolescents

Adolescence period is subject to physical and cognitive changes. The effect of pubertal growth spurt and action of hormones are needed to be to considered during formulation development for adolescents. The pattern of growth should not be altered by the medicinal products.

Adolescents constitute a challenging age subset, especially in terms of noncompliance, due to being in a period of psychological and social transition between childhood and adulthood. Although the ability to cope with dosage form increases, adolescents may reject to take the medication under the effect of peer pressure, emotional change or as they find the medication too childish. As adolescents are responsible of taking their own medication, acceptability is vital for their coordination and compliance. The matrix given in "Reflection paper on formulations of choice for the paediatric patient" (CHMP, 2006) indicated adolescents` dosage form of choice is mainly peroral and topical/transdermal formulations; rectal formulations are accepted under reserve. However, this statement does not reflect an evidence-based finding. Given the limited experience with acceptability of different dosage forms, further comprehensive research should be performed.

4.3 Conclusive remarks

Four years have elapsed since the entry into force of the Paediatric Regulation which has stimulated the conduct of high-quality research with the increase in paediatric trials.

According to "Report to the European Commission on companies and products that have benefited from any of the rewards and incentives in the Paediatric Regulation and on the companies that have failed to comply with any of the obligations in this Regulation, covering the year 2010" (EMA, 2011), the number of companies which have benefited from an extension of the Supplementary Protection Certificate in some Member States is increasing but this number may still be considered limited. The implementation of the paediatric regulation resulted in a large number of PIPs including measures for the development of age-appropriate formulations and progress should continue incrementally. Developing high quality appropriate formulations for a sensitive population with individual needs is a challenging task. Therefore, engagement between key stakeholder including companies, regulatory authorities, health professionals and society is needed. Concerted effort will pave the way for a PIP with a strongly written quality section and thereby, increase the commercial viability of medicines intended for children. Paediatric formulations should not be as an add-on to adult formulations; conversely, it should be seen as an integrated part of the overall drug development programme, unless a waiver is appropriate. The "Guideline on Pharmaceutical Development of Medicines for Paediatric Use" (CHMP, 2011) will provide additional tools for the rationale paediatric formulation development and highlight the main aspects for justification of strategy. PIP should ensure that every study contributes to the paediatric formulation development pathway. Often, problems related to formulation are identified late during the paediatric clinical trial and led to delays and complications in the conduct of the trial. For early scrutiny of pharmaceutical quality-related issues, timely submission and subsequent evaluation of potential formulations would facilitate to investigate the intended marketed product in paediatric clinical trials and would let PIP amendments related to formulation aspects. Research into novel dosage forms and administration devices along with effective utilization of existing drug delivery technologies will be pivotal to develop acceptable, safe, feasible and age-appropriate formulations for children.

5. Non clinical: The need of studies on juvenile animals in order to evaluate the toxic potential before any administration to paediatric population

The 20th century history of drug research and regulation shows that dramatic tragedies such as sulfanilamide (Wax, 1995), thalidomide (Choonara & Rieder, 2002) and TGN 1412 (Suntharalingam et al., 2006) cases facilitated the passage of stronger laws. The elixir of sulfanilamide case can be considered one of the earliest example of disaster in paediatric medicine therapy. After its safety use in adults in fact, an elixir of sulfanilamide was developed to enable administration to children, using diethylene glycol as the solvent because it was odourless, sweet, and syrupy, but without investigate its toxicological properties. Since diethylene glycol is highly toxic causing gastrointestinal, metabolic, renal and hepatic failure, more than one hundred Americans, many of them children, died and this incident was the main cause of passage, in 1938, of the USA federal Food, Drug, and Cosmetic Act.

Other incidents, since then, have induced serious adverse reactions in children (Choonara & Rieder, 2002) but in parallel multiplied efforts have been made from Regulatory agencies in the world, with the commitment of many organizations, including academy and paediatrics, to ensure that paediatric drug therapies are developed with the same level of scientific and clinical rigor as adult therapeutic agents. These efforts culminated in USA and Europe (EU)

paediatric initiatives. In Europe, the Paediatric Regulation was preceded by the ICH Guidance for conducting studies in the paediatric subjects (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use [ICH], 2000, thereafter ICH, 2000; CPMP, 2001) and it was also accompanied by guidance documents on various themes in the same field, including non clinical investigation of products in the neonatal population. In November 2008 a specific Nonclinical Working Group was created by the PDCO in order to assure consistency of paediatric investigational plan (PIP) evaluation (Silva-Lima et al., 2010). In the following paragraphs it is specifically discussed the role of juvenile animal studies in developing drugs for paediatric use.

5.1 Regulatory situation

5.1.1 Legal rules

The rules governing the clinical development of drugs and their placing on the market are described in the EU Regulations and Directives. The first are the most important legal acts after the treaties. They create the same rules for all citizens of EU Member States (MS), are uniformly valid in all MS of the Community, have direct effect, and should not be transposed into national law. Taking into account the lack of adapted children medicine in Europe, the European parliament put in place the Paediatric Regulation bringing profound changes as described above in the dedicated section of this chapter.

The Directives are binding legislative acts, directed to MS. They provide the criteria and the principles according to which individual MS govern, by means their own acts, matters for which harmonization/convergence of disciplines, in different EU countries, are necessary. Directives always needed a period in order to be transposed into national law. To simplify and harmonize the rules and administrative provisions governing clinical trials in Europe, Directive 2001/20/EC (European Parliament and the Council of the European Union, 2001), also known as Clinical Trials Directive, was enacted; its scope is wide and includes every clinical trial with every medicine on any human subject within any of the 27 MS including paediatric investigation. The Clinical Trials Directive is concretized further by Directive 2005/28/EC (Commission of the European Communities, 2005).

The Directive requires that researches/investigators and sponsors ensure ethical review, the authorization by competent national authorities before enrolling participants, the drug manufacture in compliance with Good Manufacturing Practice (GMP), and rigorous observance of the Good Clinical Practice (GCP) principles during the conduction of the trial. Furthermore, the Directive requires that any changes related to the execution of the clinical study, and its final results, be reported to the supervising authorities. The Directive recognizes that non-commercial clinical trials conducted by researchers (without the participation of the pharmaceutical industry) may be of great benefit to patients. However, after May 2004 no intervention research may be initiated without a "sponsor," defined in the Directive as "an individual, company, institution or organization which takes responsibility for the initiation, management and/or financing of a clinical trial." This means that investigators who wish to perform clinical trials without commercial backing must themselves become the study sponsors. The administrative responsibilities will be exactly the same as those of the pharmaceutical industry for commercial trials. There has been much commentary on this issue (Editorial, 2003; Meunier & Lacombe, 2003; Clumeck & Katlama, 2004). The paper of Welzing et al., 2007 discusses the consequences and implications of the

2001/20/EC Directive for independent ("no profit") investigators involved in paediatric clinical trials in relation to the first paediatric investigator-initiated trial at the University Hospital of Cologne. The authors of the paper agree that the new rule can improve quality of clinical trials, but underline that clinicians and academic researchers cannot meet the new requirements and obligations without additional financial support. Their conclusion focused to the need to develop new concepts for funding to ensure future paediatric independent investigations, for example through specific grants from the EU Community or Member States.

5.1.2 Guidelines

In addition to legally binding legislation regulatory guidelines, concerning scientific and technical requirements for develop and register medicine for human use, were released by the regulatory authority (EMA, dedicate section of the site), after discussion with industry and experts. In the contest of clinical trial authorization and medicinal product registration system, the regulatory guideline cannot be substituted by any other guideline issued by scientific organizations and societies.

Two main regulatory EU non clinical documents considers the aim of non-clinical studies to support the development of medicinal products to be used in paediatric population:

1. Non-Clinical Safety Studies For The Conduct Of Human Clinical Trials For Pharmaceuticals (CPMP, 2009).
2. Non-clinical Testing in Juvenile Animals on Human Pharmaceuticals for Pediatric Indications (CHMP, 2008).

A Food and Drug Administration (FDA) guidance on non clinical issue of paediatric medicine is also available (FDA, 2006), recognizing the importance of animal data in predicting the potential drug toxicity, and those obtained in juvenile animal studies to provide information that might not be derived from standard adult toxicology studies or from safety data from adult humans.

The original "Non-Clinical Safety Studies For The Conduct Of Human Clinical Trials For Pharmaceuticals" guideline (CPMP, 2009) was approved in 1995, and revised on December 2009. Several new sections have been introduced, one of them was dedicated to clinical trials in paediatric populations: "when paediatric patients are included in clinical trials, safety data from previous adult human experience would usually represent the most relevant information and should generally be available before initiation of paediatric clinical trials. The appropriateness and extent of adult human data should be determined on a case-by-case basis. Extensive adult experience might not be available before paediatric exposures (e.g., for paediatric-specific indications). The conduct of any juvenile animal toxicity studies should be considered only when previous animal data and human safety data, including effects from other drugs of the same pharmacological class, are judged to be insufficient to support paediatric studies".

The CHMP guideline (CHMP, 2008) provides advice on investigation of findings that cannot be fully assessed in paediatric clinical trials, with reference to specific concerns, possible aggravation of expected findings and to establish safety factors. It highlights that drugs can have different safety profiles in adults compared to paediatrics and emphasizes that studies in juvenile animals should only be performed after careful consideration of available data, age of the intended paediatric population, duration of treatment and "cause for concern" identified. Additional data to develop a medicine for children can be necessary because

additional or different risk due to immature or growing organism can be possible and because children may be included earlier in drug development, before more extensive adult data available.

5.2 Difference between adults and children

We actually know that adverse events in children may not be always predicted from adult data, because a child is not a small adult and paediatric population has immature organs rapidly developing. The use of an approved adult drug in children only considering the pharmacokinetics (PK) may be problematic, because there are many relevant PK differences age-related. Amount of metabolism and kinetics detected in children (particularly neonates) can vary in significant way to those observed in adults, these differences can cause over or under exposure in paediatric population. The CHMP guideline (CHMP, 2008) provides the list of the major systems developing in age dependent manner and for which the functional differences existing between human neonates/infants and adults should be take into account. The guideline also note that the age ranges only apply to general development and it is not applicable to all endpoints related to that organ system. This should be taken into account in the design the paediatric drug development program and the individual studies (CHMP, 2008):

- Nervous system: Development up to adulthood
- Reproductive system: Development up to adulthood
- Pulmonary system: Development up to two years old
- Immune system: Development up to 12 years old
- Renal system: Development up to one year of age
- Skeletal system: Development up to adulthood
- Organs and/or systems involved in absorption and metabolism of drugs. Development of biotransformation enzymes up to adolescence

The sensitivity and the cross-species comparative postnatal development are among the major points to take into consideration using juvenile animals. There are in fact, evidence (Baldrick, 2004) that newborn or infant animals can be more (e.g. tetracycline, sodium salicylate, morphine, chloramphenicol) or less sensitive (eg metrazol, codeine, ethanol,) to drugs than adults. It is well known that potential differences in sensitivity of juvenile animals to drug toxicity can be due to the different stage of development that varies among species, strains, organ and in some case also in subcomponent of the same organs. A range of publications have summarized functional differences in early postnatal life among species used in toxicology testing. A number of these papers have arisen out of work performed by the Developmental and Reproductive Toxicology Technical Committee of the International Life Sciences Institute (ILSI) and Health and Environmental Sciences Institute (HESI). Organ systems examined included: central nervous, reproductive, pulmonary, renal, skeletal, cardiovascular, and immune systems (Baldrick, 2004). A series of articles reviewing the current knowledge on comparative postnatal function and physiologic development are discussed (Cappon et al., 2009) observing that in general the rat can be considered the appropriate specie, because most of the major organ systems maturation that occurs postnatally in humans also occurs during the postnatal period in rat. When the rat is not the relevant species mouse, minipig, dog and primate should be considered, comparative age categories are available

for rats, minipigs, dogs, non human primates and humans based on CNS and reproductive development (Gad, 2001; Baldrik, 2004; Beck et al., 2006). However the information on developmental stage across various species is not sufficient without knowledge of functional state at these age (Baldrick, 2004).
The comparison between animals and humans generally refers to the age categories described in the ICH guideline (ICH, 2000; CPMP, 2001):

- Preterm newborn infants.
- Term newborn infants (0 to 27 days).
- Infants and toddlers (28 days to 23 months).
- Children (2 to 11 years).
- Adolescents (12 to 16-18 years). (dependent on region)

The guideline recognizes that any categorization of the paediatric population is to some extent arbitrary, but provides a basis for thinking about non clinical and clinical study design in paediatric medicine development, and neonatal population is still an issue.
An overview of comparative development of main organ systems in man and different animal species, as a basis for species selection and protocol design was one of the topic of the workshop on juvenile animal testing organized by the EMA in July 2009, involving non-clinical assessors and experts of the Non-clinical Working Group of the PDCO and of the Safety Working Party (SWP) of the Committee for Human Medicinal Product. The presentations and the examples discussed during the workshop concerned brain, reproductive system, gastrointestinal system, metabolism, skeletal system, lungs, kidneys and immune system, pointing out that the maturation of different organs in various species is a key factor to be taken into account when designing juvenile animal studies in relation to early exposure of children to medicines (Silva-Lima et al., 2010).

5.3 Non clinical aspects

Non clinical safety assessment of new medicinal product intended to be administer in adult humans is essential to support clinical trials and eventual drug product registration. Once the company has identified and manufactured consistently a promising drug candidate the next step in development is to provide evidence to the regulatory authority that it is safe for the administration to humans. This evidence must be based on a well designed programme of appropriate non clinical and clinical studies, as those illustrated in Table 1. Non clinical drug development is a complex, regulatory-driven process designed primarily to assess the safety and viability of new molecular entities. Non clinical testing is conducted throughout all phases of drug development and, when well done, can maximize the chances of success in the clinical phases. Although the terms preclinical and nonclinical are used interchangeably it should take in mind that only studies necessary before the first administration in humans [First in Man (FIM) clinical trial], can be considered preclinical unlike other studies conducted during and in parallel to Phase II and III clinical trials (see Table 1). Considering their scientific objectives, non-clinical studies should be designed to provide information regarding the primary and secondary pharmacodynamic actions, safety pharmacology and toxicology of a compound. Regulatory requirements for safety and toxicity studies are more stringent than for pharmacology ones and they must be completed in compliance with Good Laboratory Practices (GLP) following the guidelines operating at the time.

Clinical Phase	Pre-clinical/Non clinical data (CPMP, 2009)
Phase I clinical trials conventionally examine the tolerability and the pharmacokinetics of new drugs; they are often conducted in healthy subjects, but may involve patients in studies with interventions that are known to be toxic. It should be noted that Phase I clinical trials now increasingly include persons with specific diseases persons for whom all conventional therapies have failed (e.g., terminal cancer or AIDS). Such studies may be designated as Phase I clinical trials where, in fact, they properly should be designated as mixed Phase I/II or pure Phase II clinical trials	• Pharmacodynamic • Safety pharmacology (CNS; CV; Respiratory) • Toxicology studies (Single dose toxicity, Repeated dose toxicity) • Initial genotoxicity • Initial reproductive toxicology • Local tolerance • Toxic\kinetic and Pharmacokinetic Studies
Phase II clinical trials primarily examine the short-term pharmacological toxicities and, to a lesser extent, the efficacy of new drugs; they are conducted in populations with specific diseases	• Completed battery of Genotoxicity • Completed battery Reproductive toxicity (male/female) • Extended repeated dose toxicity with toxicokinetic support • Extended pharmacokinetic studies
Phase III clinical trials primarily examine the pharmacological efficacy and, to a lesser extent, the short-term toxicities of new drugs. Phase III and IV clinical trials are designed to increase the survival or the quality of life of subjects suffering from a specific disease or condition	• Chronically used drugs: • Chronic toxicity (rodent; non-rodent) • Carcinogenicity • Supplemental studies (special safety concerns, as alerted)
Phase IV clinical trials, also known as post-marketing surveillance studies, primarily examine the long-term efficacy and toxicity of already-marketed drugs	

Table 1. The clinical phase and the supporting preclinical/non clinical data

5.3.1 Information available from adult human safety data

Safety evaluation programs of a new chemical product should normally include two relevant species rodent and non rodent, however for biotechnology-derived pharmaceuticals in certain justified cases one relevant species may be sufficient (e.g., when only one relevant species can be identified or where the biological activity of the biopharmaceutical is well understood). Upon completion of non clinical studies necessary to perform clinical trials and put a new drug on the market, the following information are available.

- Rodents and non rodents No Observed Effect Level (NOEL) or No Observed Adverse Effect Level (NOAEL) in mg/kg/day obtained after repeated dose administration in rats aged from 1 to 24 months (if necessary), and in dogs aged >5-6 months of age.
- Associated drug blood levels and exposition (Cmax and AUC).
- Pre-weaning exposure is only in utero or via the milk in pre- and postnatal reproduction toxicity studies in rats, since toxicokinetic data is not normally available in pre-weaning animals.

- Genotoxicity and carcinogenicity (if it is the case) potential.

In Europe these studies are performed taking into account the European Directive on the animal welfare. In November 2010, Directive 2010/63/EU (European Parliament and the Council of the European Union, 2010), this Directive revises Directive 86/609/EEC on the same subject (Council of the European Communities, 1986). Member States have until November 2012 to transpose the Directive into their respective national legislation and the new Directive will take full effect on 1 January 2013. Moreover to take into account the 3R principles (Reduce, Refinement and Replace) they are designed so that the maximum information is obtained from the smallest number of animals. While animal tests cannot predict all of the reactions a human, at the end of regulatory assessment of all these data available, there are sufficient knowledge of the intrinsic toxicological characteristics of a given substance and their target organs of toxicity.

Until the last few years the majority of studies on juvenile animals are essentially the repetition of those conducted in the adult, and performed mainly in the environmental regulatory setting, particularly to investigated neurotoxicity (Atchison et al., 1982; Benke & Murphy, 1975; Brodeur & Dubois, 1963; Cappon et al., 1997; Rice, 1988; Rigdon et al., 1989), or to support investigations certain drug classes used in a paediatric population, e.g., antibiotic, anti-emetic, and anti-asthma drugs (Baldrick, 2004).

Since the entry into force of the paediatric European and USA legislations, the interest of the regulators and pharmaceutical industry in non clinical juvenile toxicity studies has taken on characteristics of priority as also documented by recent publications on the subject (Baldrick, 2004; Cappon et al., 2009; Bailey & Mariën, 2009; Silva-Lima et al., 2010). To avoid production of unnecessary repetitive not interpretable data and to realize robust juvenile animal studies when they necessary, represent the main objectives requiring higher consideration than in the past.

Industry conducted recently a survey to establish whether any findings, other than changed sensitivity due to pharmaco/toxicokinetics or metabolism differences, have been seen in the juvenile animal studies currently performed after specific regulatory requirements, and whether the results could not have been predicted from the adult toxicology or found in routine adult animal studies. Ten pharmaceutical companies contribute the survey and shared their experiences with over 39 juvenile animal studies (29 in rats, nine in dogs and one single dermal study in minipigs). "Novel toxicity was only observed in four studies out of the 39 compiled, these comprised a single CNS drug in the rat, two anti-infective in the rat and one other anti-infective in the dog. In only one of these cases was it felt that the results observed were predictable from the pharmacology, and none of the four were predictable from the adult data. In all cases however, the novel toxicity was observed using routine toxicological techniques and not as a result of any sophisticated or complex design. In one case this was a combination of animal observations and histopathology and in the remaining three cases the toxicity was observed at histopathology" (Bailey & Mariën, 2009).

Taking into consideration these data it might seem that we are not on the right track since juvenile animals are required by default rather than following a scientific rationale, but surely no definitive conclusions can be drawn based on so small database, and we can agree with the conclusion of Silva-Lima et al., 2010: "It is expected that with the increasing experience gained in this area by applicants and regulators, the criteria for requiring these studies will be further refined. An increased understanding of their ability to identify differences in activity between mature and immature systems, and of their predictive value for developing human organs will be gained. Research, follow-up discussions, and

experience sharing between all stakeholders will be continued and are encouraged in the EU regulatory framework". The increased database of toxicological studies in young/very young animals will contribute to better understand the level of predictability of juvenile animals for children. Currently there are examples of products, such as verapamil, phenobarbital and theophylline for which juvenile animal data help to predict age related toxicity in children (Baldrick, 2004).

In general, according both to CHMP (CHMP, 2008) and the CPMP guidelines (CPMP, 2009), the paediatric patients can be included in clinical trials when adequate pharmacokinetic, pharmacodynamic, clinical efficacy and safety data are available in human adults, this also implies, in most cases, the availability of a standard non-clinical data package. All available adult non clinical and clinical data must be reviewed and considered and their appropriateness and extent data should be determined on a case-by-case basis. These guidelines suggest that: results from repeated dose toxicity studies of appropriate duration (see also table 1 of CPMP, 2009), the core safety pharmacology package, the standard battery of genotoxicity tests, and relevant parts of the reproductive toxicity test program and also juvenile animal toxicity studies if necessary, should be available and reviewed before starting trials in paediatric population. Pharmaco- and toxico-kinetic assessment may be exceptionally useful to characterize juvenile toxicity studies since paediatric populations metabolize and respond to many substances in a very different manner than adults with mature. The data package should be justified, based on the characteristics of the clinical study and the intended paediatric population including age group(s), and the need for juvenile animal studies should be particularly taken into consideration in case of investigation of neonates (0-3 months) and when extensive adult experience might not be available before paediatric exposures (e.g., for paediatric-specific indications).

If previous animal work and clinical safety data are considered to be insufficient for a thorough safety assessment in the intended paediatric age group of interest, the juvenile studies should be run prior to the conduction of paediatric clinical trials. The need for pre-clinical juvenile toxicity testing may differ depending on the intended duration of exposure in the paediatric population. Consideration should also be given to whether pre-clinical and/or clinical studies have identified target organs vulnerable to toxicity, and whether these organ systems will be undergoing rapid development while patients are being exposed to a given drug. The children and adolescents could have higher recuperative capacities than adults, so it important to consider in the study design a period of recovery treatment-free assess the potential and timing of reversibility of any adverse effects seen. Assuming the need for juvenile study, the key elements described in the EU guideline (study design, age and duration, route of administration/doses, selection of species, pharmaco-kinetics/toxicokinetics) should be taken into account.

The analysis of published PIP decisions covering a period from August 2007 up to March 2009, revealed that, in most cases, the preferred species for juvenile animal studies was the rat; in a minority of cases, two species were required, the age animal used at the start of the study reflect the age of target paediatric population and generally, the principles stated in the guideline were followed (Silva-Lima et al., 2010). The same paper illustrates 4 case studies discussed at the EMA workshop related to a new molecular entity with a well known mode of action, an oncological new product intended to be administered to patients of all ages, a growth factor as a replacement therapy in premature newborns born after 28 weeks of gestation and a monoclonal antibody for a chronic non tumoral pathology emerging around 5 years of age.

5.3.2 Targeted approach

A targeted approach for juvenile animal study design may be used to specifically address identified toxicity concerns, and " primary factors to consider when designing a targeted juvenile animal study are: (1) ensure that the organ system of concern is undergoing similar developmental processes during the postnatal period as in the intended paediatric population; (2) define the age of exposure in the experimental species to ensure that the organ systems of concern are at the same stage of development in the animal species as in the intended human paediatric population; and (3) ensure that the appropriate endpoints are included to enable an in-depth investigation of the organ system of concern" (Cappon et al., 2009) The same authors illustrate two examples, based on real paediatric drug development programs and accepted by regulatory agencies to support those products, of targeted study, one for central nervous system and reproductive development and the other focused on liver and reproductive development. This approach support the chance to use specific case by case study design to perform suitable, interpretable and not repetitive non clinical studies in juvenile animals to support risk assessment for paediatric population.

5.3.3 Pre- and post-natal reproduction studies

The CHMP guideline the need for nonclinical testing in juvenile animals on human pharmaceuticals for paediatric indications (CHMP, 2008) supports the use of the modified pre- and postnatal development studies. It states in fact that before performing a juvenile animal toxicity study, it should be considered whether a developmental toxicity issue could be addressed in a modified pre- and postnatal development study in rats. Key factors that need to be examined include, but are not restricted to

- the amount of the active substance and/or relevant metabolites excreted via the milk;
- the resulting plasma exposure of the pups;
- which organs under development that will be exposed during the pre-weaning period;
- physical development and histopathology investigations.

The number of animals should be sufficient to draw scientifically sound conclusions, but a higher number of animals than necessary should be avoided. When a pre- and postnatal study is also being used to address a specific aspect of juvenile toxicity, such a study should be extended to include appropriate developmental endpoints: if specific developmental endpoints cannot be assessed within the context of pre- and postnatal studies, additional juvenile animal studies will be required. In the combined pre- and postnatal development toxicity study design (De Schaepdrijver & Bailey, 2009, Cappon et al., 2009) the maternal animal is dosed from implantation through post natal dose day 5. Dosing is then suspended for maternal animals and the offsprings are directly dosed until maturity (about 9 weeks). A clear advantage of this study is the reduced number of animals to be used, but it is not always suitable to investigated potentially additional end points related to target organs of concern, and excessive toxicity in the directly dosed pups can be observed (Cappon et., 2009, De Schaepdrijver et al., 2008, De Schaepdrijver & Bailey, 2009).

The guideline CHMP/SWP/169215/2005 is not rigid about the timing for performing juvenile animal studies, if these studies are considered necessary, they should preferably be available before the starting of clinical studies in paediatric populations, and pharmacokinetic data from humans and animals (including juvenile animals if available) should also be evaluated before the proposed paediatric clinical trial(s). More flexibility can

be considered for paediatric short-term investigations (PK or taste study) in which few doses are administered.

5.4 Conclusions

The needed and the efforts to develop and register medicine for paediatric population with the same level of scientific and clinical rigor as adult therapeutic products have advanced over the years through the actual legislation initiatives involving United States of America/FDA and EU Community and EMA. In the last five years many experience have been gained both by regulators and companies to assess the overall impact of non clinical juvenile studies on paediatric human pharmaceuticals risk assessment. The application of recent legislations have resulted in a significant increase in the number of juvenile animal studies request, in same case they were a simply repetition of the adult design in younger animals, but there are cases in which designs are being modified, and targeted designs were used, as we continue to gain knowledge from the submitted studies. Particularly important is to carefully consider the suitable age and the corresponding developmental stage of the children involved in the clinical trial in comparison to the development of the animals involved in the non clinical test if necessary. Children in the different interval of age considered by the ICH guideline (ICH, 2000; CPMP, 2001) can answer in different way to the same product, and neonatal population is still the main issue. As for standard adult non clinical study the benefit of harmonized design in juvenile studies should be recognized, harmonized study designs, enabling information-sharing across studies through standardized approaches to data collection and interpretation, are needed to optimize the information from these juvenile studies

At present there is general agreement that juvenile animal studies can be useful for safety determinations, especially when a problem is suspected, they are not prohibitively challenging to conduct, available data does not indicate that juvenile animal studies need to be conducted routinely to support clinical trials in paediatric patients, but might be needed under some circumstances and they should be designed on a case-by-case basis. Currently the data base of juvenile animals to investigate paediatric pharmaceutical product is still limited, however, and this conclusion might change as more studies are submitted and regulators and industry gain more experience with this issue.

6. Clinical: The need for adequate clinical trials methodologies

The benchmark for the assessment of the effect of any therapeutic intervention is the randomised clinical trial (RCT), its main assets being randomisation, to avoid bias in the allocation of subjects to treatments, blindness, to avoid bias in the evaluation of compared treatments, and the *a priori* choice of acceptable error margins, specifically type I and type II errors (Baiardi et al., 2011). However, the conduct of clinical trials in children causes several methodological, ethical and economic issues limiting paediatric research: small sample sizes, children exposure to the potential risks of a trial, and restricted paediatric medicines market are just the main examples.

Notwithstanding these multiple difficulties, understanding the effects of medicinal products in paediatric patients is an important goal that is shared by companies, regulatory authorities, health professionals and society as a whole (ICH, 2000; CPMP, 2001), and should be achieved without compromising the well-being of paediatric patients participating in clinical studies.

Traditional drug development approaches do not satisfy the requirements of research in the paediatric population. New approaches should be used to address the various practical, scientific and ethical issues that arise in paediatric research, as stated by a number of worldwide scientific and regulatory initiatives with particular reference, in Europe, to the Paediatric Regulation (European Parliament and the Council of the European Union, 2006).
Methods for extrapolation of efficacy and safety data from adults to children, modelling and simulation, adoption of innovative study design and statistics have demonstrated to be the more suitable to produce real advancement in this field.

6.1 Status of the knowledge (What has been done)

6.1.1 Extrapolation

The difficulties in performing paediatric trials obliges physicians to extrapolate data from the adult to the paediatric population, the first approach usually employed to avoid useless studies in children, provided its limitations are properly defined.
Direct extrapolation can be made on the basis of previous clinical experience and scientific knowledge that allow to assume that the disease is the same in children and in adults, and that children have reached full maturity in those pharmacokinetic mechanisms involved in drug disposition and in the involved pharmacodynamic systems. In such cases, the relationship between the two populations is linear and allometric methods based on body weight or body surface area can be used to calculate the proportional dose in children (Bellanti & Della Pasqua, 2011).
To be able to use this type of approach, paediatric PK/PD data should be known and they should demonstrate a similar exposure/concentration curve as in the adult population. However, particularly in neonates and infants, the use of the allometric approach may fail to identify the appropriate dosing range (Bouzom & Walther, 2008; Johnson, 2005), thus reducing the access to a feasible extrapolation exercise.
Much more has to be done to facilitate the extrapolation approach also in younger children, and in particular to collect data on similar disease course and outcome, and similar primary endpoints for efficacy in adults and children. A decision-tree, similar to the one developed by the US Food and Drug Administration (Fig. 1), may be of help and is currently under evaluation by the EMA Paediatric Committee, that has also created a specific 'Extrapolation Group'.

6.1.2 Modelling and simulation

As extrapolation can have limited applicability in children, modelling and simulation (M&S) may play a pivotal role in reducing the needs of specific additional paediatric trials.
Clinical trial simulation (CTS) can be used to assess the impact of a range of design characteristics on the statistical power of a clinical trial, and thus to detect a treatment effect prior to exposing patients to an experimental drug (Bellanti & Della Pasqua, 2011). CTS investigates "what if" scenarios across a different range of conditions or design features (e.g. population size, stratification levels, dose range, sampling scheme, and even different endpoints) relying on the availability of accurate model parameters and corresponding distributions (Bellanti & Della Pasqua, 2011). The possibility to predict 'trial performance', and thus identify potential limitations in study and protocol design prior to its implementation, is one of the main advantages of such a virtual or statistical experiment (Bellanti & Della Pasqua, 2011; Manolis & Pons, 2009; Laer et al., 2009; Girard, 2005; Onar et al., 2009).

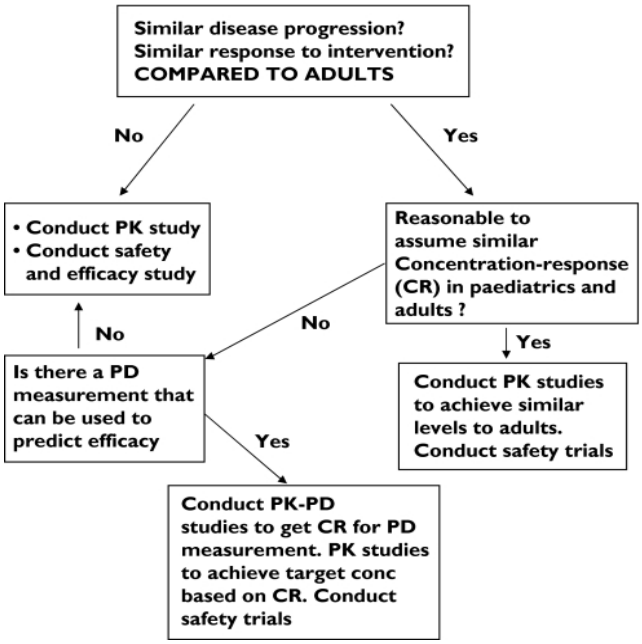

Fig. 1. Paediatric drug development decision tree for types of PK-PD studies required in children (Johnson, 2005)

CTS generally employs two types of models: first a drug–action (PKPD) model, which comprises pharmacokinetic and pharmacodynamic factors, and then a trial execution model, that simulates other important aspects of the trial (e.g. dropout, compliance and protocol deviations) (Bellanti & Della Pasqua, 2011; Santen et al., 2009).

6.1.2.1 PKPD model

PKPD models incorporate physiological differences between adults and children and between different age groups to evaluate variation in pharmacokinetics. This approach may allow conversion of the exploratory nature of first-in children studies into a confirmatory step (Johnson, 2005).

When applying bridging techniques, however, it is necessary to clearly understand the disease in question and therefore, both disease and disease progression models must be taken into account during the comparison of drug response and kinetics in adults and children (Manolis & Pons, 2009).

Disease models can also be applied to simulate treatment response; in fact, when disease models are combined with drug models, it is possible to explore the implications of different algorithms for dose adjustment (Manolis & Pons, 2009). It must be highlighted, however, that the application of sophisticated statistical methods, not achievable by standard linear regression techniques, are necessary to use disease models in the evaluation of drug–disease interactions and of the role of covariates in pharmacokinetics, pharmacodynamics and treatment outcome demands (Bellanti & Della Pasqua, 2011).

Bayesian statistical concepts are usually the basis on which these methods rely and they also include parameterisation based on hierarchical, non-linear mixed effects models that are

also known as population approaches, particularly suitable when information on individual subjects is limited (a common situation in pharmacokinetic and pharmacodynamic studies in children) as they use the population rather than the individual as the object of the investigation.

Population pharmacokinetic (pop PK) and population pharmacokinetic-pharmacodynamic (pop PKPD) models rely conceptually on pooled data across treatment cohorts or even across different studies (Anderson et al., 2006) and include the representation of three main components:

- a structural model that describes pharmacokinetics or pharmacodynamic characteristics;
- a statistical model describing between-subject variability; and
- an error model that accounts for the residual variability (Bellanti & Della Pasqua, 2011).

There are different advantages in using these types of approaches: it is possible to assess different clinical scenarios without exposing children to any risk, to explore drug, disease or covariate effects in a larger number of virtual patients compared to those enrolled in a real trial, and to assess the clinical relevance of covariates to drug exposure and to evaluate their effect on the treatment response (Anderson et al., 2006; Chatelut, 2008; Yim et al. 2005; Knibbe et al., 2002).

Moreover, the K-PD models, a specific group of nonlinear mixed effect approaches introduced into paediatric research, have been developed to describe exposure–effect relationships in the absence of drug concentration measurements (Manolis & Pons, 2009; Tod, 2008). These models are very useful if the rate-limiting step in drug disposition is the drug elimination from the biophase (Bellanti & Della Pasqua, 2011). On the other hand, data extrapolation across different scenarios (e.g. different doses, or populations) are not possible since no observations are available (Manolis & Pons, 2009).

6.1.2.2 Trial execution model

Trial execution models simulate some important aspects of the trial, such as dropout, compliance and protocol deviations (Gobburu & Lesko, 2009) and it is therefore possible to determine all possible outcomes under candidate trial designs, allowing such trial designs to be compared in a strictly quantitative manner.

6.1.3 Innovative designs

Many innovative designs have been described up to now, each design having intrinsic features that meet the requirements of the paediatric population even if there is no unique rule of thumb for choosing a specific approach (Baiardi et al., 2011).

6.1.3.1 Sequential design

This study design uses an a priori non fixed sample size and generally needs fewer patients compared to a fixed sample size design to reach a conclusion, thus guaranteeing some ethical advantages. It also ensures the possibility to stop the trial at any time during its course as soon as the scientific evidence of a superiority of one treatment against the other is proven (van der Lee et al., 2010). This approach has been developed in the 1960s, but not widely used in clinical trials: a review carried out by Goodman in 2009 showed that from 1963 to 2005, only 24 trials have been performed in the neonatal intensive care setting using the sequential design methodology, saving an average of 35% of the enrolled subjects when compared to a fixed sample size approach (Goodman, 2009). It is not usable to evaluate

survival but it could be useful to evaluate short treatments through surrogate endpoints (Baiardi et al., 2011).

6.1.3.2 Adaptive design

This approach allows modifications of the trial (e.g. sample size re-estimation, early stopping and adaptive randomization) to be made after its initiation and without invalidating the validity and integrity of the trial itself. Reports show that it has been used for dose-finding studies, for phase III trials and for phase IV trials in which the method allows the saving of up to a half of the subjects required by the traditional design (Chow & Chang, 2008). Adaptive designs are very attractive due to their flexibility and can be useful especially in early clinical development.

6.1.3.3 Bayesian design

The Bayesian design is the data dependent design *par excellence* (Schoenfeld et al., 2009).
Data from past studies are here used to form an *a priori* probability distribution for treatment effect, and then merged with the data of the current trial with the aim to provide an *a posteriori* distribution on which conclusions may be drawn (Baiardi et al., 2011).
This approach is particularly suitable for the paediatric population, since adult data can be used in designing the paediatric trial by taking advantage of past information for the sample size calculation or by directly including them into the study to generate the distribution (Goodman & Sladky, 2005).

6.1.3.4 Randomized withdrawal design

This type of design offers two main advantages to the paediatric population: first, patients have the opportunity to experience the potential benefits of the active treatment (Della Pasqua et al., 2007), and second, the individual receives the placebo for the minimum time possible. As a result, a better patient accrual may be achieved and moreover, testing the experimental drug against placebo on the responders increases the power of the comparison and requires a smaller sample size to achieve the same power of results (Baiardi et al., 2011).
This design has been recognised as the most appropriate for developing new treatments for juvenile idiopathic arthritis at the Paediatric Rheumatology Expert Meeting held in London in December 2009.

6.1.3.5 Randomized Placebo-Phase Design

The Randomized Placebo-Phase Design (RPPD) approach sets its innovation on the fact that the duration of the placebo trial is the shortest possible (Feldman et al., 2001). In fact, it is assumed that if the trial drug is active, the earlier it begins, the higher the probability to observe a response in short times, and therefore, in this type of study subjects receive placebo at different times. In more details, subjects are randomised to placebo for periods of different duration. At the end of those periods, all subjects receive the active treatment until it is possible to observe a response (Baiardi et al., 2011).
By guaranteeing the presence of a control group according to an intra-patient scheme, the blindness, and the randomisation, the RPPD trial can be classified as a RCT.

6.1.3.6 Three–stage clinical trial design

The methodological approach of the three-stage trial design combines the classical RCT and the randomised withdrawal trial with the aim to obtain the maximum level of information available from each subject.

During the first stage, subjects are randomised according to a traditional RCT. At the end of this first phase, patients responding to placebo or not responding to the trial treatment are then withdrawn from the study, while responders to the active treatment are assigned to the second phase of the trial, and non-responders to placebo are allocated to the third (Baiardi et al., 2011). Second phase subjects are then randomised again, while patients assigned to the third stage enter the following randomised withdrawal scheme: only those who respond to the drug in this phase continue the study and are again randomised to take either placebo or the active treatment (Honkanen et al., 2001).
Examples where the study may be potentially useful are in chronic conditions (for which it is expected to return to initial conditions when the active treatment is suspended), when the therapeutic efficacy in sub-populations has to be determined, in those cases in which efficacy in the general population has already been proved, or at the initial stages of the drug development when it is necessary to find dosages in small patients cohorts (Baiardi et al., 2011; Honkanen et al. 2001).

6.2 Research aim and results (What have we done)

Despite the potential of innovative research methods in collecting data on drug effects in children and/or developing clinical trials, it seems that their benefits as tools in pharmaceutical R&D has remained undervalued and sometimes ignored by key stakeholders (Cella et al., 2010; Abernethy & Burckart,2010). This attitude appears to contradict those ethical and scientific beliefs that emphasize the need for evaluation of the risk–benefit ratio in special populations, such as the paediatric one.
The Task-force in Europe for Drug Development for Young – TEDDY (Ceci et al., 2009) has performed analyses and surveys aimed at collecting data from published literature and registrative documents (i.e. EPARs [1]) to evaluate the status of paediatric clinical trials performed for drugs to be used in children (Baiardi et al, 2009), to explore the current status, limitations and perspectives of pharmacogenomic and pharmacogenetic paediatric clinical research (Krekels et al., 2009), and to at analysing the quality of the clinical trials for rare diseases.
Results showed that in the period 1995-2005, 60 drugs were licensed for use in children under the EMA centralised procedure with a total of 188 paediatric clinical trials included in their MA dossiers, mostly concerning diseases predominantly or exclusively affecting paediatric patients and serious or life-threatening diseases, occurring in both adults and paediatric patients, for which there are currently no or limited therapeutic options. PK studies are performed for almost 70% of drugs when they are intended for these diseases. Efficacy and safety studies are carried out for more than 90% in drugs intended for diseases affecting children only and for more than 75% in drugs for life-threatening diseases (Baiardi et al., 2009).
Moreover, with reference to pharmacogenomic and pharmacogenetic research in the paediatric population, a rather equal distribution of activities across the different research categories throughout the world was found. More than 50% of the research activities are related to predisposition, i.e. exploratory studies aimed establishing the connection between a given genetic trait and the risk associated with a pathology or disease (Krekels et al., 2009).

[1] The EPAR is the European public assessment report, a document provides a summary of the grounds for a EMA Committee opinion in favour of granting or refusing a marketing authorisation for a specific medicinal product.

Finally, the analysis of the clinical trials performed and published for 28 orphan medicinal products (OMP) approved by the EMA showed that even though all of these drugs can be used in children, paediatric studies were performed only in 17 out of the 28 medicines. The methodological quality of OMP dossiers is often criticised because of the several limitations identified in these dossiers: lack of controlled studies, of active comparator where available, of multicentre phase III trials with a suitable number of patients (particularly for diseases with a frequency from 5/100 000 to 5/10 000), insufficient exposure to the treatment, use of surrogate endpoints or weak proof of clinical benefit (Joppi et al., 2006). TEDDY research results, considering the number of clinical trials performed after the authorisation and the information still awaited for many of the drugs already authorised, confirm concerns raised on the quality of the OMP dossiers (unpublished material).

6.3 Ethical aspects in paediatric clinical trials

The main international reference for paediatric research is the ICH Topic E11 guideline 'Clinical Investigation of Medicinal Products in the Pediatric Population' (ICH, 2000) and was recognised by the European Union with the 'Note for guidance on clinical investigation of medicinal products in the paediatric population' (CPMP, 2001). The ICH guideline represents the methodological standard to perform scientifically correct and ethically sound paediatric clinical trials. In fact, on the basis of the assumptions that the paediatric population has the right to use medicines that have been appropriately evaluated and tested, and that it represents a vulnerable subgroup, the guideline introduces special measures to protect the rights of paediatric study participants and to shield them from undue risk.

The rights and well-being of children participating in clinical research in Europe have been for the first time assured by the provisions of the EU Directive 2001/20/EC (European Parliament and the Council of the European Union, 2001) that includes a specific article, Article 4, devoted to the protection of 'minors' and to the guarantee of their emotional, physiological and psychological specificities. This article establishes the condition for the start of a clinical trial involving minors, but does not include references to some relevant documents such as the Convention of Human Rights and Biomedicine (better known as Oviedo Convention) (Council of Europe, 1997) and its Additional protocol on Biomedical Research (Council of Europe, 2005), and ICH guideline (ICH, 2000; CPMP, 2001).

The entry into force of the Paediatric Regulation (European Parliament and the Council of the European Union, 2006a, 2006b), that is expected to increase the number of clinical trials carried out in the paediatric population, has highlighted the limits of the existing clinical trials legislation in particular with reference to children data protection, direct involvement into the 'consensus process' and respect of the children will during the entire clinical research (Altavilla et al, 2008).

In February 2008, the European Commission released updated recommendations on ethical aspects of clinical trials involving children (European Commission's Directorate-General for Health and Consumers, 2008) to tackle the weakness of the existing rules. This document provides a new regulatory context integrating principles contained in other various international ethical/legal source with the aim of ensuring the protection of subjects involved in biomedical research, while recognising the importance of benefits derived from research. The Recommendations also clarify the process of assessment of the benefit and risk balance, the processes of information and consent/assent according to age groups and level of minors' maturity, the process of ethical review of paediatric protocols, individual data protection and insurance issues. In particular, the Recommendations state that:

- the child should participate in the decision-making process together with the parents, according to his/her emerging maturity;
- information should be given by an experienced investigator, or his/her adequately trained delegate, to each parent/legal representative and to the child in language and wording appropriate to his/her age, psychological and intellectual maturity;
- minors should provide their assent. However, the minor's assent is not sufficient to allow participation in the research unless it is supplemented by the legal representative's informed consent;
- separate information sheets for adults and children, and separate consent and assent forms should be used. The child should be informed of the possibility to freely withdraw from the trial, at any time and for any reason, without any disadvantage or prejudice especially regarding medical care.

All of the above mentioned 'set of rules' valuable for protecting children during trials have represented a real advancement to assure a superior ethical context for children involved in a clinical trials. Nevertheless, some important surveys and enquires (Altavilla et al, 2008, 2011; European Forum for Good Clinical Practice (EFGCP) Ethics Working Party, 2010) demonstrate that the implementation of such provisions, and in particular of EU Directive 2001/20/EC (European Parliament and the Council of the European Union, 2001), suffered the lack of binding rules and EU guided coordination activities that led to great differences in children protection levels in different Member States. Such disparities are particularly relevant when related to consent/assent procedures, to the respect of the children's will, and to other fundamental rights (e.g., confidentiality and information). A variable lack of educational initiatives and debates involving local and national ethics committee members on the Paediatric Regulation and the European Ethical Recommendation (European Commission's Directorate-General for Health and Consumers, 2008) were also reported (Altavilla et al, 2011).
This situation claims for new initiatives devoted to implement the existing rules and to identify better ways for the transposition of legislative provisions. To accomplish this and considering the increasing number of paediatric trials in Europe, all the main interested stakeholders (e.g., sponsors, investigators, ethics committees, regulatory bodies, patient associations) should be requested to take part in the efforts.

6.4 Future research

As stated by the Paediatric Regulation, since adults data could only partially be translated to children, drugs for children should be studied according to specific plans and methodologies; in addition, special attention should be devoted to assuring that paediatric studies are conducted under the highest methodological and ethical standards.
In a field where most clinical trials utilise a conservative design, the application of innovative methodologies offers a unique opportunity to develop medicinal products tailored to children. Power calculations can be improved by using clinical trial simulation, an approach that takes into account a multitude of factors.

7. Pharmacogenomics (pgx): The need of pgx tools in the light of the paediatric regulation

Pharmacogenomics is the investigation of variations in DNA and RNA characteristics in relation to drug response; a subset of PGx, pharmacogenetics studies the influence of variations in DNA sequence on drug response (Leeder, 2003). While the traditional

approach to diagnosis and treatment has been based around phenotypic definitions of disease and the identification of broad groups of patients with similar symptoms to be included in 'standardised clinical trials', the pharmacogenomics approach is based on the identification of specific genetic/genomics characteristics and aims at identifying "the right treatment for the right patient at the right time", the so called 'personalised medicine'.
Of particular interest is the fact that these '-omics' terms have been formulated to define approaches capable of identifying groups of biomarkers to be proposed for multiple purposes, such as a) to enable the detection of states of disease, b) to stratify patients based on biochemical profiles and to monitor disease progression and, in the specific field of medicines, and c) to orient the choice of therapy, identifying responders and predicting toxicity, paving the way to a customized therapy.
The increasing introduction of translational approaches in drug development using biomarkers and better defined cohorts, has the potential to increase the manageability, efficacy and safety of clinical trials while in the longer term possibly reducing their size, duration and cost.

7.1 Omics tools identification and methodological approaches

Common methodological approaches that can be applied to pharmacogenetics and pharmacogenomics studies are described below.

7.1.1 Case-control association study

The *case-control association study* is the study design generally used to assess pharmacogenetic effects. This approach examines the active treatment arm of a clinical trial and divides subjects into two groups: those with positive response and those with negative or no response. The groups are then genotyped for a particular candidate gene considered to be related to the treatment phenotype (Russo et al., 2011).
These types of studies are easy to perform, but they also present a number of potential biases or difficulties in interpretation. It is therefore necessary to ensure a good match between the genetic background of cases and controls to avoid biased sampling; techniques that can be employed to detect or eliminate the potential bias of population stratification are the match of cases and controls for ethnicity or the use of multiple unlinked markers (Pritchard & Rosenberg, 1999). Moreover, it is also indispensable to consider additional aspects such as sample size (Campbell et al., 1995), replication selection of candidate gene polymorphism (bioinformatic tools), observation bias (phenotyping and genotyping methods), linkage disequilibrium, allele or genotyped analysis, multivariate analysis, gene-gene and gene-environment interaction, and correction for multiple comparisons to guarantee the quality of the study and preventing false positive associations (Russo et al., 2011).
Efficient and powerful tools to identify inherited DNA sequence variations that contribute to phenotypic expression and variability are available to geneticists thanks to very high throughput DNA analysis technologies (e.g., *single nucleotide polymorphism [SNP] array*) and databases (HapMap project) harboring information about the genomic positions of DNA sequence variations. In fact, it is now possible to test a great amount of polymorphic markers for association with a particular phenotype in a single study, the *genome-wide association study* (GWAS).
GWASs are an important approach for revealing polymorphisms accounting for individual differences in drug efficacy and drug safety (Gurwitz & McLeod, 2009), as shown by

Crowley et al. and by the NHGRI GWAS catalog that summarized the results of 12 published pharmacogenomics GWASs (Crowley et al., 2009; National Human Genome Research Institute, n.d.) and showed that 6 of these GWASs evaluated the association of genetic variation with drug efficacy, five assessed adverse effects, and one examined a dose-response relationship (Russo et al., 2011). Despite their efficiency and potential for leading to useful clinical medicine and public health applications, however, genome-wide association studies have been used in only two drug clinical trials so far, each nonetheless providing relevant insights for future research (Russo et al., 2011, Maitland et al., 2007).
A new and promising field of research is *pharmacogenomics of miRNA* (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee & Ambros, 2001), defined as the study of microRNAs and polymorphisms affecting miRNA function with the aim to predict drug behaviour and improve drug efficiency (Mishra et al., 2008; Mishra & Bertino, 2009). MiRNAs, small, single-stranded, 21-23 nucleotide-long, independent functional units of noncoding RNA, are drug targets that regulate expression of several important proteins in the cell and are differentially expressed in malignant versus normal cells, thus providing MiRNA pharmacogenomics with strong clinical implications (Mishra et al., 2007; Calin et al., 2002; Hon & Zhang, 2007; Iorio et al., 2005). MiR-polymorphisms, in fact, have the potential to be employed predictors of drug response and may lead to the development of more accurate methods of determining appropriate drug dosage based on a patient's genetic makeup, thus decreasing the likelihood of drug overdose (Russo et al., 2011; Mishra & Bertino, 2009).

7.1.2 Other tools

Other pharmacogenomic research areas are transcriptomics, metabonomics and proteomics.
Transcriptomics is the study of gene transcripts, generally analyzed by cDNA expression microarrays which led to a number of exciting breakthroughs (Kieckle & Holland-Staley, 2003; McGregor, 2003). This approach has the advantage to include all genes of potential importance and therefore, provides the possibility to identify new therapeutic and diagnostic targets. On the other hand, its main disadvantage is to be a non-targeted genome-wide approach and therefore influenced by noise (i.e. expression signals of irrelevant genes) and increases the number of false positives (i.e. unimportant genes that are identified by chance) (Russo et al., 2011). Since acute lymphoblastic leukemia (ALL) is a 'liquid' tumour, relatively homogeneous and easy to isolate and characterize, it is ideal to assess global gene expression in cancer. Robust gene-expression profiling is a less labour-intensive and more automated alternative to the multiple methods that are currently used (e.g., immunochemistry, cytogenetics and molecular diagnostics), despite the possible limited availability of the appropriate source of sampling (e.g. blood, excreta, tissue), which is the major limitation of microarray studies (Armstrong et al., 2002; Golub et al., 1999; Ramaswamy & Golub, 2002; Moos et al., 2002; Ross et al., 2004).
Metabonomics or *metabolomics* is the study of metabolite profiling (Plumb et al., 2002; Reo, 2002). It has sampling limitations similar to transcriptomics, with one significant difference: metabonome represents an integrated response, in real time, to all endogenous plus all exogenous stimuli (e.g. drugs, chemical exposures, occupation, lifestyle, nutrition, age, gender) and it might offer the means to follow an individual patient's phenotype—as a function of age, nutrition, course of disease, or therapy (Russo et al., 2011). Metabolomics and "liver profile" test can therefore be considered analogous, except that metabolomics also includes measurement of metabolites and thus provides greater sensitivity. It is true that metabolite profiling can be performed only on easily available samples, nonetheless this

approach may be regarded as an extension of the present practice of clinical pharmacology (Nebert et al., 2003).

Proteomics is the study of all proteins encoded by the genome (Campbell & Ghazal, 2004) and has also been successful in certain areas of basic research. Although a recent study (Xing et al., 2004) estimated an average of 3.0 human proteins per gene, others have estimated that the true number of proteins per gene might be considerably higher (Russo et al., 2011). The limitation of proteomics—like transcriptomics and metabolomics—is represented by the types of source that must be sampled, e.g., blood, excreta or biopsy or tumour tissue in which relevant proteins exist.

7.2 The need to use pharmacogenomics tools to develop paediatric medicines

Recent data indicate that the general interest in the use of pharmacogenomics tools is increasing and in particular, it has been reported that today in some major companies up to 90% of molecules reaching the clinical development stage are associated to a biomarker strategy (Scarpa et al, in press).

The 35% of US approved drugs have pharmacogenetic information in their labels, and currently, 3 pharmacogenetic based drugs are licensed in the EU: herceptin (trastuzumab), glivec (imatinib mesilate) and erbitux (cetuximab). The marketing approved drug camptosar (irinotecan hydrochloride) is considered for re-labelling, based on post-approval research and the FDA decision to re-label the drug on the basis of the research's results.

Notwithstanding these considerable efforts and the claimed interest, the translation of 'omics' results into clinical practices is apparently growing at a very disappointing rate; in particular, there is for example no consolidated source of information on industry activities with enough detail to allow accurate estimates. This gap is probably due to proprietary and patent issues, which prevent public disclosure of information (Scarpa et al, in press).

In addition, it has been demonstrated that throughout the world more than 50% of the 'omics' research is related to predisposition, i.e. the investigation of the correlation between genetic traits and the probability or susceptibility for a given pathology or disease, and this type of exploratory studies provide no insights into the mechanisms of the disease or the drug action, neither it can be used to improve medical practice or support therapeutic solutions.

While many populations could take advantage from the introduction of 'omics' science aimed at developing personalised medicines, this approach could result of paramount importance particularly in 'small populations' for which the current trials approach, based on randomised controlled trials are not always applicable. Children represent the most significant example of such a case: in the paediatric field, in fact, few specific information are available because of the small number of patients and few resources invested to increase specific knowledge, and drugs are commonly used off-label or unlicensed and clinical trials result to be more difficult, longer and more expensive.

As explained in other sections of this chapter, there is a tangible need to base paediatric trials on new methodological approaches that take into account the peculiarities of this population, providing at the same time the highest scientific evidence from each enrollable subject and protecting as much as possible the patients exposed to the trial.

'Omics' approaches, by definition, aim at capturing the essence of the developmental processes that characterize maturation from birth through to adulthood, a particularly appealing methodology for the paediatric research context. However, as explained for

traditional research, it should be underlined that children should not be considered as small adults when they approach pharmacogenetic or pharmacogenomic studies. Many patterns of ontological development in the systems of the body illustrate how paediatric patients can differ from adults. Genotypes do not always correspond with expected phenotypes, making the exercise of deciding how to apply genomic research to paediatric medicine all the more complex (Scarpa et al, in press).

7.3 Pharmacogenomics tools in the paediatric populations: State of the art

Preliminary data on this issue have been provided within the context of the TEDDY Network of Excellence, an EU-funded project that dedicated a survey to the advancement of pharmacogenetic/pharmacogenomic application with the aim to explore the current status, limitations and perspectives of pharmacogenomic and pharmacogenetic clinical research in the paediatric population from an academic, regulatory and industrial perspective (Krekels et al., 2009). The main results reported by TEDDY showed that:

- innovative PGx research in the paediatric field is ongoing, but generally suffers from a non-standardized methodology, and a scarcity of recognized source of data (databanking tailored for children) and industrial interest and funds;
- few pharmaceutical companies declared ongoing pharmacogenomic- or pharmacogenetic-related research involving paediatric indications (Abbot, Altana Pharma, BD, Boherninger Ingelheim, Bristol-Myers Squibb, Ferring Pharmaceuticals, Genentech, GlaxoSmithKline, Isis Pharmaceuticals, Janssen-Cilag, Lilly, Merck, MGI Pharma Biologics, Novartis, Novo Nordisk, Roche, Sanofi Aventis, Servier, Taj Pharmaceuticals);
- the translation of pharmacogenetics and pharmacogenomics into the clinic is very slow. In addition, often pharmacogenetic and pharmacogenomic studies show contradictory results that reflect inconsistent research methods, small sample sizes, no replication studies, non-standardized outcome measures, or little consideration of potential covariates such as co-morbidity.

Pharmacogenetic studies in childhood conditions have been mainly developed in the following most common childhood conditions.

7.3.1 Attention-deficit/hyperactivity disorder

A multifactorial disorder characterized by physical hyperactivity and behavioural disinhibition, the attention-deficit/hyperactivity disorder (ADHD) usually appears during childhood or adolescence and often persists into adulthood. The usually prescribed psycho-stimulant is methylphenidate (MPH) that presents an estimated 70% response rate in ADHD affected children (Elia et al., 1991; Spencer et al., 1996).

According to pharmacogenetic studies, the inter-individual differences in stimulant-response may be related to genetic influences (Kirley et al., 2003; Langley et al., 2005; Gilbert et al., 2006; Winsberg & Comings, 1999; Roman et al., 2002; Purper-Ouakil, 2008; Kereszturi et al, 2008; da Silva et al., 2008) and the search for candidate genes associated with ADHD focused on the catecholamine system. Genes associated with increased risk for ADHD are the dopamine transporter (DAT1) (Purper-Ouakil, 2008), the dopamine receptors (DRD4 and DRD5) (Van Tol et al., 1992), serotonin transporter (5-HTT), and synaptosomal-associated protein (SNAP-25) (Husain et al., 2007; Faraone et al., 2005; McGough et al., 2006). Other genes of potential interest in pharmacogenetic studies include catehol-O-

methyltransferase (COMT) (Kereszturi et al, 2008), the adrenergic α2-receptor (ADRA2A and ADRA1A) (da Silva et al., 2008; Polankzyk et al., 2007; Elia et al., 2009) (Fig. 2). However, some pharmacogenetic studies show conflicting results. For example, in some of them individuals homozygous for the DAT1 10-repeat 480 bp-VNTR showed poorer outcome (Winsberg & Comings, 1999; Roman et al., 2002), whereas others report improved clinical outcome (Kirley et al., 2003) or no effect (Langley et al., 2005) on MPH response (Russo et al., 2011) (Fig. 2).

7.3.2 Growth hormone deficiency

GH deficiency (GHD) causing short stature is usually treated in children with GH replacement, carried out with fixed doses of human recombinant GH (hGH) adjusted for body weight or surface (Jorge et al., 2006).

Two of the most common isoforms of GHR in humans are generated by retention (full-length GHR, GHRfl) or exclusion of exon 3 (exon 3-deleted GHR, GHRd3) (Pantel et al., 2000). These isoforms present a widespread distribution in humans, with the frequency of each allele ranging from 68–75% for GHRfl and 25–32% for GHRd3 (Pantel et al., 2000; Dos Santos et al., 2004) (Fig. 2). It has been demonstrated that, among children with idiopathic short stature or who were born small for gestational age, patients with at least one GHRd3 allele presented 1.7 to 2 times more growth acceleration induced by hGH therapy than patients homozygous for the full-length isoform (Dos Santos et al., 2004). The study conducted by Jorge and colleagues demonstrated that patients carrying at least one GHRd3 allele had a significantly better growth velocity in the first year of hGH replacement and achieved a taller adult height when compared with patients homozygous for GHRfl alleles (Jorge et al., 2006) (Fig. 2).

7.3.3 Acute lymphoblastic leukemia

Cancer chemotherapy is the therapeutic class that could benefit more from PGt and PGx: anticancer agents, in fact, are often given at doses near to those that produce toxicity, show wide inter-patient variability in disposition and effects, and should therefore be administered at optimal doses for the best chance of cure (Russo et al., 2011).

In the USA, the leading cause of death by disease in children between 1 and 15 years of age is cancer and leukemia accounts for 33% of these deaths (Cheok & Evans, 2006). Twenty-five percent of all cancers in children is represented by Acute lymphoblastic leukemia (ALL). Treatment of ALL has undergone a significant progress, nonetheless long-term event-free survival rates are currently almost 80%, with 20% of patients who do not respond to standard therapy [Russo et al., 2011; Husain et al., 2007).

Polymorphisms in genes encoding enzymes that metabolize chemotherapeutic agents can modify treatment response. The thiopurine methyltransferase (TPMT) genetic polymorphisms and mercaptopurine toxicity are one of the best-studied examples in pharmacogenetics. Although 23 variant alleles have been identified to date (Ujiie et al., 2008), 3 variant alleles (TPMT*2 [Ala80Pro], TPMT*3A [Ala154Thr and Tyr240Cys] and TPMT*3C [Tyr240Cys]) account for >95% of low or intermediate TPMT enzyme activity: patients with TPMT deficiency are at very high risk of severe hematopoietic toxicity if treated with conventional doses of thiopurines (Cheok & Evans, 2006; Lennard et al., 1990; Yates et al., 1997). Patients who are heterozygous at the TPMT locus are at intermediate risk of dose-limiting toxicity and might require a modest dose reduction of approximately 35–

50%, whereas TPMT-deficient patients require a dose reduction of >90% (Cheok & Evans, 2006). Other important genes involved in ALL therapy are those codifying for the enzymes of the glutathione-S-transferase (GST) family. The polymorphisms of these genes have been associated with increased cancer incidence, therapy-related cancers and toxicity following chemotherapy (Cheok & Evans, 2006; Hayes et al., 2005). Polymorphisms of GSTM1, GSTP1, and GSTT1 exist in all populations. The GSTM1*0 (GSTM1 null) and GSTT1*0 (GSTT1 null) alleles represent deletions of GSTM1 and GSTT1 genes respectively and result in a loss of enzymatic activity (Rebbeck, 1997). The 1578 A > G transition in GSTP1 gives rise to the Ile105Val polymorphism, which confers reduced enzyme activity (Ye & Song, 2005); it is associated with high etoposide clearance in African-Americans treated with steroids (Kishi et al., 2004). Methotrexate (MTX) is also an important chemotherapeutic drug in the treatment of ALL. Methylenetetrahydrofolate reductase (MTHFR) is an essential enzyme in the folate/methotrexate metabolism pathway. About 10% of Caucasians show a genotypic variant of MTHFR (677 C >T; Ala222Val), which encodes a protein with about 30% of the wild-type activity (Frosst et al., 1995). This SNP has been linked to hepatotoxicity following methotrexate treatment (Ulrich et al., 2001). Another low-function variant of MTHFR results from the 1298 A >C (Glu429Ala) substitution; it has been reported to be protective for adult acute lymphocytic leukemia (Skibola et al., 1999; Wiemels et al., 2001) but not to altered effects of MTX in leukaemia (Krajinovic et al., 2004) (Fig. 2).

7.3.4 Asthma

The most common chronic disease among children, asthma affected in 2002 more than 30 million individuals in the USA reported having been diagnosed as having asthma, including 122 per 1000 children (Mattke et al., 2009).

The response to shortacting albuterol therapy in children with asthma is influenced by a common polymorphism in the coding region of ADRB2 gene (Fig. 2). Bleecker and colleagues have recently showed no pharmacogenetics affect of this genetic variant on therapeutic response when the patients were treated with inhaled corticosteroids plus longacting β2-agonists (Bleecker et al., 2007).

The other two modality of asthma treatment are corticosteroids and leukotriene modifiers, and polymorphisms of the genes (CRHR1, LTC4, ALOX5) involved in their modulation have been described (Fig. 2). In this example, the LTC4S -444 A>C promoter polymorphism has been associated to a reduced risk for asthma exacerbations when compared with individuals homozygous for reference allele (Sampson et al., 2000; Whelan et al., 2003; Husain et al., 2007); in other studies this observation was not consistent (Currie et al., 2003; Kedda et al., 2004) (Fig. 2).

8. Pharmacovigilance: The need for ad hoc measures

According to the World Health Organisation (WHO), pharmacovigilance is defined as "the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem".

In adults pharmacovigilance predominantly refers to post-marketing surveillances.

The situation is different in children. Because of the large extend of unlicensed and off-label drug uses and the small population numbers intensive monitoring of adverse events has to be performed before and after marketing authorisation.

Childhood disease	Gene	PharmGKB ID	Genetic polymorphisms	[1]HGVS names	Polymorphism location and function	Biochemical and clinical effect(s)	PMID
Asthma	ADRB2	PA39	SNP (rs1042713)	NM_000024.4: c.46G>A	Coding region (Arg16Gly)	Arg-allele improves albuterol response	9399966, 10340917
			SNP (rs1042714)	NM_000024.4: c.79G>C	Coding region (Gln27Glu)	No association with response to albuterol	
	AC9	PA30	SNP (rs2230739)	NM_001116.2: c.2316A>G	Coding region (Ile772Met)	Met-allele improves albuterol response	15879435
	CRHR1	PA26874	SNP (rs242941)	NM_004382.3: c.122-1310C>A	Intron	Allele G improves lung function in responses to inhaled corticosteroids	15128701; 19210659
	TBX21	PA36362	SNP (rs2240017)	NM_013351.1: c.99C>G	Coding region (His33Gln)	Gln-allele enhances the effects of inhaled corticosteroid on airway responsiveness	15604153
	LTC4S	PA235	SNP (rs730012)	NT_023133.12: g.24030224A>C	Promoter (-444 bp from start site)	Allele C has a reduced risk for asthma exacerbation	10992553; 14520724
	CYSLTR1	PA38453	SNP (rs320995)	NM_006639.2: c.927C>T	Coding region (Phe309Phe)	Allele C is associated with persistent asthma in males	19080797
	ALOX5	PA46	DIP (rs71921156)	–	5' near gene (-/GGGCGG)	Homozygous for mutant alleles (3,4,6 tandem repeats) reduces lung function responses to ABT-761 treatment	10369259; 12911785; 10369259
	GSDML	PA162390303	rs7216389	NM_001042471.1: c.236-1199G>A	Intron (locus controlling ORMDL3 expression)	T allele is associated with the risk of exacerbations of asthma	17611496; 18395550
ADHD	DRD4	PA27480	VNTR (2-11 repeats)	–	Coding region (48 bp sequence)	The shorter forms (2-4 rep alleles) have a blunted response to dopamine	1319557; 17979513; 19336242
	DAT1	PA311	VNTR (3, 9-10 repeats)	–	3'UTR (40 bp sequence)	Homozygosity of the 480-bp allele (10 rep) is associated with a poor response to MPH	10596245; 12172219; 18563707; 7717410
	5-HTT	PA312	DIP (rs12720056)	–	Promoter (-1212 to -1255)	The 44 bp deletion reduces transcription and protein levels	11425009; 18200432; 8632190
	SNAP-25	PA35980	SNP (rs3746544)	NM_003081.2: c.*239G>T NM_130811.1: c.*239G>T	3'UTR (Conserved element)	Allele T improves dose responses in comparison with the allele G	17979513; 15950004; 17023870
	COMT	PA117	SNP (rs4680)	NM_000754.2: c.472G>A	Coding region (Val158Met)	Val-allele or Val/Val genotype have a good MPH response	18214865
	ADRA2A	PA35	SNP (rs1800544)	NT_030059.12: g.31585029G>C	Promoter (-1291 bp from start site; maps to CpG island)	Allele G improves effects of MPH on inattentive symptoms after 1 month of treatment	17283289; 18200436
ALL	TPMT	PA356	SNP (rs1800462)	NM_000367.2: c.238G>C	Coding region (Ala80Pro)	TPMT*2, TPMT*3A and TPMT*3C alleles have low or intermediate TPMT enzyme activity with high risk of severe hematopoietic toxicity after thiopurines treatment	1973780, 9103127, 16491071
			SNP (rs1800460)	NM_000367.2: c.460G>A	Coding region (Ala154Thr)		
			SNP (rs1142345)	NM_000367.2: c.719A>G	Coding region (Tyr240Cys)		
	GSTT1	PA183	GSTT1*0	–	Large deletion	Loss of enzymatic activity	8198545, 9298582
	GSTM1	PA182	GSTM1*0	–	Large deletion	Loss of enzymatic activity	9298582, 9057653
	GSTP1	PA29028	SNP (rs1695)	NM_000852.3: c.313A>G	Coding region (Ile105Val)	Ile-allele confers high etoposide clearance in African-Americans treated with steroids	15862746; 12969965
	MTHFR	PA245	SNP (rs1801133)	NM_005957.3: c.665C>T	Coding region (Ala222Val)	Val-allele is a low-finction variant (30% of the wild-type activity). It has a higher hepatotoxicity following MTX treatment	7647779, 10536004; 11418485; 11274424
			SNP (rs1801131)	NM_005957.3: c.1286A>C	Coding region (Glu429Ala)	Ala-allele is a lower-activity variant. It not alters MTX effects	
	GGH	PA432	SNP (rs115450789)	NM_003878.1: c.452C>T	Coding region (Thr151Ile)	Ile-allele is associated with 67% lower catalytic activity in the degradation of long-chain MTXPG, but not short-chain	16491071
GHD	GHR	PA28674	GHRfl (NM_000163)	–	Full lenght isoform	GHRd3 isoform had a better growth velocity in the first year of hGH replacement	10764769; 15208626; 16291702
			GHRd3 (AF210633)	–	Exon 3-deleted isofrom		

Legend: ADHD=attention-deficit/hyperactivity disorder; ALL, acute lymphoblastic leukemia; GHD= growth hormone deficiency, SNP,=ingle nucleotide polymorphism; DIP=deletion/insertion polymorphism; VNTR=variable number tandem repeat; MPH=methylphenidate; MTX=methotrexate; MTXPG=methotrexate polyglutamate; HGVS=Human Genetic Variation Society; PMID=PubMed Identifier

Fig. 2. Genetic polymorphisms in the most common childhood diseases (Russo et al. 2011)

Adverse drug reactions (ADRs) in children differ from those manifested in adults in terms of frequency, nature and severity due to their distinct pharmacokinetics and pharmacodynamics (Kearns et al., 2003).

There are also other issues to be considered with respect to the detection of adverse drug reactions in the paediatric population. Children can often not express their own drug therapy experience, which makes the determination of ADRs harder and takes more efforts and sensitivity towards the subject. The involvement of parents and carers therefore is even more important.

8.1 Sample size considerations

In the developed world, the paediatric population is small in general and relatively healthy when compared with for instance the elderly. Children and adolescents account for less than 25% of the population of 0-80years old only. Thus, the number of patients needing a

treatment is always going to be smaller hence, the power to detect adverse drug reactions is far more limited.
Furthermore, the paediatric population ranges from birth to the completion of the 18th year of age. With respect to the safety and efficacy of medicine, this cannot be seen as one homogenous population. Growth and development during these first years of life effect many physiological processes depending on age in different ways. This results in wanted and unwanted reactions to medicines that are not seen in adults and/or other paediatric sub-groups. To accommodate these differences the paediatric population has been sub-classified by the ICH into different age groups (Table 2).

< 37 weeks	Pre-term neonates
< 28 days	newborn and neonates
1months – 2years	infants
>2 - < 11years	children
>11	adolescents

Table 2. Paediatric age groups according to ICH and EMA guidelines

Developing medicines for children means that each age group needs to be studied separately. This poses another challenge, as certain numbers of participants are necessary in order to identify ADRs with statistical power. Many serious and severe ADRs are infrequent occurring in not more than 1 per 10,000 patients.
Therefore, in paediatric pharmacovigilance methods, which can systematically capture large populations, are essential.

8.2 Spontaneous reporting

Spontaneous ADR reporting is the most common method in pharmacovigilance. It has been introduced after the Thalidomide scandal in the late 1960s. Today spontaneous reporting is part of the legal duties of health care professionals in many countries around the world.
The system is well established and has many advantages but also disadvantages. Spontaneous ADR reporting is particularly useful for the detection of signals for new and unknown ADRs as it covers large populations, has low costs and is widely implemented.
However, it cannot be used to study incidence and prevalence as there is a significant under-reporting and unknown denominators, i.e. size of the population exposed and therefore at risk for ADRs. Also it does not allow for an early detection as by the time ADRs are reported the outcome of the ADR is already established, hence there is no possibility of any intervention for the individual patient.
Furthermore the data generated by the system are often biased because of both under-and over-reporting. The introduction of a drug to the market or promotion within the media may result in an increased awareness and thus increased reporting.
The highest value of spontaneous reporting is signal detection is. However, to detect a signal a minimum number of case report is needed. For the spontaneous ADR reporting this ranges between 3 and 9 cases (Edwards et al., 1990). How fast this number of cases can be collected depends on various factors such as the number of users, the frequency of the ADR and the reporting rate. This means that compared to adults, for the relatively small population of children in different age groups it will take much longer to receive the

appropriate number of cases to identify signals. Reports for children with rare disease, or relating to drugs not commonly used, result in even lower reporting rates and hence may never be identified as potential safety signal. Therefore increasing the awareness of ADR reporting in the paediatric population and the compilation of ADR reports on an international basis is crucial to retrieve relevant and important information on adverse drug reactions.

Despite all the limitations of spontaneous reporting, without the continuous reporting on suspicions of ADRs new hypothesis cannot be raised and consequently be tested by using other measures of pharmacovigilance. Therefore, spontaneous reporting will continue to be a crucial part in ADR reporting and efforts should be made to further improve it.

8.3 Active ADR surveillance

Stimulating clinicians and health care professionals regularly to report suspected ADRs and reminding them about the importance of reporting can significantly improve reporting rates. This may be a simple reminder sent on a weekly basis or the contribution of for instance pharmacists on the ward who will regularly ask for ADRs and document them accordingly. Furthermore tools can be made available to health care professionals which will make the reporting easier and also assure data collection is complete.

Various pilot-projects were set up in the past and have shown the effectiveness of active ADR surveillance (Carleton et al., 2009; Clarkson et al., 2004; Menniti-Ippolito et al., 2000). Unfortunately resource limitations usually have not allowed to implement these in general practice.

However, the results of these studies proof that if active surveillance is put in place the number of ADR reports significantly increases. Thus active ADR surveillance can help to overcome underreporting. Furthermore, active surveillance enables a more thorough data collection process and hence improved data quality. Establishing standardized methodologies can further improve and optimize the detection of ADRs.

Overall, active pharmacovigilance poses an important feature in the pharmacovigilance process which particularly in the paediatric population should be utilized more and replace the simple standard of spontaneous reporting.

8.4 Targeted ADR surveillance

Targeted pharmacovigilance focuses on monitoring the safety profiles of specific drug groups in relation to specific ADRs. Depending on the set up it can reach large populations under observation and at the same time provide denominator data to allow for incidence calculations. The AMSP study in Germany has proven that targeted pharmacovigilance in psychiatry is a valuable tool for the evaluation of ADRs as well as educating clinicians in the field of ADRs (Grohmann et al., 2004). Another study from the UK determined the feasibility of conducting a prospective targeted pharmacovigilance study to monitor adverse drug reactions associated with atypical antipsychotic therapy in children (Rani et al., 2009).

The projects showed that targeted pharmacovigilance can be very powerful but at the same time is resource intensive requiring a considerable amount of commitment from the participating clinicians including appropriate training. Nevertheless, the focused collection of practice based data is important. Methods accommodating all methodological issues still need to be developed using the continuously enhancing technological possibilities. The

payment of incentives or free access to all data collected during a surveillance could further enhance reporting.
The potential of this method has been realized by various stakeholders such as the EMA and the Royal College of Paediatric and Child Health which recommend in their guidelines on the conduct for paediatric pharmacovigilance the use of "targeted pharmacovigilance" to monitor drug safety in children (CHMP, 2007b; Royal College of Paediatrics & Child Health, 2004).

8.5 Computerized ADR surveillance

The use of computerized methods to improve the detection of adverse drug reactions in hospitalized patients is a promising approach in both paediatric and adult patients.
Laboratory parameters outside the normal range or the change of such within a certain period of time would allow to generate signals which can inform the clinician that an ADR has potentially developed or may develop in a particular patient. A simple example is the decrease of potassium in a patient receiving medication impacting on potassium levels such as loop diuretics or beta-mimetics.
Pilot studies have shown that such systems are feasible and the sensitivity is high. However, currently there is still a significant lack of specificity for those systems. The definition of rules is a particular challenge in the paediatric population as the normal ranges quickly change within different developmental stages (Haffner et al., 2005; Neubert et al., 2006).
Another promising approach which has also been implemented on paediatric wards are "trigger tools". Trigger tools use screening criteria to identify possible harm in a patient followed by an in-depth review of the patient chart for actual harm. The Canadian Association of Paediatric Health Centers Trigger Tool (CPTT) recently introduced the first validated comprehensive trigger tool to detect AEs in children hospitalized in acute care facilities (Matlow et al., 2011). Trigger tools do not solely report adverse drug reaction but also capture medication errors. With respect to medication safety in paediatric patients this is of importance; with respect to gaining effectively information on long-term safety outcomes there are clear limitations.

8.6 Education and increasing awareness of ADRs

The commitment and awareness of clinicians and other health care professionals involved with the paediatric patient is crucial for the detection and reporting of safety issues. This should be communicated early in the educational process and be part of the general training.
The involvement of paediatric pharmacologists is important to improve the understanding of general principles of how drugs are acting. However, to date there are only a few experts specifically trained in this field.
Within the recent activities around the paediatric drug development this need has been recognized. GRIP –Global Research in Paediatrics has been commissioned by the EU. One of its primary goals is the establishment of educational programmes in paediatric clinical pharmacology.
In addition to health care professionals knowledgeable in paediatric pharmacology and pharmacovigilance families and carers have to be actively involved and encouraged to report adverse reactions. Especially in the paediatric population as children cannot express theirselves, parents and carers play an important rule. They are often very closed to the

children and get the impression of how the child feels and whether changes in behaviour and/or well-being could be related to drug therapy. This information may not be gathered from the responsible health care professionals.
The new European regulation on pharmacovigilance supports this issues as it will allow that adverse drug reactions can be reported to the regulators and pharmaceutical companies by lay people such as patients, parents and carers.

8.7 Conclusions

In summary, pharmacovigilance is important in paediatric drug development. The need to further improve its methods has been realized by different stake holders including health care professionals and regulators. Various methods for ADR detection and reporting are available; to optimize ADR reporting in children a combination of methods needs to be used. Targeted pharmacovigilance is particularly to be promoted. It generates information on certain drugs or drug groups for short as well as long-term outcomes in large populations.
In addition, training of health care professionals and increasing the awareness towards paediatric drug safety is a key element to generate a sustainable drug safety culture in paediatrics.

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