Topics in Medicinal Chemistry 22

Claudiu T. Supuran
Clemente Capasso *Editors*

Zinc Enzyme Inhibitors

Enzymes from Microorganisms

Springer

22
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Claudiu T. Supuran • Clemente Capasso
Editors

Zinc Enzyme Inhibitors

Enzymes from Microorganisms

With contributions by

E.A. Ambrose · H. Brandstetter · C. Capasso · J. Dessolin · M.M. González · S. Köhler · R. Lehneck · S. Pöggeler · E. Schönauer · C.T. Supuran · A.J. Vila · J.-Y. Winum

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Preface

Among the transition elements, zinc together with iron is among the most abundant metal ions in biological systems including microorganisms, plants and animals. Zn^{2+} is highly stable as a dication having Lewis acid properties and lacking redox activity, due to its full d-shell (complete d^{10} orbitals). Thus, zinc is a ubiquitous, essential, non-toxic element possessing crucial biochemical roles in many proteins, including enzymes, transcription factors, and biological membranes. Zn(II) is highly regulated under normal physiological conditions, as this metal ion plays a key role in a wide variety of processes such as DNA and RNA synthesis, transmission of the genetic message, growth and development, signal transduction, apoptosis and metabolism. At the molecular level, the intracellular Zn^{2+} is most often tightly bound to proteins, being an essential co-factor for hundreds of enzymes and thousands of metabolic and regulatory proteins, fulfilling both structural and catalytic roles. In this volume of *Topics in Medicinal Chemistry* series, we propose a series of reviews regarding several such zinc enzymes present in pathogenic microorganisms belonging to Bacteria, Fungi and Protozoa, and which are drug targets by themselves or are involved in drug resistance problems.

The first chapter by Vila and González presents an updated review on metallo β-lactamases, which are binuclear Zn(II)containing enzymes involved in bacterial drug resistance to many types of antibiotics such as penicillin, cephalosporins, carbapenems and monobactams. Although X-ray crystal structures are available for many of these enzymes from pathogenic bacteria, and although a rather large number of in vitro inhibitors have been reported, belonging to a range of chemical classes, the clinical use of such an inhibitor remains a demanding, yet unmet, goal in the fight of bacterial infections. Identification of specific inhibitors against histidinol dehydrogenase, the bacterial metalloenzyme responsible for the catalysis of the last step of L-histidine biosynthesis, which has opened new opportunities for the development of novel anti-infective agents, is reviewed in the second chapter by Köhler et al.

The third chapter, by Ambrose, deals with three zinc-bearing toxins that pose a particular threat to the civilian and military populations as potential biological warfare agents: the botulinum neurotoxins, the tetanus toxin, both of which are clostridial Zn(II)-containing enzymes, and the anthrax toxin lethal factor, a zinc hydrolase.

The structural biology and inhibition of bacterial collagenases, many of which are also present in various *Clostridium* spp., as the proteins dealt with in the previous chapter, are discussed in chapter "Inhibition and Activity Regulation of Bacterial Collagenases" by Schönauer and Brandstetter. Although the crystal structure of the *C. perfringens* enzyme was recently reported by these authors, and even if effective hydroxamate inhibitors are available for many years, also for this orphan target no clinically useful inhibitors were so far developed.

The last three chapters deal with a superfamily of ubiquitous zinc enzymes, the carbonic anhydrases (CAs), present in organisms all over the phylogenetic tree, including microorganisms such as fungi (chapter "Fungal Carbonic Anhydrases and Their Inhibition" by Lehneck and Pöggeler), protozoa (chapter "Protozoan Carbonic Anhydrases" by Supuran and Capasso) and bacteria (chapter "Bacterial Carbonic Anhydrases" by Capasso and Supuran). CAs catalyse a very simple reaction, having as substrates carbon dioxide and bicarbonate, but they are involved in many crucial biological processes, among which pH regulation and several biosynthetic pathways involving carboxylation/decarboxylation reactions. In mammals, many CAs are established drug targets for decades, with many clinically used drugs available. For the anti-infective field, the CA inhibitors met with a sceptical view from the scientific establishment for the moment, although several proof of concept studies showed their efficacy as antifungals, antiprotozoan or antibacterial agents. It should be mentioned that a huge number of such enzymes belonging to several genetic families seem to be present in many pathogenic organisms but only a very limited number of them were for the moment cloned in various such organisms, such as the fungi *Candida* spp., *Cryptococcus* spp., and *Malassezia* spp.; the protozoans *Plasmodium falciparum*, *Trypanosoma cruzi*, and *Leishmania* spp.; or bacteria such as *Neisseria* spp., *Helicobacter pylori*, *Escherichia coli, Mycobacterium tuberculosis*, *Brucella* spp., *Streptococcus pneumoniae*, *Salmonella enterica, Haemophilus influenzae*, *Legionella pneumophila, Vibrio cholerae, Porphyromonas gingivalis, Streptococcus mutans, Clostridium perfringens,* and *Pseudomonas aeruginosa*. Many of these microorganisms provoke serious diseases affecting millions of patients and in many cases a significant drug resistance problem was reported for many of them with the clinically used agents (antifungals, antibiotics, antimalarials) whereas for some of the protozoan diseases the available agents are very toxic and ineffective. Thus, the CA inhibition from pathogenic organisms may offer new yet poorly explored opportunities for developing agents with an alternative mechanism of action to classical anti-infectives.

As outlined here and throughout this volume, this is a rather new research field and no clinically used agents belonging to these zinc enzyme inhibitors are

available. We are confident that the present volume may represent an important ring bell to those working in the anti-infective field, where few crucial developments were achieved for decades although the need for new, effective and less toxic therapies is urgent.

Naples, Italy — Clemente Capasso
Florence, Italy — Claudiu T. Supuran
May 2016

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An Elusive Task: A Clinically Useful Inhibitor of Metallo-β-Lactamases

Mariano M. González and Alejandro J. Vila

Abstract Multidrug-resistant bacterial infections have become in recent years an increasingly worrisome problem in the medical community. The β-lactams are the most used antibiotics consisting in more than 60% of the prescribed antibacterials. Indeed, carbapenems are considered as "last resort" antibiotics for the treatment of several pathogens that are difficult to eradicate. The most widespread bacterial resistant mechanism against β-lactams consists in the expression of β-lactamases which inactivate these compounds by hydrolyzing the β-lactam bond. Metallo-β-lactamases (MBLs) are metal-dependent enzymes that are able to coordinate one or two Zn(II) ions in their active site which are essential for the catalytic mechanism. In view of this scenario, the search and identification of inhibitors against these enzymes is of outmost importance for the rescue of the antibiotic activity of the β-lactams. Here we present a critical analysis of the different chemical motifs that had been reported as MBL inhibitors, inspected within the context of mechanistic and structural information with the goal of identifying common aspects that can be used for the development of more efficient and broad-spectrum leads. We also suggest possible future directions for the development of this exciting research field.

Keywords Bacterial resistance, Inhibitors, Metalloenzymes, Zinc

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1 β-Lactams and Mechanisms of Resistance

β-lactam antibiotics are the most widespread drugs employed to combat the action of opportunistic and pathogenic bacteria. These compounds, originally known as "magic bullets," are losing their potency due to the worldwide emergence of bacterial infections that are highly resistant to the antibiotics available in the clinic [1]. Specifically, the resistance towards β-lactam antibiotics has recently prompted several efforts for the identification of new antibacterial compounds that could be able to palliate this situation [2]. β-lactam drugs include penicillin, cephalosporins, carbapenems, and monobactams (Fig. 1). They all act through binding and inhibiting the bacterial penicillin binding proteins (or PBPs), triggering several events that finally produce the bacterial death [3]. One of the most important resistance mechanisms against β-lactams is the expression of enzymes capable of inactivating them by hydrolysis, named β-lactamases.

The first β-lactamases identified are evolutionary related to the PBPs, and contain an activated site serine residue which is essential for the catalytic mechanism and were thus named as serine-β-lactamases (SBLs) [4]. This group of enzymes was classified as β-lactamases of classes A, C, and D based on sequence homology [3]. On the other hand, during the decade of 1960s another type of β-lactamase was identified by E.P. Abraham from a non-pathogenic *Bacillus cereus* strain that was dependent of Zn(II) ions as essential cofactor and was inhibited by the chelating agent ethylenediaminetetraacetic acid (EDTA) [5]. This enzyme appeared to be a mere biochemical curiosity at the time. However, in the 1980s these kind of enzymes, known as metallo-β-lactamases (MBLs) or class B

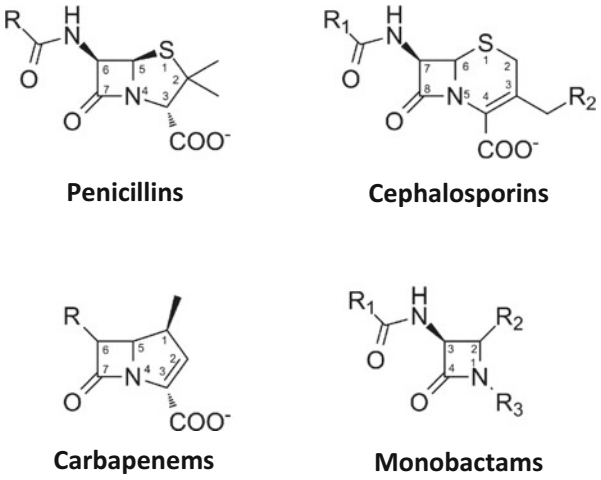


Fig. 1 Structure of β-lactams

β-lactamases, were found chromosomally encoded in several pathogenic bacteria such as *Bacteroides fragilis* [6], *Stenotrophomonas maltophilia* [7], and various *Aeromonas* [8, 9] and *Chryseobacterium* strains [10–12]. Among Gram-negative bacteria, a silent gene coding for an MBL was found in *Bacillus anthracis* [13]. The situation became more worrisome when genes coding for MBLs were found in mobile genetic elements (which also harbor other resistance cassettes) in several Gram-negative pathogens including members of the Enterobacteriaceae species, *Pseudomonas aeruginosa*, *Serratia marcescens*, and the *Acinetobacter* species [14, 15]. These mechanisms of genetic transference have facilitated the dissemination of these enzymes among prevalent pathogens, thus delineated a threatening scenario for the health systems. MBLs are able to hydrolyze equally well penicillins, cephalosporins and (of major concern) carbapenems, usually last option antibiotics. Outbreaks of pathogens producing the MBLs VIM-2 (Verona Integron-encoded MBL) and NDM-1 (New Delhi MBL) are rising in incidence all over the world, with high rates of death due to the lack of therapeutic options [16, 17].

2 MBLs Classification

MBLs constitute a family of proteins belonging to an ancestral superfamily of metallohydrolases, which includes more than 30,000 genes coding for enzymes that hydrolyze thiol esters, phosphodiesters, and sulfuric ester bonds, but also encompassing oxidoreductases [18]. In contrast to serine-dependent lactamases, which are exclusive to the bacterial world, the MBL fold is ubiquitous in all living organisms. However, enzymes showing lactamase activity within the MBL fold are clearly restricted to bacteria. The understanding of similarities and differences

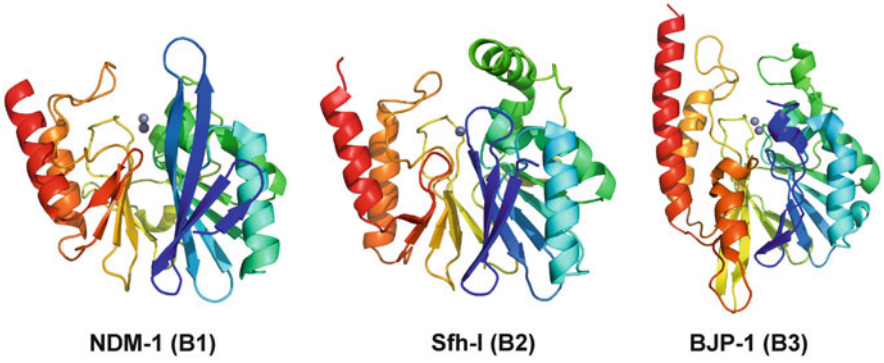


Fig. 2 Global fold of representative metallo-β-lactamases of the three subclasses

between enzymes with a similar fold catalyzing different reactions is of fundamental importance for medicinal chemistry efforts targeting exclusive inhibition of bacterial MBLs [19].

MBL enzymes share a common αβ/βα sandwich scaffold (Fig. 2) and a metal binding motif (His/Asn116-X-His118-X-Asp120-His/Arg121, His196, Cys/Ser221, His263, according to the standard BBL numbering scheme [20]) located in the interface of the two αβ domains. Most of the non-β-lactamase hydrolases present binuclear sites containing Zn(II), Fe(II)/Fe(III), or Mn(II) ions, with an Asp/Glu221 residue as a bridging ligand between the two metals. On the other side, MBLs lack a bridging protein ligand; instead a water/hydroxide molecule occupies the bridging position while at position 221 a Cys or Ser residue is present [21]. Although MBLs are divergent, with sequence identities as low as 10% or less in some cases, they have been grouped into three subclasses, B1, B2, and B3, based on sequence alignment guided by common structural features [20].

Subclass B1 and B3 MBLs are binuclear Zn(II) enzymes with a broad substrate profile (penicillins, cephalosporins, and carbapenems) [22–26]. The smaller subgroup B2, albeit phylogenetically closer to B1 enzymes [27], includes mononuclear Zn(II) enzymes capable of hydrolyzing exclusively carbapenems [28]. Subclass B1 enzymes exhibit sequence identities higher than 23% between their members [21]. This group includes almost all the clinically relevant MBLs: the aforementioned NDM [29], the VIM variants [30], the IMP (Imipenemase) variants [31], and SPM-1 (São Paulo MBL) [32], acquired by pathogens through mobile genetic elements, apart from other endogenous MBLs like chromosome-borne *B. cereus* BcII [33], *B. fragilis* CcrA [34], or *Elizabethkingia meningoseptica* BlaB (β-lactamase B) [10]. The exclusive carbapenemases from subclass B2 share 11% sequence identity with B1 enzymes [21]. This group includes endogenous MBLs like *A. hydrophilia* CphA (Carbapenem-hydrolyzing MBL) [35], *A. veronii* ImiS (Imipenemase from *A. veronii bv. sobria*) [9], and *Serratia fonticola* Sfh-I [36]. Finally, subclass B3, the most distant in phylogenetic terms [27], comprises endogenous enzymes sharing only nine common residues with the rest of MBLs.

Members of this group include chromosome-borne MBLs *Stenotrophomonas maltophilia* L1 [7], *E. meningoseptica* GOB [37], and *Legionella (Fluoribacter) gormanii* FEZ-1 [38]. The recently reported AIM-1 (Australian Imipenemase) represents the first B3 enzyme encoded in a mobile genetic element, suggesting that gene dissemination may not be limited to subclass B1 [39].

3 Structural Diversity in MBLs

The crystal structures of B1 and B3 enzymes have revealed binuclear metal centers in the active site, comprised of two Zn(II) ions: one in a tetrahedral coordination sphere (Zn1 site) and one in a trigonal bipyramidal coordination sphere (Zn2 site) [23, 24, 40–43]. In B1 enzymes (Fig. 3), the Zn(II) ion at the Zn1 site is coordinated to residues His116, His118, and His196 and a bridging water/hydroxide molecule (Wat1); while the Zn(II) ion at the Zn2 site is coordinated to residues Asp120, Cys221, and His263, the bridging water and an apical water molecule (Wat2). Mutational analyses on B1 enzymes have shown that all metal binding residues are essential to provide full activity [44]. The active sites in these enzymes are surrounded by several loops that define the substrate binding cavity: L3, L7, L9, L10, and L12 (Fig. 4). Loop L3 may not be fully defined in the crystal structures (by the lack of electron density) or it has been reported with high crystallographic B-factors, both in the absence or presence of inhibitors or reaction products in which it is closed over the active site, suggesting that this loop is a flexible one whose conformation is modulated by binding of substrates and inhibitors [45–47].

In B3 enzymes, the arrangement of the Zn2 site differs with respect to B1 enzymes (Fig. 3). These enzymes present a Ser residue at position 221 (instead of the Cys) that does not participate in metal coordination. Instead, a His residue at position 121 completes the coordination sphere of the Zn2 site. B3 enzymes are characterized by longer loops flanking the active site, which appears less open and exposed, despite they also display a broad substrate profile.

B2 enzymes (Fig. 3) are fully active with a single Zn(II) ion localized in the Zn2 site, sharing the same ligand residues as in B1 enzymes, i.e., Asp120, Cys221, and His263 [28, 48]. A naturally occurring His116Asn substitution at the Zn1 site precludes Zn(II) from binding with high affinity, whilst in the presence of excess

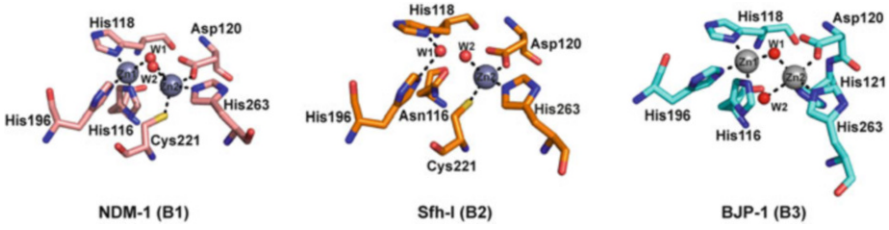


Fig. 3 Active site structure of representative metallo-β-lactamases of the three subclasses

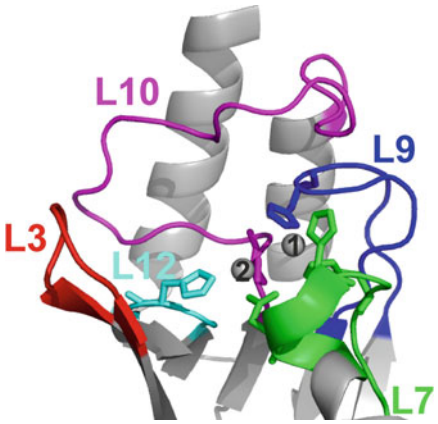


Fig. 4 Loops flanking the active site in B1 metallo-β-lactamases. Zn (II) ions are depicted as *gray spheres*. Metal ligands are shown as sticks

Zn(II), binding to this site can be inhibitory [28]. A mutational analysis of the B2 enzyme CphA showed that metal ligands Asp120, Cys221, and His263 are essential for the enzyme activity, as well as residues His118 and 196 [49]. The presence of residue Cys221 in B1 and B2 MBLs contrasts with the ubiquity of an Asp/Glu221 bridging ligand in other members of the MBL superfamily devoid of lactamase activity. The Cys ligand (despite being absent in B3 enzymes) is a hallmark of the active site of MBLs, since Cys residues are rarely found as Zn(II) ligands in exposed catalytic sites [50]. This is most striking considering that MBLs bind the Zn(II) cofactor in the oxidizing periplasmic space. The observation that B3 enzymes, lacking a Cys ligand, are phylogenetically closer to other members of the MBL superfamily [21] suggests that this residue confers an evolutionary advantage. Indeed, we recently demonstrated that a Cys residue in the metallic centers of B1 MBLs is critical for ensuring metal uptake in the periplasmic space of Gram-negative bacteria, leading to the active binuclear enzyme [51].

4 Substrate Binding and Catalytic Mechanism

The active site diversity of MBLs has precluded the finding of common features describing substrate binding and the catalytic mechanism, despite several encouraging hints have appeared in the last decade. We refer to recent reviews specifically covering these aspects [52–56]. Remarkably, despite broad-spectrum B1 and B3 enzymes bind and hydrolyze penicillins, cephalosporins, and carbapenems, aztreonam (lacking a carboxylate moiety) does not even bind to MBLs [57], suggesting the presence of some minimal recognition elements that may yield information for inhibitor design.

Substrate binding experiments in solution performed with BcII have revealed that the apoenzyme is not able to bind β-lactam substrates, indicating that substrate binding in B1 MBLs is largely driven by electrostatic interactions with the two

metal ions [58]. Particularly, the interaction of the invariant β-lactam carboxylate moiety (at C3 in penicillins and carbapenems and C4 in cephalosporins) with Zn2 has been demonstrated by the crystallographic structures of enzyme-product (EP) complexes of MBLs representative of the three subclasses [43, 59, 60]. This carboxylate moiety also interacts with a highly conserved charged residue at position 224 in B1 enzymes and residues Ser221 and Ser223 in B3 enzymes [43, 59, 60].

The exclusive carbapenemases from B2 subgroup not only harbor a single Zn (II) ion, but they also present a more occluded active site, since one of the flexible loops is replaced by a helix that restricts substrate access. However, the positively charged residue in position 224 is also preserved in B2 enzymes, being a common substrate anchoring point reinforcing the electrostatic recognition provided by the Zn(II) ion.

The catalytic mechanism of MBLs differs radically from that of serine-dependent enzymes in several features: (1) the active nucleophile is a Zn(II)-bound hydroxide, (2) MBLs do not accumulate a covalent intermediate after C-N cleavage, and (3) instead, an anionic intermediate stabilized by the Zn2 ion can accumulate depending on the particular combination of enzyme and substrate. B1 enzymes strictly require two metal ions to be active in the bacterial periplasm [51]. This information is of great relevance since it identifies the active species to be inhibited in vivo.

5 MBLs Inhibition

Since the mid 1990s, there have been several reports of MBLs inhibitors but, despite all of these efforts, no clinically useful molecules have been yet developed [61]. The first difficulty in this aim lies in the absence of a covalent intermediate during the catalytic mechanism carried out by these enzymes [62], which precludes the development of suicide inhibitors as clavulanic acid, sulbactam, or tazobactam, effective against SBLs. Furthermore, the lack of a common intermediate between members of the different subclasses has impeded the development of Transition State Analogues with the capacity of function as broad-spectrum inhibitors with submicromolar potency. Generally, the compounds reported display inhibitory activity against one or two MBLs, being less effective against others. Interestingly, several reports identified that thiol compounds are capable of coordinating the active site metal center and inactivating MBLs of different subclasses with good potency, proving that MBLs broad-spectrum inhibition is feasible [63–65]. Regardless of how promising the characteristics of a candidate molecule (e.g., nM K_i, low IC_{50}s, low MICs) are, a successful inhibitor must prove its worth in the clinical setting. For example, the compound must be in sufficient concentration in serum and periplasm. Otherwise, optimization of pharmacokinetics, pharmacodynamics, and safety issues of the compound are essential to reach a useful drug. There is a concern about cross reactivity with zinc-dependent enzymes, not only with the

MBL fold, but also with the angiotensin-converting enzyme (ACE), which is inhibited by thiol-containing drugs such as captopril [66–71].

Notwithstanding a large number of articles reporting MBL inhibitors to date, there are a few molecules designed with structural analogy to substrates, reaction intermediates, or products. In fact, most inhibitors discovered belong to several families of compounds unrelated with β-lactam molecules, but in most cases with the capacity of binding the active site. Other compounds have been tested due to inhibitory activity against related zinc metalloenzymes. Screening techniques have been applied with satisfactory results and several potent and selective compounds were identified against a selected target [72]. It must be noted that in order to compare the potency between diverse inhibitors, the experimental conditions in which the inhibition constants are estimated for different MBLs must be homogeneous. Unfortunately, in vitro inhibitor assays and MIC tests reported in the literature encompass a large variety of experimental conditions, substrates, and bacterial strains, precluding a direct comparison of the real potency of different compounds. Most studies make use of the non-clinical chromophoric cephalosporin nitrocefin as substrate in the in vitro inhibition assays. This compound, together with other chromogenic or fluorogenic probes, is of great utility to screen libraries for MBL inhibitors. However, final tests with a selected group of clinically relevant antibiotic should always be reported.

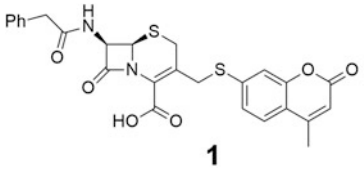
As all subclasses of MBLs hydrolyze carbapenems (nowadays, the most clinically important β-lactam antibiotics), members of this β-lactam group should be selected as reference substrates during an inhibitor discovery program. Here we present a survey of the most promising molecules reported with a focus on the latest published experimental works.

5.1 Screening Methods

Several interesting reports described the utilization of different screening methodologies for the identification of MBL inhibitors [73]. Electrospray-ionization mass spectrometry (ESI-MS) was used to study the inhibition by a set of mercaptocarboxylates of two representatives MBLs, BcII (B1 subclass) and CphA (B2). The results for the BcII enzyme demonstrated that the inhibitors bind to the binuclear form of the enzyme. The results for the CphA complex unexpectedly revealed an increased affinity for the binding of a second metal ion in the presence of thiomandelic acid [74]. Lienard and colleagues reported the implementation of a dynamic combinatorial mass spectrometry approach for the identification of thiol-containing compounds as inhibitors of MBLs [75].

The large scale application of β-lactams in automated HTS screening for MBL inhibitors is largely hampered by their lengthy and often difficult synthesis and the high costs associated with these substrates. More importantly, these substrates suffer from poor substrate recognition by MBLs as a result of the high diversity of this enzyme family varying significantly in sequence, structure, and substrate

specificity, thus making it hard to use a single substrate for broad MBL activity screening. The development of new assays for broad range MBL activity screening, based on hydrolysis of chromogenic or fluorogenic β-lactams would, however, significantly facilitate inhibitor identification. Schofield and coworkers reported a series of new inexpensive and stable fluorogenic and chromogenic β-lactams (**1–2**), which were used to develop a platform for the identification of inhibitors of MBLs [76–79].



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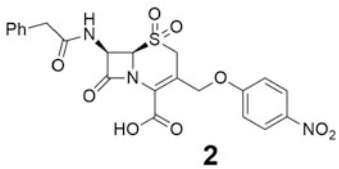

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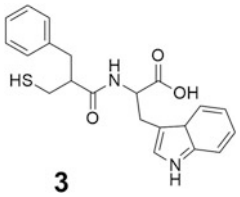
An interesting report developed at Astra Zeneca showed the utility of combining target-based whole-cell Screening and NMR spectroscopy in order to identify compounds inhibiting a specific enzymatic reaction in bacterial cells. With this approach they could find the compound (**3**) which showed strong NDM-1 inhibition with cellular IC_{50} of 0.51 μM [68, 80]. Finally, the possibility of virtual screening for the identification of MBL inhibitors was recently exploited [81]. The recent development of a new force field, named AutoDock4Zn [82], and databases of commercially available compounds suited for structure-based virtual screening such as ZINC [83] can be an interesting possibility for the identification of new metalloenzyme inhibitors in the future.



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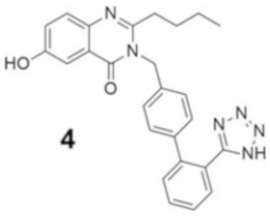
5.2 Biphenyl Tetrazoles

Screening of a Merck collection and molecular modeling studies on the MBL CcrA from *Bacteroides fragilis* provided the early identification of several biphenyl tetrazoles as potent competitive inhibitors. The crystal structure of the CcrA:L-159 061 (**4**) inhibitor complex revealed one of the N atoms of the tetrazole moiety interacting with the Zn2 site. This Zn2-N interaction has been proposed to be crucial in stabilizing the anionic intermediate in the catalytic mechanism [84, 85], and has been identified in several enzyme-product complexes characterized by X-ray crystallography [25, 59, 86], therefore providing basis for more rationale redesign that has not been further explored.

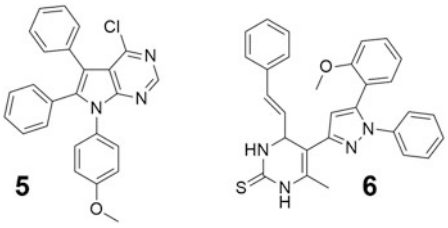
These compounds exhibit low micromolar IC_{50} and K_i values, the substitution of the biphenyl group being essential to the strong binding observed. Molecular dynamics simulations suggested that, in the presence of the inhibitor, Trp64 in the mobile flap L3 blocks the entrance of the active site channel, thereby contributing to the stabilization of the enzyme-inhibitor complex. Other interactions as the hydrogen bonding of Lys224 to the inhibitor carbonyl group and hydrophobic contacts between the inhibitor and side chains of loop residues surrounding the active site prove to be significant binding forces of the enzyme-inhibitor complex [87–89].



4

5.3 Pyrrole Derivatives

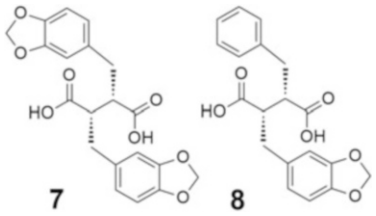
Several reports have addressed the diverse biological applications of pyrrole nucleus and pyrrolo[2,3-d]pyrimidines, due to their antibacterial and antiviral activities, among others [90]. With the aim of developing novel MBLs inhibitors, Mohamed and coworkers synthesized, tested, and performed in silico docking analysis of compounds based on pyrrole using IMP-1 as target [90]. The inhibition constant (K_i) values of six compounds which displayed the most potent inhibitory activity were in the low micromolar range (10–30 μM). In silico docking suggests that compound (**5**) binds within the active site of IMP-1 parallel to the flexible loop with the oxygen atom of the methoxy group close to the two Zn(II) ions (Zn1: 3.4 Å, Zn2: 2.7 Å) [90]. A second set of pyrrole and thiopyrimidine derivatives were synthesized and tested as IMP-1 inhibitors. Five compounds displayed K_i values around 20 μM. In silico docking of (**6**) with IMP-1 suggested that the thione moiety would be bridging the two Zn(II) ions. In addition, a nitrogen atom of the pyrimidine ring forms a hydrogen bond to the nitrogen atom of Lys224. Hydrophobic interactions between the isopropyl group of Val67 and the methoxybenzene moiety and between the benzene ring of Phe87 and the isopropyl group of Val61 with the benzene ring of the styryl group contribute to the inhibitor binding [91].



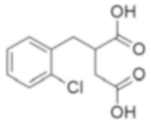
5.4 *Dicarboxylic Acid Derivatives*

In a search through the Merck chemical collection, it was possible the discovery of several (2S,3S)-disubstituted succinic acids as potent inhibitors of IMP-1 MBL, which exhibit IC_{50} values in the low nanomolar range [92]. The (S,S)-isomers are far more active than (R,R) or (R,S)-isomers. Also, two substituents of significant hydrophobicity on the succinic acid core have been found necessary for IMP-1 inhibition.

The crystal structure of IMP-1:(**7**) reveals that this compound experiences strain because of unfavorable steric interactions with the side chain of Ser119. The complex of IMP-1 with inhibitor (**8**) shows that the smaller benzyl substituent present in this compound binds in an unstrained conformation, giving rise to a slightly more potent inhibitor. Importantly, the carboxylate groups interact with the Zn(II) ions, displacing the bridging and apical water molecules. One oxygen of the right-hand carboxylate group interacts with both Zn1 and Zn2, whereas the second oxygen on the same carboxylate interacts with Nδ2 of Asn233. Another oxygen of the left-hand carboxylate interacts with Zn2, and the second interacts with the main chain nitrogen of Asn233, as well as with the Nε of Lys224.

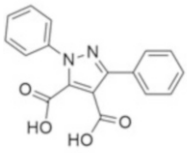


A screening of the National Cancer Institute diversity set identified further succinic acid derivatives that were mixed inhibitors of the IMP-1 MBL. The most potent compound (**9**) within this series displayed a K_i value of 3.3 μM, due in part to the presence of the chlorine atom which proved to be crucial for potency [93]. Actually, an analog compound devoid of the chlorine atom displayed no inhibition at 40 μM compared to the >80% inhibition for (**9**), which also displayed an in vitro antibacterial activity lowering the minimum inhibitory concentration (MIC) of meropenem against an IMP-1-expressing *E. coli* strain.



9

An interesting work showed the utility of pharmacophore generation, database searching, docking studies, and enzyme kinetics for the discovery of new MBLs inhibitors [94]. Based on the crystal structure of IMP-1 in complex with succinic acid and mercaptocarboxylic acid inhibitors [23, 92], three 3D pharmacophores were generated. Then, a database was searched for compounds mapping to the pharmacophores and 74 hits were obtained. Based on a score plot calculated from a set of descriptors that define possible good leads, 12 compounds were selected for experimental determinations of the IC_{50} value. With this approach, three compounds (**10**), (**11**), and (**12**) with IC_{50} values less than 15 μM were identified.



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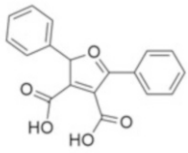

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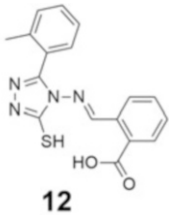
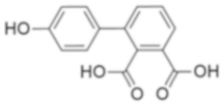


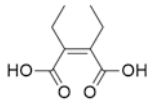
12

From a library screening it was possible to obtain several active compounds against IMP-1 [95]. The most potent of these compounds, a phthalic acid derivative, was chemically modified and a structure–activity relationship study identified a set of molecules able to inhibit IMP-1 with submicromolar IC_{50} values. Notably, compound (**13**) also showed a decrease of the biapenem MIC of a *P. aeruginosa* strain from 64 to 128 μg/mL to 1 μg/mL.



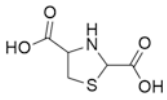
13

The in vitro inhibition activity of a novel malic acid derivative, ME1071 (**14**), indicated that this compound displays the capacity of potentiate the activity of ceftazidime and carbapenems against MBL-producing strains of *P. aeruginosa* [96]. The K_i values of IMP-1 and VIM-2 for ME1071 were 0.41 μM and 120 μM, respectively. Despite this selective inhibitory behavior, it was shown that ME1071 still exerts a synergistic effect with carbapenems for VIM-2 producers, suggesting that a low K_i is not the most important characteristic to reach a compound with potent in vitro and in vivo activity.



14

N-heterocyclic dicarboxylic acid derivatives were synthesized and characterized as inhibitors against MBLs representatives of the three subclasses: CcrA (B1), ImiS (B2), and L1 (B3). Five compounds were identified as inhibitors of at least two enzymes, being (**15**) a broad-spectrum inhibitor with low micromolar K_i values. Also, the in vitro antibacterial activities of these compounds were investigated by determining the effect of them on the MIC of cefazolin or imipenem [97]. Compound (**12**) was the most effective in lowering the MIC values of β-lactam antibiotics on all tested Gram-negative bacteria.



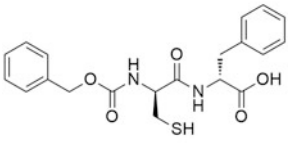
15

Overall, these efforts indeed confirm that the flexible nature of the MBL active site (both in terms of the low steric restrictions and the flexible coordination geometry of the Zn(II) ions) allows efficient binding and inhibition of a rather disparate range of dicarboxylate chelating agents. However, the systematic analysis of different compounds, mostly by Toney, strongly suggests that, in addition to the metal binding capabilities of the carboxylates, some subtle stereochemical differences can be tuned to enhance the inhibitory potency.

5.5 Thiol Compounds

Zn(II) is a thiophilic metal ion, therefore thiol compounds are appealing candidates as MBL inhibitors, and stand as the largest group of tested MBL inhibitors. Indeed, a recent article surveys the usefulness of approved thiol-based drugs as MBL inhibitors [98] (see below). Several substituted thiols and cysteinyl dipeptides were early assayed as BcII inhibitors [99]. Interestingly, some of these compounds, such as (**16**), showed low micromolar K_i values and the dependence of the pK_i with pH indicated that the thiolate could displace the hydroxide anion from the active site of BcII. It was also demonstrated that similar compounds but with opposite stereochemistry can differ by an order of magnitude in their relative potencies. In a latter study, Sun and coworkers described the synthesis of a library of analogs based on the structure of cysteinyl dipeptides using combinatorial chemistry [100]. Analogs with different Zn chelating moieties were synthesized and tested as inhibitors of L1 enzyme. The K_i values demonstrated that homo-cysteinyl peptide (**17**) was a

more potent inhibitor than cysteinyl peptide (**18**). Based on these results an optimization procedure in order to obtain homo-cysteinyl derivatives with distinct chemical groups and higher potency allowed the identification of a potent inhibitor (**19**) of L1 with a K_i of 2 nM [100].



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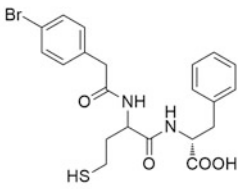

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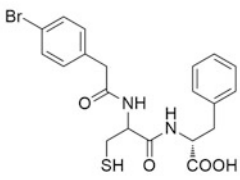

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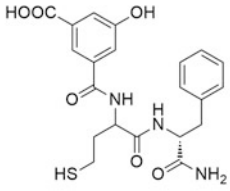
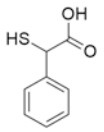


19

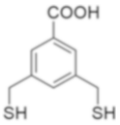
Based in the fact that the Zn(II) ions in the active site, as well as coordinating water molecules, are essential structural and chemical factors in the catalytic mechanism of MBLs of all subclasses, it was reported that thiol compounds exhibiting Zn chelating capacity are effective inhibitors of MBLs. One of these compounds, thiomandelic acid (**20**), was the first broad-spectrum inhibitor reported [63]. This inhibitor exerts a potent inhibitory activity against MBLs of B1 and B3 subclasses (with submicromolar K_i values), although it is less effective on B2 CphA enzyme. The inhibition profile of several derivatives allowed to determine that the thiol group is an essential feature for the inhibitory activity whereas the carboxylate increases the inhibition potency. It was suggested through NMR experiments on BcII that the thiol group binds both Zn(II) ions and its carboxylate is H-bonding Arg121. In addition, the contribution of the "flapping loop" surrounding the active site to the stabilization of the enzyme-inhibitor complex was suggested.



20

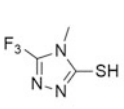
Using ESI-MS under non-denaturing ionization conditions, several thiols were screened for binding of representative MBLs of three subclasses: BcII and IMP-1 (B1), CphA (B2), and L1 and FEZ-1 (B3) [101]. Two of these compounds were found to inhibit all tested MBLs with submicromolar K_i values, being (**21**) the first

reported broad-spectrum inhibitor with K_i values lower than 1 μM for all MBLs. Modeling studies indicated that the same compound can display different binding modes between enzymes of different subclasses. These results showed that potent inhibitors can be design to inhibit MBL from all subclasses.



21

Vella and colleagues described the discovery of ten promising leads for the development of IMP-1 inhibitors using a 500 compound Maybridge™ library suitable for fragment-based screening [102]. From the thiol-containing compound (**22**), which displays a $K_i \sim 1$ mM, Faridoon and coworkers synthesized several analogs of this compound with the aim of optimize its activity [103]. Although the structure–activity analysis was instructive, the authors could not succeed in improving the potency of compound (**22**). However, some acylated thiosemicarbazide precursors obtained during the synthesis of the analogs were serendipitously identified as potent IMP-1 inhibitors. In particular, thiosemicarbazides acylated with aromatic groups displayed strong inhibition with $K_i \sim 10$ μM. The interaction of the most potent of these inhibitors (**23**) with IMP-1 was examined by in silico docking. Interestingly, modeling unexpectedly suggested that the oxygen atoms of the nitro group, instead of the sulfur, were interacting with the Zn(II) ions. Other interactions include nitro-aromatic ring with Trp64 on the flexible flapping loop of IMP-1 and two N-H bonds between the terminal thio-urea group and the backbone carbonyl oxygen from Tyr227.



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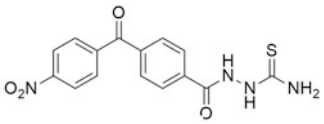
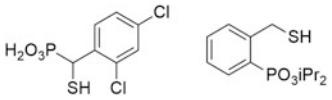


23

Mercaptophosphonate derivatives were found to be potent broad-spectrum inhibitors of MBLs [64]. These compounds exhibit also a good inhibitory effect on the subclass B2 CphA enzyme. Crystallographic studies of CphA complexed with (**24**) and (**25**) showed that both sulfur or phosphonate group can interact with the Zn2 ion, which indicate that similar compounds can adopt distinct binding modes.



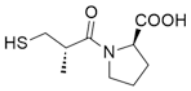
24 **25**

Two systematic studies of a series of thiol compounds as MBL inhibitors are available. The first, reported by Dmitrienko and coworkers [104], gave several important conclusions: (1) the availability of two thiol compounds did not elicit a higher inhibitory potency compared to mono-thiols, (2) in general, inhibition did not occur by metal removal, and (3) strong binding compounds show a first, slow binding phase, followed by a rearrangement in the active site. This information might be exploited to identify further recognition events by MBLs. A more recent work examined a series of approved drugs with thiol groups against different MBLs [98]. These authors conclude that not all thiol-containing compounds are able to inhibit MBLs and, furthermore, the inhibition potency of a given compound against different homologous B1 enzymes can differ notably.

5.5.1 Captopril

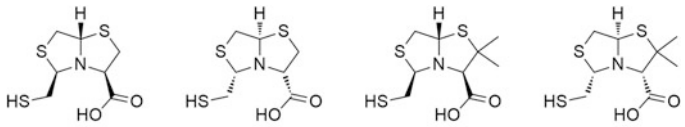
D-Captopril (**26**) is a thiol-based commercially available drug used as inhibitor of the angiotensin-converting enzyme for the treatment of hypertension. This compound was tested as inhibitor of MBLs, being more effective against B1 and B3 subclasses than against CphA (B2) [101]. The crystal structures of BlaB (B1) [105], L1 (B3) [42], and FEZ-1 (B3) [106] in complex with this inhibitor were obtained. D-Captopril is bound into the active site of these enzymes by its thiolate group, which is bridging both Zn(II) ions, and in this way displaces the nucleophilic hydroxide. In the structure of NDM-1 (B1) in complex with L-captopril [25] a similar binding mode was featured. These data are in contrast with the coordination mode of D-captopril with the monozinc CphA (B2), in which the inhibitor carboxylate group coordinates a tetrahedral coordinated Zn(II) ion and the side chains of His196 and Asn233, whereas the sulfhydryl group forms interactions with the side chain of Asn233 as well as with a hydrophobic pocket [101].

Brem and coworkers carried out a systematic study of captopril stereoisomers as inhibitors of MBLs [66]. These efforts are very important for identifying the rationale of the inhibition of a given structural motif which permits to obtain hints that can be used for the development of more potent and effective inhibitors [67, 107].



26

We recently reported the design, synthesis, and the inhibition assays of four bisthiazolidine compounds (**22–25**) bearing thiol and carboxylate moieties as broad-spectrum inhibitors of MBLs of subclasses B1, B2, and B3 [108–110]. These scaffolds are bicyclic mimics of the penicillin core (Fig. 1), with the conserved carboxylate group that is present in all β-lactams, and the bridging N atom that binds Zn2. The four member beta-lactam ring is replaced by a five-member ring that can be decorated with several metal binding groups, such as a thiol moiety, with different stereochemistries. The scaffold can also include the gem dimethyl group present in penicillins (**27–30**).



27 **28** **29** **30**

These compounds were able to penetrate the bacterial periplasm and inhibit NDM-1 in situ. In addition, they were also able to restore the bactericidal activity of imipenem against MBL-expressing clinical strains. The crystal structures of MBLs in complex with these compounds showed that they bound to the active site by coordination of the thiol group between both Zn(II) ions (with the exception of the complex between (**27**) and Sfh-I in which the Zn(II) ion is coordinated by the carboxylate). In all cases, the metal-coordinated waters present in the free enzyme forms were displaced. The carboxylate interacts, either directly or through water intermediacy, with the structurally analogs positions 224 (in B1 enzymes) or 223 (in B3 enzymes), which stabilize the compounds in the active site. The stereochemistry on the bisthiazolidine scaffold did not dominate the binding mode. Instead, inhibition to B1, B2, and B3 enzymes was possible based on the multiple binding modes that can be adopted by these compounds, mimicking the enzyme substrates (Fig. 5).

5.6 Irreversible Inhibitors

Kurosaki and colleagues described the design, synthesis, and biochemical and structural characterization of two irreversible inhibitors (**31**) and (**32**) of IMP-1 [111]. These compounds have in their structure an ester and a thiol group, the latter being responsible of coordinating the binuclear Zn(II) center in the active site. The conserved Lys224 residues, located at 6.0 Å from the two Zn(II) ions attack the activated ester, thus forming a covalently bound inhibitor-enzyme complex that irreversibly inhibits the enzyme. The enzyme-inhibitor structure confirmed the formation of a covalent amide bond between the ester and side chain N^{ζ} atom of Lys224.

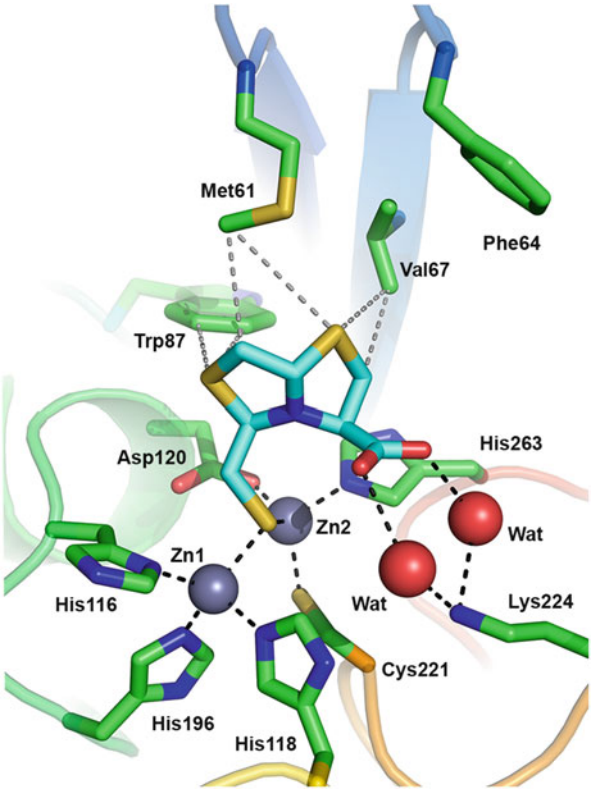
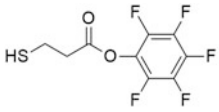


Fig. 5 Structure of compound (**27**) in the active site of NDM-1

A similar strategy targeting Lys 224 has been exploited by Fast and coworkers, who showed that NDM-1 is inhibited by covalent derivatization upon reaction with cefaclor, bound to Lys224 [112]. On the other hand, cephalothin and moxalactam also inactivate NDM (albeit at very high concentrations) by covalently modifying Cys221.



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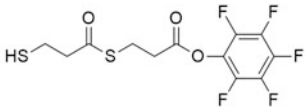
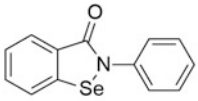


32

Upon Zn(II) dissociation, the free thiol of Cys221 can be covalently modified, giving rise to irreversible inhibition. This inhibition has been reported for the first time by Galleni and coworkers upon treatment of the exclusive carbapenemase CphA with cephamycin or moxalactam [113]. In both cases, a covalent adduct is being formed with the thiol moiety, inactivating the enzyme. Ebselen (**33**), an organoselenium compound with anti-inflammatory activity, was also identified in

a HTS study as a covalent inhibitor forming a Se-S bond [114]. Mercaptoacetic esters (**34**) are also mechanism-based inactivators of BcII, by the generation of mercaptoacetic acid that is irreversibly bound to the enzyme, forming a disulfide bridge with the side chain of Cys221 in the active site [115]. However, this strategy [116] is highly dependent in the event of metal dissociation, which is not necessary coupled to inhibitor binding, and it has been discarded [117].



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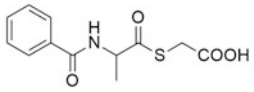
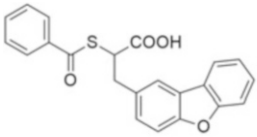


34

5.7 Thioester-Based Compounds

Several thioester derivatives such as (**35**) were identified as inhibitors of IMP-1 [118]. These compounds act as substrates for the enzyme, yielding thiol hydrolysis products which are themselves competitive inhibitors. These observations suggested that thiol compounds rather than thioesters were responsible for the inhibition. In addition, they showed weak inhibition of CcrA, which highlights the structure variability between members of the same MBL subclass. The reversible nature of the inhibition also suggested that there was not a covalent modification of the enzyme, as has been previously observed for mercaptoacetic acid thioester derivative (**36**) with BcII [115]. Thus, these classes of molecules are able to display different inhibition mechanisms against similar MBLs. In addition, thioesters could reverse carbapenem resistance in an IMP-1-producing *E. coli* strain. In another recent example, Liu and coworkers synthesized and assayed several amino acid thioester derivatives as L1 inhibitors, which showed K_i values ranging between 0.11 and 0.95 μM [119].



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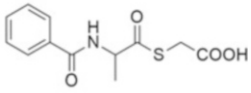

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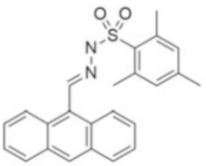
5.8 Sulfonyl Compounds

N-arylsulfonyl hydrazones displaying structural similarities with cephalosporins were synthesized and assayed as inhibitors of IMP-1 [120]. Several compounds exhibited IC_{50} values in the low micromolar range and were optimized by chemical modification at different positions. Then, two compounds (**37**) and (**38**), with the lowest IC_{50} values were selected to identify the inhibition mechanism carried out against IMP-1 and BcII. They resulted as mixed-type inhibitors of IMP-1 with K_i values of 0.7 and 6.6 μM, while they only exerted marginal inhibitory activity against BcII.



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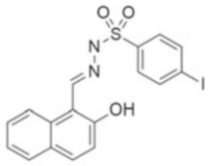

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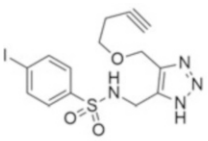
In an effort to identify VIM-2 inhibitors, a high-throughput screening (HTS) study was performed. VIM-2 was screened against a library of pharmacologically active compounds (LOPAC) as well as a novel click-chemistry library enriched in metalloenzyme inhibitors [121]. The four most potent inhibitors were selected to perform a more detailed analysis of their inhibition mechanism. From the LOPAC library, mitoxantrone was found to be a pure non-competitive inhibitor of VIM-2 with $K_i = K'_i = 1.5$ μM, whereas the other compound from this library, pCMB, was a slowly reversible or irreversible inhibitor. Two sulfonyl-triazoles derivatives, (**39**) and (**40**), from the click-chemistry library were identified as VIM-2 inhibitors. These compounds were competitive inhibitors of VIM-2 with K_i values of 0.41 and 1.4 μM, respectively. Then, MICs values for a VIM-2-producing *E. coli* strain were determined in the presence of each inhibitor. Mitoxantrone (MIC = 8.4 μg/mL) and pCMB (MIC = 17.9 μg/mL) exhibited antibacterial activity, whereas sulfonyl-triazoles were inactive. In inhibitor/imipenem synergy assays, potentiation activity of imipenem against VIM-2-producing *E. coli* was observed for mitoxantrone and pCMB but not for click compounds. In a recent study, with the intention of potentiate the in vitro antibacterial activity of (**40**), Weide and colleagues described the synthesis and characterization of several compounds with different moieties in the triazole group [122]. It was possible to identify some very potent inhibitors (K_i values between 0.01 and 0.39 μM against VIM-2) with hydrophobic groups emanating from the C4 methyl of triazole, which can be accommodated in a hydrophobic cavity in VIM-2. By otherwise, all compounds tested were inactive against IMP-1. Interestingly, compound (**41**) improved the MIC of imipenem by threefold at 10 μM against a VIM-2-expressing BL21 strain.



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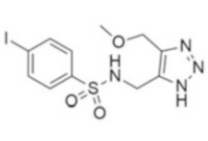

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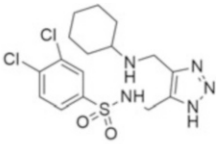

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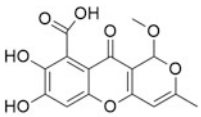
5.9 Natural Products

In an effort to identify MBLs inhibitors, natural product extracts were screened against BcII [123]. Using this approach three tricyclic inhibitors from an extract of *Chaetomium funicola* were detected and successfully purified. The most active of these compounds, SB238569 (**42**), showed K_i values of 79, 17, and 3.4 μM against BcII, IMP-1, and CcrA MBLs, respectively. The crystal structure of CcrA complexed with SB236050 (**43**) demonstrated that the compound has key interactions with Lys224 and Asn233. Also, two rings of the inhibitor form stacking interactions with hydrophobic amino acids present in the "flapping" loop. Finally, both compounds at a concentration of 8 and 32 μg/mL exerted significant drops in the MICs of meropenem for *B. fragilis* organisms producing CcrA.



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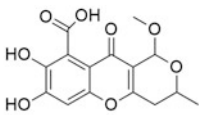
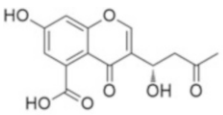


43

Two new polyketide compounds (**44–45**) were identified from the cultures of a Penicillium strain isolated from the rhizosphere soil of the plant *Picea asperata* from Kanas Lake, Xinjiang, China [124]. These compounds displayed inhibitory activity against New Delhi MBL 1 with IC_{50} values of 94.9 and 87.9 μM, respectively. To further evaluate their antibacterial synergy with meropenem, the MICs of meropenem, alone and in combination with (**44**) or (**45**) against an NDM-1-producing strain of *K. pneumoniae* were measured. These compounds had no inherent antibacterial activity at 256 μg/mL, and the MIC of meropenem (128 μg/mL) for this strain was not reduced even in combination with (**44**) or (**45**) at a concentration of 128 μg/mL.



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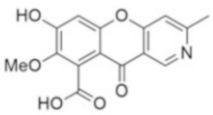

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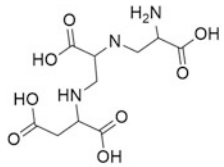
5.10 Metal Chelators

Zn(II) removal is an efficient strategy for inactivation of zinc-dependent enzymes in the test tube. However, chelation therapy is of limited use (generally prescribed for heavy metal intoxication) due to the risky side effects of massive metal depletion in the organism [125]. However, after the discovery of aspergillomarasmine A (see below), this area of research gained some consensus in the community.

Several chelating compounds have been reported as MBL inhibitors, due to their capacity of removing the essential metal ions from the active site. Recently, King and colleagues using an in-house collection of dimethylsulphoxide (DMSO)-dissolved natural product extracts derived from environmental microorganisms, identified an inhibitor of the NDM-1 MBL named aspergillomarasmine A (**46**) [126]. This compound possesses structural similarity with EDTA (**47**), it has the capacity of inhibit NDM-1 and VIM-2 and it is able to restoring the antibacterial activity of meropenem in a murine model system. In addition, it was also reported that other chelating agents such as EDTA-Ca(II), NOTA (**48**), TPEN, DPA, and NODAGA peptide derivatives present interesting inhibitory characteristics and the ability to reduce the MIC against meropenem for several highly resistant bacterial strains [127–129].



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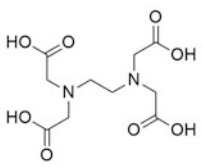

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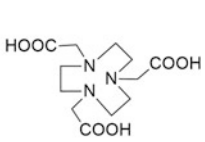

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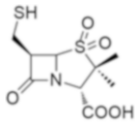
5.11 β-Lactam-Derived Inhibitors

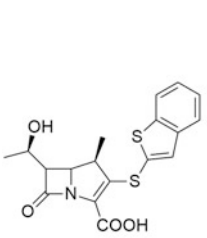
A series of penicillin-derived compounds were identified as MBLs inhibitors [130]. Compound (**49**) has the ability to inhibit both BcII and L1 MBLs and was

also active in vitro, displaying synergy with piperacillin against β-lactamase-producing strains, including a strain of *P. aeruginosa*.



49

Some interesting carbapenem derivatives were described as potent MBLs inhibitors and were also capable of exert in vitro antibacterial activity. Compound J-110,441 (**50**), that possess a benzothienylthio moiety at the C2 position of 1-β-methylcarbapenem, showed K_i values lower than 1 μM against IMP-1, CcrA, L1, and BcII MBLs and also presented good inhibition against class A and class C SBLs with K_i values of 2.54 and 0.037 μM, respectively [131]. Furthermore, the antibacterial activity of imipenem or ceftazidime against several strains of *S. marcescens*, *P. aeruginosa*, and *E. cloacae* was potentiated by the addition of J-110,441, with FIC indexes lower than 0.5. Another related 1β-methylcarbapenem derivative (**51**) was able to inhibit the transferable IMP-1 MBL with a K_i value of 0.18 μM [132]. It was also observed a synergistic effect with imipenem against IMP-1-producing *S. marcescens* and *P. aeruginosa*, with FIC indexes of 0.38 and 0.5, respectively.



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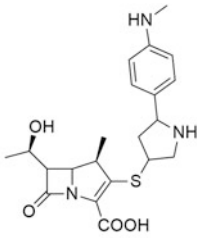
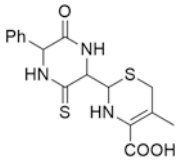


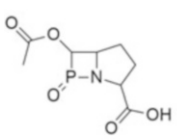
51

Tsang and coworkers reported that thioxocephalosporins are poor substrates for the BcII MBL and act as weak competitive inhibitors (K_i of 700 μM). The hydrolysis product of thioxocephalosporins, a thioacid, also inhibits the enzyme competitively with a K_i of 96 μM, whereas the cyclic thioxo-piperazinedione (**52**), formed by intramolecular aminolysis of thioxocephalexin, has a K_i of 29 μM [133].



52

Yang and collaborators synthesized a phospholactam analog (**53**) of carbapenem and assayed as inhibitor of several MBLs. The phospholactam proved to be a weak, time-dependent inhibitor of IMP-1 (70%), CcrA (70%), L1 (70%), NDM-1 (53%), and Bla2 (94%) at an inhibitor concentration of 100 μM. The phospholactam activated ImiS and BcII at the same concentration. Docking studies were used to explain binding and to offer suggestions for modifications to the phospholactam scaffold to improve binding affinities [134]. Fast and coworkers reported that NDM-1 was irreversibly inactivated by three β-lactam substrates: cephalothin, moxalactam, and cefaclor, albeit at supratherapeutic doses (e.g., cefaclor $K_i = 2.3 \pm 0.1$ mM; $k_{inact} = 0.024 \pm 0.001$ min^{-1}). Inactivation by cephalothin and moxalactam was mediated through Cys221. Inactivation by cefaclor (**54**) proceeded through multiple pathways, in part mediated by Lys224 [112].



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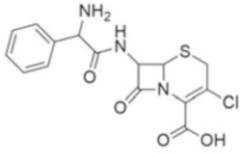

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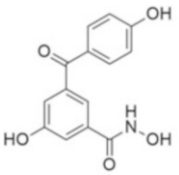
5.12 Hydroxamic Acid Derivatives

Hydroxamates have been exploited as metalloenzyme inhibitors for decades. Lienard and colleagues [135], starting from benzohydroxamic acid structure–activity studies, led to the identification of selective inhibitors of the FEZ-1 MBL, e.g., 2,5-substituted benzophenone hydroxamic acid (**55**) has a K_i of 6.1 ± 0.7 μM against the FEZ-1 MBL but does not significantly inhibit the IMP-1, BcII, CphA, or L1 MBLs. Ganta and collaborators reported several cephalosporin-derived reverse hydroxamates (**56**) that showed submicromolar inhibition of the GIM-1 MBL and were also slowly hydrolyzed by serine β-lactamases, although the degree of inactivation was not enough to be clinically useful [136]. The authors noted that the substitution of the amide N–H bond by N–OH in these compounds may represent a useful strategy for the inhibition of β-lactamases.



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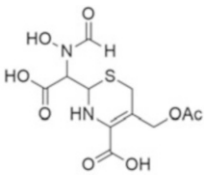

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5.13 Sulfonamide Derivatives

Sulfonamides are well-known inhibitors of the Zn-dependent enzyme carbonic anhydrase. Despite the Zn1 site of B1 and B3 MBLs closely resembles the metal binding site of carbonic anhydrases, sulfonamide inhibition has been scarcely explored. A potent sulfonamide inhibitor of NDM-1 was identified by multi-step virtual screening using a focused NDM-1 inhibitor library containing 298 compounds from ZINC database [81]. Sulfonamide inhibition of the B3 enzyme BJP-1 was studied and validated by X-ray crystallography of the EI adduct. The sulfonamide ligand replaces the bridging hydroxide [26] (the attacking nucleophile) and the water molecule acting as the proton donor, i.e., directly altering the catalytic machinery of the active site. However, inhibition was poor, and this family of compounds clearly deserves more attention.

5.14 Rhodanine Derivatives

Several studies confirmed the inhibitory capacity of diverse rhodanine derivatives against PBPs, SBLs, and more recently, MBLs. In a study reported by Schofield and collaborators, they showed that the rhodanine ring of (**57**) is hydrolyzed to give rise a thioenolate that bridges both Zn(II) ions in the active site of VIM-2 [72]. The crystal structure (Fig. 6) indicated the formation of a ternary complex between the MBL, the thioenolate, and the rhodanine, that also would be formed in solution.

5.15 Thioacetamide Derivatives

Yang and coworkers developed new diaryl-substituted azolylthioacetamides by conjugation of thioazoles with arylacetamide in an attempt to find broad-spectrum inhibitors of MBLs [137]. In this study they reported that 18 of these compounds were inhibitors of the MBL L1 from *Stenotrophomonas maltophilia* with K_i values

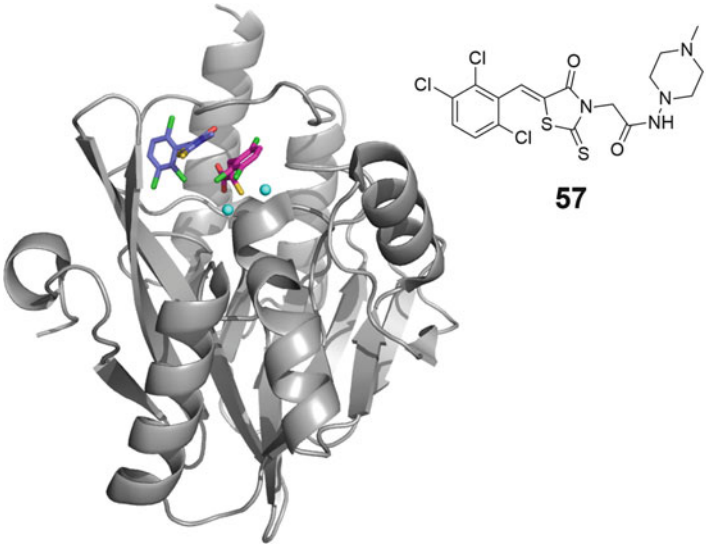
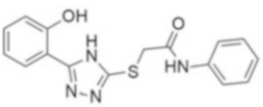


Fig. 6 Structure of VIM-2 in complex with the thioenolate fragment and the rhodanine (**57**). Zn (II) ions are depicted as *cyan spheres*

less than 2 μM, 13 were mixed inhibitors of NDM-1 ($K_i < 7$ μM), and four were broad-spectrum inhibitors of all four tested MBLs CcrA from *Bacteroides fragilis*, NDM-1, and ImiS from *Aeromonas veronii*, and L1 ($K_i < 52$ μM), which are representative of the B1, B2, and B3 subclasses, respectively. Docking studies revealed that the azolylthioacetamides (**58**) coordinate to the Zn(II) ion (s) preferentially via the triazole moiety, while other moieties interact mostly with the conserved active site residues Lys224 (CcrA, NDM-1, and ImiS) or Ser221 (L1). Recently, the same group reported new azolylthioacetamides that were highly effective as inhibitors of ImiS, but did not inhibit CcrA, NDM-1, and L1 in vitro [138]. Finally, the inclusion of diverse azolylthioacetamides, such as (**59**)**,** resulted in lower MIC values when using *E. coli* BL21(DE3) cells expressing CcrA or ImiS or *P. aeruginosa*. In another recent report, this group informed biological activity assays which indicated that amino acid thioester derivatives are very potent inhibitors of L1, exhibiting an IC_{50} value range of 0.018–2.9 μM and a K_i value range of 0.11–0.95 μM using cefazolin as substrate [119]. Partial thioesters also showed effective inhibitory activities against NDM-1 and ImiS with an IC_{50} value range of 12–96 and 3.6–65 μM, respectively. Also, all these thioesters, such as (**60**), increased susceptibility of *E. coli* cells expressing L1 to cefazolin, indicated by a two to fourfold reduction in MIC of the antibiotic. Docking studies revealed potential binding modes of the two most potent L1 inhibitors to the active site in which the carboxylate group interacts with both Zn (II) ions and Ser221.



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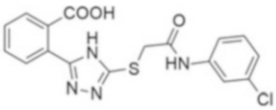

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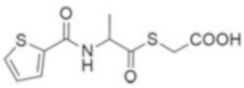

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5.16 Peptides

Rotondo and coworkers used enzyme kinetic assays in combination with fluorescence spectroscopy and stopped flow UV–Vis spectrophotometry to explore the structure–activity relationship of arginine-containing peptides as inhibitors of VIM-2 [139]. This study showed that the inhibitory potency of the investigated peptides was mainly dependent on the number of arginine residues in the center of the peptide sequence, and on the composition of the N-terminus. The most potent inhibitors were found to curtail enzyme function in the mid-to-low nanomolar range. Salts generally reduced peptide-mediated inhibition. Analysis of the mode of inhibition suggests the peptides to act as mixed-type inhibitors with a higher affinity for the enzyme-substrate complex. Stopped flow UV–Vis and fluorescence studies revealed the peptides to induce rapid protein aggregation, a phenomenon strongly correlated to the peptides' inhibitory potency. Inhibition of IMP-1 (another subclass B1 MBL) by the peptides was found to be much weaker than that observed with VIM-2, a finding which might be related to subtle molecular differences in the protein surfaces.

6 Perspectives

Several classes of molecules bearing different zinc binding functionalities (ZBF) able to bind and inhibit mono and di-Zinc MBLs were reported during the last 20 years. Some of these compounds were derived from the natural substrates of MBLs (β-lactam derived inhibitors). Others were obtained from library screening or based in their known ability to inhibit metalloenzymes. However, taking into account that subclasses B1, B2, and B3 share carbapenems as common substrate, the identification of a carbapenem-derived reaction intermediate could serve as a structural scaffold to develop potent and broad-spectrum inhibitors of MBLs. The combination of several of the strategies here presented could also present some perspective, such as the bisthiazolidine scaffold, that mimics a beta-lactam core and includes strong zinc binding groups. Finally, the impact of the use of chelators, such as AMA, could also increase in the near future.

Acknowledgements The work in Rosario has been supported by grants from NIH (1R01AI100560) and ANCPyT. MMG is recipient of a PhD fellowship from CONICET, and AJV is a Staff member from CONICET.

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Inhibitors of Histidinol Dehydrogenase

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Abstract The widespread emergence of resistance and multi-resistance to antibiotics among bacterial pathogens represents a major threat and necessitates a permanent race for finding new molecular targets. Inhibition of enzymes catalyzing particular steps of bacterial amino acid biosynthesis has been considered for several years as a pertinent strategy for drug intervention. Identification of specific inhibitors of the original target histidinol dehydrogenase (HDH, EC 1.1.1.23), the bacterial metalloenzyme responsible for the catalysis of the last step of L-histidine biosynthesis, has opened new opportunities for the development of novel anti-infective agents. This chapter gives an up-to-date overview of the role played by this crucial enzyme in intracellular bacteria and describes the different approaches explored to identify inhibitors of this metalloprotein with potential application as novel antimicrobial drugs.

Keywords Antibacterial, Histidinol dehydrogenase, Inhibitors, Intracellular bacteria, Metalloenzyme, Virulence factor

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1 Introduction

Emergence of resistance and multi-resistance to antibiotics among bacterial pathogens constitutes currently an alarming health problem worldwide. There is a growing need to identify and validate rational drug targets in bacteria in order to develop effective new classes of antibacterial agents that could circumvent the established resistance mechanism [1, 2]. Bacterial and mammalian metabolism show significant differences which are characterized by the fact that most microbial species have the capacity to synthesize all of their important metabolic building block molecules, while mammals must acquire many of these from dietary sources.

Within this context, targeting key enzymes involved in the amino acid biosynthesis has been a long-lasting strategy used by medicinal chemists for the development of new agents with selective antibacterial properties [3].

Histidine biosynthesis pathway, which consists in the conversion of 5-phosphoribosyl-1-pyrophosphate to L-histidine in ten enzymatic steps, has attracted attention of researchers, as this pathway is found in bacteria, fungi, and plants, but is absent in mammals [4]. This pathway thus provides a substantial number of highly attractive potential protein targets to be examined for the development of selective antibacterial agents.

L-histidinol dehydrogenase (HDH, EC 1.1.1.23) is a 4-electron oxidoreductase involved in the last two steps of L-histidine biosynthesis, where it catalyzes the sequential NAD-dependent oxidations of L-histidinol to L-histidinaldehyde and then to L-histidine using a Bi-Uni-Uni-Bi PingPong kinetic mechanism [5–8] (Fig. 1).

HDH, a well-conserved protein in plants, archae, and bacteria [9], is a metalloprotein of 47 kDa which presents a homodimeric structure with one Zn^{2+} cation in each subunit. The presence of a divalent metal ion is essential for the enzymatic activity: the replacement of Zn^{2+} cation causes either a decrease of enzymatic activity (replacement with Mg^{2+}, Ni^{2+}, Co^{2+} or Cu^{2+}) or an enhanced activity (replacement with Mn^{2+} or Cd^{2+}) [5, 6].

This metalloenzyme has been thoroughly investigated and characterized in plants [10] but also in bacteria such as *Escherichia coli* and *Salmonella typhimurium* [11, 12]. In addition, various studies identified HDH as a virulence factor in pathogenic bacteria with intramacrophagic development such as

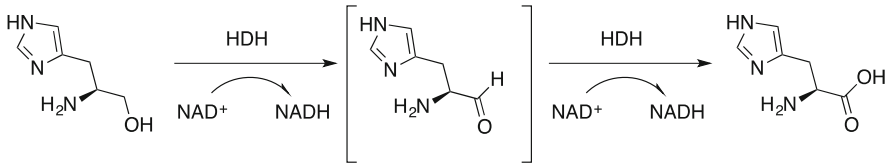

Fig. 1 Sequential 2-step oxidation catalyzed by L-HDH

Burkholderia pseudomallei [13], *Mycobacterium tuberculosis* [14], and, more recently, *Brucella suis* [15]. Therefore, the possibility to selectively inhibit the bacterial enzyme HDH inside the host cell has opened a new route towards the discovery of new antibacterials with reduced risk of secondary effects on the host itself.

This chapter is dedicated to review the importance of histidinol dehydrogenase as a target for the development of new antimicrobial agents.

2 HDH in Bacteria

Histidinol dehydrogenase in bacteria has been studied for more than 50 years, following the establishment of the biochemical reaction catalyzing the last step of histidine biosynthesis, with production of histidinal as an intermediary product [16]. The first bacterial species, from which HDH has been purified, was *S. typhimurium*. In this bacterium, the histidine biosynthesis pathway had been well characterized, and many mutants were available. Purification confirmed that a single functional enzyme catalyzes the two oxidation steps from histidinol to histidine, and stimulation of the enzymatic activity by addition of Mn^{2+} was observed [17]. A few years later, the amino acid composition of HDH was reported, and several lines of evidence suggested that this enzyme of *S. typhimurium*, encoded by the gene *hisD*, was a dimer of identical subunits with an estimated molecular weight of 40 kDa each [12]. More recently, based on gel filtration, SDS-PAGE gel separation techniques and gene sequence information, this value was corrected to approximately 46–47 kDa per subunit [18].

In 1982, Andorn and Aronovitch published the purification and characterization of HDH from a derepressed mutant of *E. coli*, aiming at the comparison of the enzymes from the two species [19]. The molecular weight of the monomer, obtained by SDS-PAGE, has been determined to be about 52 kDa. Despite slight differences in molecular weight, structures and catalytic properties of the two enzymes are very similar: Native HDH forms a dimer of identical or nearly identical subunits; both enzymes are highly specific for histidinol and NAD^+, have a pH-optimum of 9.5 for the reaction, and can be activated by Mn^{2+}, which stabilizes the enzyme in its catalytically active form.

Furthermore, stereochemical and active site studies of HDH have been performed on the enzyme from *S. typhimurium*, yielding several important results:

(1) Stereochemistry at the cofactor NAD, i.e., geometric relation of NAD to substrate, is the same in both steps of histidinol dehydrogenase reaction; (2) An active site base occupies the same location in both steps; (3) The imidazole ring of histidinol binds to a specific portion of the active site by close interactions via the 1-, 2-, and 3-positions and does not rotate freely in the active site; (4) The use of side chain analogues such as ethanolamine or of molecules in which the alpha-amino group has been replaced yielded a dramatic decrease in catalytic activity of HDH; the same was true for the removal or modification of the imidazole ring [20]. Kinetic isotope effects studies with deuterated histidinols on *S. typhimurium* HDH revealed that the rate constants obtained at pH 9.0 allowed kinetic simulations indicating a thermodynamically unfavorable but relatively fast hydride transfer from histidinol, and an irreversible and slower second hydride transfer from a histidinal derivative [21]. The authors concluded that both partial reactions participate in the overall rate limitation. Site-directed mutagenesis of *hisD*, resulting in the replacement of the five His-residues with asparagine or glutamine, caused an important decrease in k_{cat} for His-261 and His-326 [22]. Experiments suggested that both residues are involved in proton transfers during catalysis, and that His-261 and His-418 are candidates for zinc ion ligands, as affinities for metal ions decreased with substitutions at these residues.

More recently, HDH of the facultative intracellular pathogen *B. suis* has been identified as being essential for infection of the host cell, as a Tn5-mutant affected in *hisD* is strongly impaired in intramacrophagic replication [23]. At 48 h post-infection, the number of viable intracellular bacteria is 1,000-fold lower than that of the wild-type strain. In parallel, it has also been described for *M. tuberculosis* that human THP-1 macrophage-like cells are able to restrict growth of a *hisD* mutant auxotrophic for His [14]. This led to the conclusion that in both pathogens bacterial biosynthesis of His is crucial for intracellular growth and that these vacuole-borne pathogens have no access to this amino acid produced by the host cell. Based on these fundamental findings, we then made the choice to focus on the *hisD*-encoded enzyme HDH of *Brucella* sp. as a potential target of novel antibacterial agents that are currently under development and study (see below). To this end, *hisD* of the human pathogen *B. suis* was cloned and overexpressed in *E. coli* and the recombinant, His-tagged HDH was produced and purified as a stable 49 kDa-protein, as evidenced by SDS-PAGE [24]. The activity assay used directly monitored the reduction of NAD^+ to NADH at 340 nm in the presence of histidinol, as described previously [17], and the Km-value for histidinol is approximately 12 μM. This was the first description of the biological activity of HDH of *Brucella* [24].

The fourth bacterial species of which the HDH enzyme has been studied is *M. tuberculosis*. This pathogen, responsible for tuberculosis, represents a major concern in public health, as it causes approximately 1.3 million deaths per year worldwide, and an increasing number of multi-drug-resistant cases have been reported. As a first step towards the possible development of antibacterial drugs targeting HDH of *M. tuberculosis*, following cloning and overexpression of the *hisD* gene, the enzyme has been purified in an untagged form by anion exchange

and gel filtration chromatography [25]. Analytical gel filtration, inactivation by chelating agents, and activation by divalent metal ions confirmed the homodimeric metalloenzyme character of the protein. Primary sequence comparisons and molecular homology modeling of *M. tuberculosis* HDH led to the proposal of the amino acids residues involved in substrate binding.

Following fundamental work that has been achieved with *S. typhimurium* and *E. coli* HDH on comprehension of the structure and mechanisms of enzyme action, studies on HDH of *B. suis* and later of *M. tuberculosis* opened up new strategies for the targeting of these bacterial virulence factors, essential during the interaction with their hosts, in the search of novel antibacterial agents.

3 HDH Crystallographic Studies: Modeling

Histidinol dehydrogenase is a homodimeric metalloenzyme with one Zn^{2+} per monomer. Despite the fact that it has been known and studied for many years [26], the number of deposited structures is still limited since only seven entries can be found in the Brookhaven Protein Data Bank. Among these, four were obtained with different or no ligands from *E. coli* [18] and two were the *B. suis* [27] protein with and without an 'in house' inhibitor. The last one was the *Methylococcus capsulatus* homolog in its apo form and has not been associated with a publication to this date (PDB code: 4GIC). While the informations pertaining to HDH three-dimensional structure are limited, the enzyme mechanism and role of the different residues have been much studied along the years.

Kinetics of the enzyme was explored by Grubmeyer [6], allowing insights for the comprehension of the mechanism and was followed by mutations of the protein [28] that quickly led to a complete site mapping of the active site [20]. Indeed, the role of Zn^{2+} and the presence of NAD^+ in the active site were elucidated, along with the number of active sites of the dimeric, active enzyme [5]. The presence of two identical active sites, one in each subunit of the dimer, was ascertained, allowing a better understanding of the enzyme. This work also led to the identification of two histidine residues critical for the activity. In *S. typhimurium* [22], His-261 and His-418 mutations suggested that both amino acids were zinc ligands, while another team agreed with this statement in *Brassica oleracea* [29] by the mean of biochemical experiments. ^{113}Cd NMR studies allowed confirmation of most of the site mapping and mechanism previously enunciated with suggestions about the coordination of the metal ion by the substrate [30]. The data suggested that one nitrogen atom from the imidazole ring and an oxygen from the substrate/inhibitor would bind the metal ion, while the nitrogen atom from the amine in the ligand would not. The same technique demonstrated that the essential Zn^{2+} could be substituted by other cations like Cd^{2+} or Mn^{2+} [31], which justify the use of NMR when X-ray data are not available for metalloenzymes.

The first crystalline HDH forms, with or without inhibitor/substrate, were then reported but not divulgated, due to crystal instability [32]. Even the purported

inhibitor structure was not shown while four different crystal structures were discussed. At this time, the enzymatic reactions mechanism was still debated, until it was clearly proposed by Grubmeyer some time later [22].

However, inhibitors related to L-histidinal were disclosed along with a computational model which aimed to study the binding conformation of the intermediate aldehyde [33]. Ligand-based method using these inhibitors allowed a pharmacophore mapping of the cabbage HDH active site. The obtained L-histidinal conformation was compared to already published crystals from the Cambridge Structural Database. This, in turn, confirmed that ligand molecules were able to bind the metal ion with or without NAD^+, confirming the NMR data already mentioned [30, 31], and the crucial presence of Zn^{2+} for positioning of the ligand. Particularly, the model was completely coherent with the substrate binding proposed in the ^{113}Cd studies and was similar to the Zn^{2+} binding site of a published carbonic anhydrase structure (PDB code 1H4N).

The first published crystals were obtained with the *E. coli* HDH in its native state and with several substrates and Zn^{2+} [18]. The dimer layout resulting in an active site displayed a domain swapping between the monomers and allowed a complete mapping of the Zn^{2+} and substrate binding by the involved residues. More important, the NAD^+ molecule was crystallized with L-histidinol into the active site, allowing the very first view of the cofactor in the enzyme in one of the four obtained PDB structures. However, the electronic density was not well defined for NAD^+, allowing different binding modes, but mostly not accounting for the enzymatic activity because of its distance to L-histidinol, which was slightly superior to what was expected. This resulted in a lack of activity that probably explained why both substrate and cofactor were trapped simultaneously in the active site.

The substrate position was more decisive, since obtained from three similar structures and related molecules: L-histidinol, L-histamine, and L-histidine. In all cases, contrary to the NMR studies discussed previously, the amine function was directed unambiguously toward the metal ion. A detailed analysis of the role of the active site residues was possible and confirmed the enzymatic mechanism proposed in a previous work [22]. Another crucial finding of this study was the discovery of an incomplete Rossmann-fold in two domains of the protein, which could not be discerned by homology before this work. Indeed, one strand-helix hairpin was missing to allow recognition by sequence alignment [18].

The *B. suis* HDH was the latest published structure with a nanomolar inhibitor and an unbound form [27]. Only a C366S mutant allowed crystallization to process, probably forbidding oxidation/reduction of the native enzyme at this position. Because of the sequence identity with the *E. coli* enzyme, molecular replacement was used to solve the structure of the apo form, which in turn was used to refine the structure of the inhibitor/enzyme complex. As expected, the similarity between the *B. suis* and *E. coli* proteins was rather high, since only one insertion and two deletions were noted between the sequences, allowing only two new secondary structure elements, far from the active site. Consequently, the RMSD value between the apo structures was rather low, and the residues responsible for the substrate and NAD^+ binding were highly conserved between the two species. Once again, the

amine group was found responsible of the substrate orientation by interacting with the Zn^{2+} while the overall stabilization of the cation changed between the unbound and bound forms. In the apo structure, the Zn^{2+} coordination was tetrahedral, while it was found to be octahedral in the inhibitor/enzyme complex. There was also some change of residues involved in the cation binding, since the Zn^{2+} position shifted of 1.5 Å when the inhibitor was present.

With the availability of three-dimensional structures, new perspectives will open up in terms of drug design that should rapidly lead to new inhibitors and enzymatic mechanism determination.

4 HDH Inhibitors

Inhibition study of bacterial L-HDH was first reported by Grubmeyer in 1989 [20]. In this paper, the authors described the inhibition study of a library of histidinol analogues against *S. typhimurium* HDH. One of the most potent inhibitors tested was found to be the α-methyl ketone **1** showing an inhibitory activity in the micromolar range ($K_I = 5$ μM).

Based on the structure of **1**, Dancer et al. [34] reported in 1996 the synthesis of novel inhibitors by increasing the length of the side chain in order to target a potent adjacent lipophilic pocket close to the active site. Among the small inhibitors described, compounds **2a** and **2b** showed the best activity against *E. coli* (IC_{50} of **2a** = 1 μM) and cabbage (IC_{50} of **2b** = 0.04 μM) L-HDH (Fig. 2).

In line with the structures previously described by Grubmeyer and Dancer research groups, Abdo et al. investigated in 2007 new inhibitor series [24]. Inhibitory activities obtained on purified *B. suis* L-HDH varied from 3 to 200 nM with 11 highly potent compounds (IC_{50} below 30 nM), the best being compound **3** with an IC_{50} of 3 nM. In order to improve the potency of this model inhibitor, another library of inhibitors was designed by the same group and reported in 2011 [35], in which the structure was extended by a second aromatic ring with length and geometry variabilities at the linkers level. Good to very good inhibitory activities against *B. suis* HDH were observed with $IC_{50} \leq 70$ nM, the most potent inhibitor being compound **4** (Fig. 2).

Always in the context of the pathogen *B. suis*, the same group reported in 2008 [36] and 2014 [37] two new series of inhibitors having respectively: or a sulfonyl hydrazide moiety as linker of the side chain with aromatic rings, or an oxo- and thiooxo-imidazo[1,5-c]pyrimidine ring in place of the aminomethyl imidazole part. Despite the variety of structure, these modifications did not provide more potent inhibitors, inhibitory activity against *B. suis* HDH staying in the micromolar range with the best inhibitors **5** and **6** having respectively an IC_{50} of 25 and 5 μM (Fig. 2).

Other interesting series of inhibitors were also reported in 2008 [38] and 2010 [39] by Pahwa et al. against fungal histidinol dehydrogenase. Two new compounds **7** and **8** designed from docking results turned out to be potent micromolar range

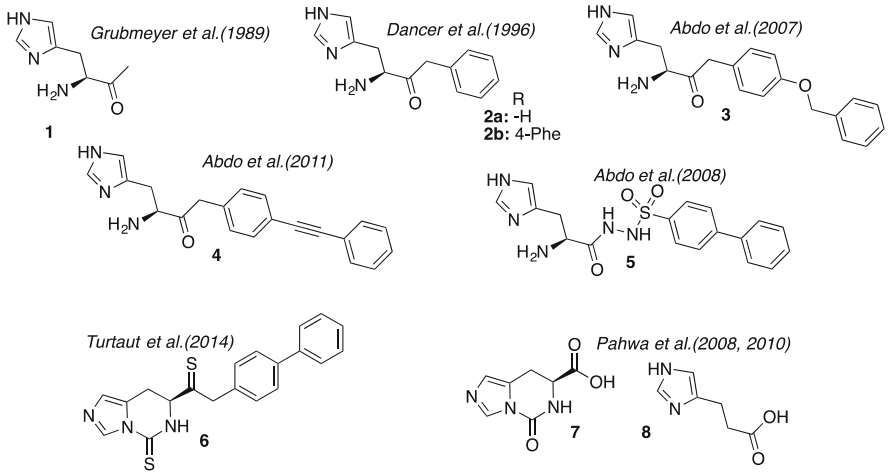

Fig. 2 Structure of bacterial and fungal L-HDH inhibitors

inhibitors of *Geotrichum candidum* histidinol dehydrogenase, showing IC_{50} values around 3 μM (Fig. 2).

5 Applications: HDH as Therapeutic Target for the Development of Anti-infectious Compounds

Among human pathogens, intracellular bacteria constitute a major class, characterized by their capacity to multiply within the host cells. This family of bacteria, to which belong also *M. tuberculosis* and *Brucella* sp., are usually sensitive to the antibiotics currently used in therapy, but multi-, extremely, or even totally drug-resistant clinical isolates of *M. tuberculosis* have been reported these past years. As *Brucella* sp. has been considered as a potential bioterrorism agent in the USA and in certain European countries and classified as such, the requirement for the identification of new bacterial targets and the development of the corresponding novel antibacterial drugs is essential to avoid situations where certain populations would be facing failure of treatments due to the incapacity to handle pathogen resistance. To cope with these risks, two approaches are possible: (1) targeting of conserved bacterial functions, in general essential for bacterial replication and growth; (2) targeting of virulence factors such as those involved in intracellular replication within the host organism [40]. Analysis of the intramacrophagic virulome of *Brucella* by Tn5-mutagenesis allowed indirect characterization of this niche, and a number of genes essential for intramacrophagic replication were identified [23]. The HDH-encoding gene *hisD* figures among these genes, indicating that *Brucella* needs to synthesize His in order to multiply within the macrophage. Such a target-based approach, aiming specifically at affecting intracellular replication, has

several advantages: (1) Absence of impact on the extracellular, natural commensal flora of the host; (2) Limitation of the possible selective pressure on the intracellular niche; (3) Possible lower mutation rate in bacteria exposed to the inhibitor, as selective pressure occurs only in the host cell; (4) Novel inhibitors may be used as antibacterial adjuvants, in combination with classical antibiotics, thereby diminishing the risk of appearance of resistance to antibiotics [40].

Besides its obviously essential role in intracellular replication of *Brucella*, HDH has been chosen as a potential target of novel antibacterial agents for the following reasons: (1) Lack of the presence of amino acids biosynthesis pathways in mammals, hence reducing the risk of undesired secondary effects on the metabolism and the physiology of the host cell; (2) Possibility of overexpression and purification as a recombinant protein from *E. coli*; (3) Straightforward measurement of enzymatic activity by spectrophotometry, allowing the convenient screening of a large number of molecules; (4) Accessible synthesis of substituted benzylic ketones as substrate analogues, based on previously described scaffolds [34].

The efficiencies as HDH inhibitors of the series of substrate analogues described in the previous paragraph (see Fig. 2) were evaluated by different approaches: First, the molecules were tested on the purified enzyme and the corresponding IC_{50}-values were determined. This allowed the identification of at least 15 inhibitors with IC_{50}-values in the range of 3–200 nM, of which some are more efficient on *Brucella* than on cabbage HDH [24]. Then, the biological effects of the same inhibitors on the in vitro growth of *B. suis* in minimal medium and on the replication of the pathogen in human macrophage-like THP-1 cells were investigated. Minimal medium mimicks the nutrient-poor *Brucella*-containing vacuole in the macrophage, and the bacteria have to synthesize His and the other amino acids under these conditions. Inhibition of HDH is therefore expected to abolish bacterial growth. The drugs most effective in strongly inhibiting bacterial growth in minimal medium in the μM-range were also the most active ones in the inhibition of HDH activity, possessing the lowest IC_{50}-values obtained [41]. The specific action of these inhibitors on the His-biosynthesis pathway was validated by the relief of growth inhibition upon addition of His to the cultures. The drugs most active on Brucellae in minimal medium were tested for their capacity to block intramacrophagic growth of *B. suis*: At 24 h post-infection, replication of the bacteria inside the cells was reduced 50–2,500-fold [41]. Altogether, these results also led to the conclusion that the benzylic ketones have the capacity to cross the cytoplasmic and the bacterial membranes to reach their target and may be concentrated in the vacuoles containing *Brucella*. The data obtained therefore confirmed for the first time the purification of active HDH from *Brucella* and the suitable character of this enzyme as a target for the potential development of novel, non-classical antibacterial therapy against a facultative intracellular pathogen.

The initial, most active hit compound **3** was the starting molecule for the optimization of HDH inhibitors by Suzuki and Sonogashira reactions, introducing substituted aryl or alkynyl aryl tails [35]. A second, major hit compound **4** with an IC_{50}-value of 3 nM was discovered in one of these new series, where IC_{50}-values of all the molecules were also in the nM-range. Growth of *Brucella* in minimal

medium was affected to a variable degree, ranging from 30 to 100% inhibition. Obviously, subtle structural modifications in the tail parts of the molecules influenced the degree of enzyme inhibition. The effect on intramacrophagic bacteria was even more pronounced than with the first hit, as a concentration of 5 μM reduced survival by a factor 1,000 [35], confirming that development of these inhibitors may be a promising step towards novel anti-virulence drugs.

6 Conclusion: Future Prospects

As a non-human enzyme involved in the last two steps of histidine biosynthesis, HDH can be considered as a very promising target in bacteria. This enzyme was proven to be a virulence factor in several intracellular pathogens and its inhibition constitutes an innovative antibacterial approach. Preliminary interdisciplinary approaches involving bacteriology, structural studies, and medicinal chemistry have allowed to identify very potent inhibitors with interesting activity in vitro against *B. suis*. Complementary studies on these drugs must be undertaken in vivo to evaluate their potency in the murine model of *Brucella* infection. Studies on the activities of these compounds have to be extended to *M. tuberculosis* and its purified HDH. Deepening the knowledge on the interactions between inhibitors and the active site of HDH of pathogenic bacteria will result in a better understanding of mechanisms of inhibition and will lead to the design of new potential drugs with improved activity.

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Botulinum Neurotoxin, Tetanus Toxin, and Anthrax Lethal Factor Countermeasures

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Abstract Bioterrorism constitutes the deliberate release and dissemination of biological agents to incapacitate, maim, or kill individuals, groups, or populations, including humans, animals, and/or plants, in acts of terrorism. When carried out in the context of war, these acts are also termed biological warfare. Agents can be of bacterial, fungal, or plant origin; bacterial toxins are of particular concern as bioweapons due to their ease of production and weaponization, and their high level of toxicity to humans. Most macromolecular toxin-based biowarfare agents are metalloenzymes, featuring a catalytic metal in their active site(s) that is chiefly responsible for proteolytic activity leading to host cytotoxicity. This chapter outlines recent work on three metalloenzyme toxin types which exhibit high potential for deployment as bioterror agents: the botulinum neurotoxins (BoNTs), the tetanus toxin (TeNT, also known as tetanospasmin), and the anthrax toxin lethal factor (LF). All of these enzymes are deadly to humans; additionally, they are challenging to detect, and their toxic effects are difficult to treat. Although a great deal of research effort in this area has resulted in key steps forward that are discussed here, reliable and effective post-exposure countermeasures to these toxins remain elusive. A comprehensive national security and anti-bioterrorism strategy in the twenty-first century must therefore prioritize continuing research to combat these threats.

Keywords Anthrax toxin lethal factor, Bioterrorism, Biowarfare, Botulinum neurotoxins, Tetanus toxin, Toxins, Zinc metalloenzymes

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1 Introduction

Zinc metalloenzyme-based toxins have emerged as popular therapeutic targets, in various human and non-human disease modalities as well as in bioterror scenarios [1–6]. This review addresses recent efforts to target three zinc-bearing toxins that pose a particular threat to the civilian and military populations as potential biological warfare agents (BWAs): the botulinum neurotoxins (BoNTs), the tetanus toxin (TeNT), both of which are clostridial metal-bearing neurotoxins, and the anthrax toxin lethal factor (LF), a zinc hydrolase. BoNTs and TeNT function via similar yet distinct mechanisms, entering neurons and interfering with neurotransmitter release by cleaving key components of neuroexocytosis systems [7, 8]. LF engenders anthrax-related cell death by disabling mitogen-activated protein kinase kinase (MAPKK) enzymes that play key roles in the host immune response; this zinc-binding enzyme also functions by a number of other pathways that are less well understood, including one or more mechanisms that result in the disruption of endothelial cells, leading to eventual circulatory shock and host death [3–6, 9–12]. Any or all of these toxins could be deployed in a bioterrorism attack, to cause illness, death, social disruption, and/or saturation of medical facilities, and targeting individuals, groups, nations, or populations. Importantly, beyond life-threatening disease, bioterror attacks involving toxins such as these can result in wide-ranging panic, economic losses, contamination of food and water supplies, and interference with energy and transportation networks. Risks to warfighters and to the civilian population continue to be significant. Notably, detection of BWAs, especially toxins, is often challenging, and in the case of anthrax, definitive symptoms of illness may not manifest for hours to days, at which point treatments may be of limited efficacy. Early detection and treatment therefore continue to be key.

The three pathogens discussed in this manuscript have emerged as popular therapeutic targets over the last two decades, especially BoNT serotypes and LF. The work reviewed here incorporates a variety of existing and novel pre- and post-exposure treatment design strategies, involving computer simulations, fragment screening, high-throughput screening (HTS) of molecular libraries, antibody design, structural biology approaches, and cell-based assays, which taken together have led to several promising new drug scaffolds, antibodies, and vaccine modalities that are currently under investigation. In silico techniques have been particularly popular and useful methodologies to aid and guide the design and optimization

process of metalloprotein-targeted small molecules, specifically pharmacophore mapping, support vector machine (SVM) modeling, and three-dimensional quantitative structure–activity relationship (3D-QSAR) modeling [4, 6]. Newer "lead-hopping" techniques such as topomeric searching, where a highly active but pharmacokinetically compromised compound is used as a template to "leap" to new scaffolds that manifest similar three-dimensional shapes but feature different functional groups, have shown particular promise for identifying small molecules that can be "built" or optimized into new drugs, with the overall goal of retaining biological activity while avoiding impediments to effective in vivo metabolism [1, 13–15]. Advances in experimental fragment and HTS technologies have also facilitated new compound identification. The research reviewed here outlines several unprecedented steps forward in terms of therapeutic design and optimization; however, given the complex nature of anti-BWA drug discovery and mechanistic research, greater strategic and financial commitments in this area, and continuing focus on medicinal chemistry approaches will be critical in order to obtain highly effective countermeasures against toxin-related biowarfare.

2 Botulinum Neurotoxin Inhibitors

The botulinum neurotoxins (BoNTs) constitute a class of highly lethal neurotoxins, which act inside peripheral nerve terminals and are considered to be the most lethal toxins to humans in existence today; the estimated intravenous human LD_{50} of BoNTs is as low as 1–2 ng/kg [16]. As the primary cause of botulism-related neurotoxicity, BoNTs are generated by the anaerobic, Gram-positive bacterium *Clostridium botulinum* and are classified into seven antigenically distinct serotypes (BoNT/A–BoNT/G) based on specific immunological properties [17–20]. All of these toxins act by the same mechanism, in which they interfere with the release of acetylcholine at peripheral cholinergic nerve terminals, resulting in a rapid-onset neuronal paralysis of long duration, which in turn leads to respiratory failure and subsequent host death. BoNT/A has garnered a great deal of attention as a therapeutic target due to its particularly potent toxicity, as well as its potential use as a BWA. The Centers for Disease Control and Prevention (CDC) classifies BoNTs as Tier 1 Select Agents with high potential to be weaponized. Although a bioterror attack involving BoNT/A is yet to be successfully carried out, evidence exists that Iran, Iraq, North Korea, and Syria, among other nations harboring terrorist groups, have constructed and stockpiled biological weapons containing various BoNT serotypes [21].

Structurally, BoNTs consist of two chains (L = Light, ~50 kDa and H = Heavy, ~100 kDa), joined by a disulfide bond; these chains in turn comprise three approximately 50-kDa domains, each of which exhibits a specific, fairly well-characterized yet complex functionality [17–20, 22]. BoNTs are highly flexible, capable of adopting a fairly broad variety of conformational states, which, together with their unusually large catalytic substrate binding sites, has historically

complicated the therapeutic design process. The N-terminal domain of BoNT, on the L chain, is involved in zinc-catalyzed metalloprotease activity; residues in the C-terminal domain on the H chain are responsible for neurospecific interactions with neuronal presynaptic membranes; and the "intermediate" domain on the H chain acts to translocate the L chain through the neuronal membrane. When the toxin enters the cytosol, the L chain cleaves a soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) protein that is necessary for vesicle fusion with the neuronal plasma membrane, resulting in the lethal toxic buildup of acetylcholine within the cell [23].

Current treatments for botulism are mostly limited to supportive therapy and antitoxins of limited efficacy. Standard treatment protocols require early diagnosis, immediate treatment with the equine antitoxin heptavalent botulism antitoxin (HBAT) [24], intensive care hospitalization, and aggressive ventilation. However, the effectiveness of this protocol is limited; like other antitoxins, HBAT can clear BoNTs already in circulation, but is unable to inhibit the action of toxins that have already translocated into neurons. HBAT also presents several undesirable side effects, including serum sickness. Newer work toward countermeasures against BoNTs has therefore focused on medicinal chemistry approaches, particularly the design and optimization of small molecules to directly inhibit one or more domains of BoNTs, taking advantage of recent discoveries in mechanistic pathways. Work continues to proceed on vaccine-based therapeutic options for botulism; [25] however, this review will focus chiefly on small-molecule approaches, with a brief discussion of recent advances in concurrent antibody-based therapeutic development.

Smith and coworkers reported a series of potential small-molecule inhibitors of BoNTs in 2009, and in 2015 followed up with further work focusing on the two most potent inhibitors from that study [26]. Via molecular modeling, peptide cleavage assays, and ex vivo assays, these researchers demonstrated that the two new molecules not only inhibited BoNT/A but also exhibited varying potency against other serotypes. These compounds were found to inhibit BoNT/B at ~30–31%, as well as serotypes C, E, and F at somewhat lesser potency [26]. This manuscript was the first to report small molecules that were able to inhibit four BoNT serotypes in an ex vivo assay; however, the authors note that the compounds are currently not feasible as therapeutics as they cannot cross the cell membrane; further work will be necessary to increase bioavailability as well as binding affinity to the BoNTs [26].

Also in 2015, Jin and coworkers found that oral intoxication of BoNT/A can be inhibited by carbohydrate receptor mimics [27]. This approach appears favorable due to the low cost, high stability, ease of administration and transportation, and lack of adverse effects demonstrated by these molecules; however, potency against BoNT remains a challenge. The authors focused on lactulose as a prototype inhibitor against BoNT intoxication, establishing that lactulose blocks host galactose-containing carbohydrate receptor binding to BoNT and thereby interferes with BoNT translocation into cells [27]. Although the simple carbohydrates reported in this paper demonstrated rather weak inhibition of BoNT/A, the authors

recommend the development of multivalent carbohydrate inhibitors that are capable of targeting multiple sites on the BoNT chain complex, for potential use as adjunct and/or preventive therapeutics.

The Floriano group subsequently developed a series of molecular probes which bind to alternate and/or potential allosteric sites on BoNT/A [28]. These workers used scintillation proximity assays (SPAs) to evaluate five probe candidates that had been predicted in silico (using the authors' Protein Scanning with Virtual Ligand Screening [PSVLS] approach) to demonstrate affinity for various non-orthosteric binding sites on both chains of BoNT/A. Although these small molecules were not designed as therapeutics, they were able to help pinpoint multiple, structurally distinct binding regions on BoNT/A that are not conserved across the BoNT serotypes, elucidating key structural features that could be exploited to increase small-molecule selectivity and preference for BoNT/A in particular. This work constitutes an important first step toward potentially highly selective botulism treatments that are less likely to exhibit off-target effects. Also, the authors note that these probes could be used to monitor treatment response via medical imaging without interfering with standard treatment protocols [28]. The authors do observe, however, that the PSVLS approach does not sufficiently address BoNT structural flexibility, and recommend that further in silico studies in this area incorporate molecular dynamics to explore potential conformational states of the enzyme [28].

The Janda group at the Scripps Research Institute has conducted extensive studies toward inhibition of BoNTs; in 2014, these workers reported a series of benzoquinones that irreversibly inhibit BoNT/A in the low micromolar range [29]. These workers established key structure–activity relationships for a series of benzoquinone and naphthoquinone-based compounds bound to BoNT/A, and pursued a fragment-based design strategy to target a key cysteine residue they pinpointed. Although the fragment approach was largely unsuccessful, the researchers found that substitution at the two and three positions on the naphthoquinones in particular was found to enhance binding, and the overall discovery process resulted in identification of three new small molecules that could be modified further to increase activity. In 2015, the Janda group investigated Dyngo-4a™, an endocytic inhibitor of BoNT/A via dynamin inhibition [30], and found that it also exhibits activity against the metalloproteinase itself, likely through an exosite.

Swaminathan and colleagues employed high-throughput computational screening to evaluate a library of commercially available compounds from the online ZINC database for potential activity against the light chain of BoNT/A [31]. Further in vitro and cell assay work led to the identification of two lead compounds with potency against the toxin in the low micromolar range; the researchers also synthesized a subset of lead analogs, with one exhibiting higher potency than the original leads, with a BoNT IC_{50} of ~16 μM. These workers also reported a cyclic peptide inhibitor of BoNT/A light chain, with an extremely promising biological activity of 12.3 nanomolar [32]. X-ray studies reported in this work illustrated that the peptidic inhibitor adopts a helical conformation and occupies the majority of the

active site, blocking the substrate binding path at the BoNT/A surface as well as the catalytic zinc region in the active site. It was also found that a key disulfide bond was responsible for inhibitor stability. The authors suggest that a useful approach for further work would be to convert cyclic peptides such as that reported in this manuscript to hydrocarbon-stapled peptides, permitting cyclization using a hydrocarbon chemical "brace" to lock the inhibitor into an active conformation, rather than relying on disulfide bonds alone – which would also be challenging given the flexibility of the BoNT enzyme. New peptide-based inhibitors targeting BoNTs were also developed by Chen and coworkers [33], targeting the two BoNT structural regions involved in substrate recognition and protein cleavage, in a similar "double-barreled" approach. With activity in the low nanomolar range, these are among the most active BoNT inhibitors discovered to date, and also demonstrate favorable toxicity profiles and excellent activity in an in vivo murine model; however, as peptides they are unlikely to find application in the clinic.

The Dickerson group at Scripps has identified a series of quinolinol-based BoNT/A light chain inhibitors [34], with two compounds exhibiting activity below 10 μM, and one of these active in the nanomolar range. This work drew heavily on structural biology information and established interesting structure–activity relationships at the BoNT/A active site. The researchers pinpointed specific requirements at binding subsites and found that although their compounds did not exhibit optimal solubility at physiological pH, they were highly soluble at low pH which suggests a possible oral dosing regimen. The compounds also displayed favorable ADME properties, including high stability in human and rat plasma, and half-lives similar to existing hydroxamate-based BoNT/A inhibitors that had displayed far poorer selectivity [34]. Given the size and flexibility of the BoNT/A catalytic site, these workers rather surprisingly found that a wide range of substituents on their scaffolds was tolerated, unlike what was previously found in the case of hydroxamate-based inhibitors that demonstrated observed inconsistency in SAR. The authors are currently working on further pharmacokinetic analysis and optimization of these compounds; one awaits these results with interest.

Recently, Heath and coworkers identified a substrate-mimicking macrocyclic peptide as a direct inhibitor of BoNT, and developed a second peptide macrocycle that binds to an active-site-adjacent epitope [35]. From these, the researchers created a Trojan Horse-type divalent inhibitor that inhibits BoNT/A at the picomolar level; it penetrates cells easily by means of the intact holotoxin, and proved highly effective in a human neuron model. The applicability of this type of inhibitor as a therapeutic is yet to be proven, but the authors note that this approach of identifying and joining together peripheral binding units is likely to find applicability in the design of small-molecule-based BoNT inhibitors that also function in a Trojan Horse-like fashion.

In 2013, Jadhav and colleagues reported novel BoNT/A exosite inhibitors based on a previously identified compound, D-chicoric acid [36]. Targeting BoNT exosites may constitute a feasible alternative to direct inhibition of the catalytic zinc site, but structural features of these exosites have not been fully explored, they are among the most flexible regions of the BoNT enzymes, and their suitability for

small-molecule binding remains uncertain. In this work, the researchers employed virtual screening, molecular dynamics simulations, and experimental (FRET and gel-based) assays to assess the binding requirements of BoNT/A exosites and identify potential inhibitors thereof [36]. The MD simulations indicated that the alpha-exosite of BoNT/A was likely more conformationally stable than the beta-exosite, and even more so than the catalytic site itself; these and other computational methodologies pointed to loops 200 and 250 in the closed conformation as associated with the active form of BoNT/A. Exosite inhibition was found to be synergistic, with over 40 residues of BoNT/A contributing to substrate binding interactions. These researchers found that these "hotspot" residues were largely clustered around four binding sites: the active site, the alpha-exosite, the beta-exosite, and a fourth exosite termed the anchor site [36]. Virtual screening led to two new inhibitors, C1 and C2, with the latter exhibiting binding in the nanomolar range. Cocrystallization was not successful, but predicted binding modes via docking indicated that C1 favored the alpha-exosite and C2 the beta. The authors do note that some of the inhibitors identified were Michael acceptors and could achieve inhibition by forming covalent bonds with cysteine residues at various locations; specific binding configurations for these compounds remain elusive. The authors state that the inhibitory mechanisms of these compounds are currently under ongoing study.

Antibody-based antitoxins have also been explored as therapeutic options for neutralizing BoNT serotypes. However, major caveats exist regarding any antibody-based treatment: high cost, uncertain efficacy, large required doses, rare but serious adverse effects, pharmacokinetic liabilities, limited tissue accessibility, and impaired interactions with the immune system [37–39]. To overcome some of these roadblocks, and in a unique and creative approach, the Barrera group developed and investigated three antitoxins based on camelid heavy chain-only antibodies, expressed in the chloroplasts of green algae, which accumulate as soluble proteins that bind and deactivate BoNT/A [40]. The authors note that this antitoxin production process is favorable due to the photosynthetic nature of algae, which are highly scalable and economically produced, and that adverse effects are likely to be rare. Green algae are also suitable for oral administration and can be stored at relatively low cost. In 2013, the Hust group identified a series of scFv antibodies from a macaque immune library that protected against BoNT/A ex vivo, demonstrating the strongest neutralization in a murine phrenic nerve-hemidiaphragm assay to date for a monoclonal antibody [41]. The Hust group also identified a human-like scFv-Fc that neutralizes the E serotype of BoNT [42]. Arnon and coworkers also recently published a combined antibody that neutralizes BoNT/H [43] and could be used for the prevention and treatment of botulism resulting from serotype H toxicity.

Other approaches to combating BoNT activity have focused on interfering with various host biochemical pathways rather than direct toxin inhibition. For example, the Bavari group recently found that certain existing Src family kinase inhibitors were able to inhibit BoNT serotypes A, B, and E via targeting host SRK pathways necessary for toxicity [44]. These researchers also identified a series of phosphatase

inhibitors that function as BoNT antagonists in murine and human embryonic stem-cell-derived motor neurons (ES-MNs), again not by directly inhibiting BoNT, but by modulation of host neuronal processes [45]. In 2015, Pirazzini and coworkers reported a new druglike small molecule, EGA, which interferes with the translocation procedures of various BoNT serotypes into cells [46]. Effective both in vitro and in vivo, EGA was found to inhibit BoNT neuroparalysis at low doses, with no discernible toxicity. It was not possible for these workers to identify the specific target of EGA, but they did establish that rather than exerting a direct inhibitory effect on BoNTs, EGA likely blocks an intracellular target that plays a role in BoNT cell entry. This mechanism would render EGA a more effective pre-exposure rather than post-exposure treatment [46]. The authors note, however, that in cases of infant botulism, the toxin often enters continuously into neurons from *Clostridia* species colonizing the gastrointestinal tract; in these scenarios, EGA could also be useful as a post-exposure treatment. The authors also present a straightforward synthetic scheme for potential EGA analogues, that could be optimized as potential drug leads [46].

3 Tetanus Toxin Countermeasures

The toxin responsible for tetanus, TeNT (tetanospasmin), is another potent zinc-bearing neurotoxin which is also synthesized by a species of *Clostridium* (in this case, *Clostridium tetani*), exhibiting an estimated human LD_{50} of approximately 2.5 ng/kg [47]. Although technically not as lethal as the BoNT family of neurotoxins, TeNT still poses a significant threat as a bioterror agent and also in infectious disease scenarios in developing nations. TeNT functions by suppressing inhibitory circuitry in the spinal cord, resulting in rapid paralysis and muscle spasticity; mortality rates from tetanus can approach 50% [47]. The tetanus holotoxin is a 150-kDa enzyme, featuring three domains: the N-terminal catalytic, zinc-bearing light chain (L) domain; the internal or intermediate heavy chain domain (HN); and the C-terminal domain that is responsible for receptor binding to its receptor ganglioside on neurons (THc domain) [48–50]. Notably, TeNT belongs to the thermolysin family of Zn endopeptidases and features the signature HEXXH zinc metalloenzyme consensus sequence found in many zinc hydrolases. In a multistep process, TeNT binds to a target neuron and is endocytosed, followed by retrograde transport through axons and subsequent neurotransmitter blockage via interference with formation of the synaptic SNARE complex [51]. Specifically, TeNT cleaves the SNARE protein synaptobrevin at the Gln76 – Phe77 peptide bond, resulting in blockade of glycine and GABA release from inhibitory interneurons [52]. Countermeasures against tetanus have traditionally concentrated on pre-exposure vaccines, with recent work (including by Sun and coworkers [53]) focusing on improving vaccine production, safety, and efficacy while reducing adverse effects. Other studies have resulted in new, second generation vaccine candidates (Leclerc and coworkers [54]) of higher potency than currently available

pre-exposure treatments, and that also display antitumor activity, with newer combination vaccines such as DTaP-IPV (Kinrix) and DTaP-IPV/Hib (Pentacel) also showing promise [55].

The strong emphasis on successful tetanus vaccine research, and the availability of effective vaccines to the general public in developed nations, has resulted in a general dearth of medicinal chemistry research effort toward small-molecule inhibitors of the toxin itself. A very small body of work exists regarding the development of small-molecule TeNT inhibitors; in 2012, the Skariyachan group reported computational analyses of the suitability of new plant-based natural products as TeNT countermeasures [56]. They identified berberine, curcumin, coumarin, catechol, and diosphenol as potential starting scaffolds for a small-molecule TeNT inhibitor; however, this work was based on homology modeling of TeNT and was not followed up by experimental validation. In a more traditional medicinal chemistry approach, Roques and colleagues investigated a series of beta-aminothiols designed to mimic key residues in the synaptobrevin protein targeted by TeNT [57], specifically the peptide bond cleaved in the proteolytic process. They designed and synthesized combinatorial libraries of pseudotripeptides, with experimental in vitro activities (Ki values) against TeNT in the 3–4 μM range. These workers identified key structure–activity relationships between their small-molecule ligands and the catalytic zinc active site of TeNT, focusing on extending interactions into the S' subsites of the receptor [57]. However, none of these compounds has entered the clinic as a therapeutic.

Other research has explored antibody-based approaches to TeNT neutralization. Petrusić and coworkers reported the production and application of a monoclonal antibody (MoAb) specific to TeNT in 2011 [58]. With the objective of developing a model system to study the mechanism of TeNT internalization, these workers used hybridoma technology to design the MoAb, which is likely to find use in the detection of reverse toxicity in vaccine production. The authors also suggest that this antibody could be useful for researchers studying intracellular TeNT transport and delivery of polypeptides through the blood–brain barrier. Notably, in 2010, Indrawattana and colleagues developed a human monoclonal ScFv that directly interferes with TeNT endocytosis and metalloprotease activity [59]. The authors observe that traditional animal-derived immunization therapy, in addition to potentially inducing serum sickness and allergic reactions, blocks entry of the toxin into nerve cells but does not interfere with catalytic metalloprotease activity and is therefore incapable of reversing tetanus-related symptoms [59]. Antibody-based therapeutics may constitute an important step forward in the development of post-exposure treatments; however, widespread implementation of such a solution is, unlikely, again due to the effectiveness and availability of the tetanus vaccine. However, inhibition of TeNT proteolytic activity is still required in order to reduce mortality rates post-exposure, in unvaccinated populations, or in the event of a bioterror attack where the target population may no longer be fully immune. The tetanus toxin can therefore be considered a "sleeper" biowarfare threat for which the civilian population may not be prepared.

4 Anthrax Toxin Lethal Factor Inhibitors

Anthrax is a highly lethal infectious disease caused by the Gram-positive, rod-shaped anaerobic bacterium *Bacillus anthracis* [12, 60]. The lethality of anthrax is attributable primarily to two factors: its highly protective polysaccharide capsule, and its ability to synthesize anthrax exotoxin comprising lethal factor (LF), calmodulin-activated edema factor adenylate cyclase (EF), and protective antigen (PA), encoded by the pXO1 plasmid [61]. Most critical for pathogenesis is LF, an 89-kDa Zn metalloprotease which combines with PA to form the anthrax lethal toxin [62]. Once translocated by PA into the cytoplasm of host target cells, LF cleaves members of the mitogen-activated protein kinase kinase (MEK) family, including MAPKKs 1–3, in the proline-rich N-terminal area directly adjacent to the kinase domain [63, 64], interrupting MAPKK phosphorylation that, in turn, interferes with cellular immune/inflammatory defense mechanisms against pathogens [65–68]. In the later stages of the disease, LF also targets endothelial cells and causes disruption of vascular barriers [69–71]. While many studies have been conducted toward the design of small molecules that target the LF binding site (see below), there is currently no effective small-molecule therapeutic on the market that can counteract LF-mediated cell death. The anthrax vaccine currently used in the USA, AVA (anthrax vaccine adsorbed), is obtained from a toxigenic, non-virulent strain of *B. anthracis* [72–74], and a newer vaccine prepared with purified, recombinant PA is now under investigation [75]. However, widespread civilian immunization is unlikely because anthrax is perceived by the public as a high-risk but rare disease. Moreover, although LF is known to structurally alter members of the MEK family, the substrate requirements that determine LF specificity are unknown. Antibody-based anthrax treatments have emerged as an encouraging step forward, but key potential caveats exist as outlined in the previous sections of this review. All anti-anthrax antibodies developed to date target PA binding to host cells, to interfere with toxin translocation therein. The human monoclonal antibody MDX-1303 (Valortim) has now been incorporated into the CDC Strategic National Stockpile; however, post-exposure studies performed on non-human primates resulted in only 70% efficacy [76]. In 2012, the FDA approved raxibacumab (Abthrax) with antibiotics for the treatment of inhalational anthrax. Nevertheless, efficacy studies on *Cynomolgus* macaques and New Zealand white (NZW) rabbits showed that raxibacumab may be inferior to currently approved antimicrobials [77, 78]. More recently, the FDA approved Anthrax Immune Globulin Intravenous (Human), a purified human IgG, for the same purpose. Similar non-human primate efficacy studies, however, demonstrated that even in combination with antimicrobials, complete protection from *B. anthracis* by this IgG remains challenging [79]. The lethal factor has therefore been the subject of many medicinal chemistry-based studies, toward the design and optimization of small-molecule inhibitors. However, none has yet reached the clinic, and an urgent need still exists for a post-exposure treatment that can be administered concurrent with antibiotics to increase the likelihood of host survival.

The anthrax toxin lethal factor comprises four domains: the N-terminal domain (I); the large central domain (II); a small helical domain (III); and the C-terminal catalytic domain (IV) [3–6]. Although the N-terminal domain is not catalytic and does not appear to interact with LF substrate(s), it plays a major role in anthrax pathogenesis, as it directly binds to the protective antigen (PA) and is required for PA-enabled translocation of LF into cells. The exact functions of domains (II) and (III) are unknown, but the large central domain may play a mechanical role in LF specificity for MAPKKs. The C-terminal domain forms the LF catalytic active site, and has therefore been the primary target of LF inhibition studies. This domain contains a Zn^{2+} atom coordinated to three active-site residues: His686, His690, and Glu735. The two histidines are located on an alpha helix near the bottom of the LF substrate binding site, and form part of the signature Zn metalloproteinase HEXXH motif that is also present in the matrix metalloproteinases (MMPs) as well as TeNT [80]. Glu735 is located on a separate but closely adjacent helix near the top of the active site. The binding cleft itself comprises three general subsites: the deep, strongly hydrophobic, and sterically constrained S1′ subsite; the largely hydrophobic but less restricted S1–S2 region, which is an open-ended, partly solvent-exposed tunnel; and the less well characterized and somewhat more electrostatically complicated S2′ area.

Mechanisms of anthrax toxin uptake by cells, LF substrate specificity, and LF Zn catalysis continue to be the subjects of intense research. Cytotoxicity is considered to commence when the protective antigen (PA) binds to one of two homologous membrane-bound receptors on the surface of the cell. PA is then cleaved extracellularly by furin-like convertase enzymes, releasing a small N-terminal fragment and leaving a larger, 63-kDa C-terminal fragment bound to the cell [63–68]. The N-terminal fragment does not appear to contribute further to the disease process, but the membrane-bound PA fragment spontaneously oligomerizes to form stable, symmetric heptamers, which bind up to three LF or edema factor (EF) units. Once LF and/or EF are bound, the entire complex is enclosed in a lipid membrane and endocytosed in a partly understood sub-mechanism involving the TEM8 and CMG2-interacting transmembrane protein LRP6 [81]. The acidic interior of the intracellular LF/EF/PA vesicle induces a conformational change in the PA heptamer, causing it to dissociate from the receptor and form a porelike channel in the vesicle membrane [67, 68, 81]. LF and/or EF then partly unfold and proceed through the pore into the cytosol, where LF targets and cleaves MAPKKs at amino-terminal sites. LF cleavage is thought to disrupt a downstream MAPKK substrate binding site, shutting down three major MAPKK pathways [63, 64] in host cells and thereby impairing the function of various immune cell types including macrophages, neutrophils, and T cells. However, the mechanisms by which LF specifically binds and cleaves MAPKKs are not well understood; the LF large central domain (II) may ensure specificity for MAPKKs by obstructing access of other proteins to the LF active site. Hypotheses of Zn catalysis mechanisms leading to substrate cleavage have been formulated largely based on structural similarities to enzymes in the MMP family in addition to the conserved HEXXH sequence. In one such hypothesis, the LF catalytic Zn is believed to induce MAPKK substrate

cleavage by activating a water molecule occupying the fourth Zn-cation coordination site; this water consequently attacks the MAPKK substrate scissile amide bond [63, 74] and donates a proton to Glu687 in the HEXXH sequence. The proton is in turn delivered to the amino group of the leaving substrate. The negative charge on the scissile bond carbonyl oxygen is thought to be stabilized by an important LF active-site tyrosine residue (Tyr728) that is also common to the astacin-family metalloproteinases [82–84]. However, many open questions remain, and mechanisms of MAPKK specificity and Zn catalysis in particular are expected to be further elucidated by more structural details of the LF active site and by closer examination of key LF-substrate and LF-inhibitor interactions.

The first potent LF inhibitors reported were, like the early MMP inhibitors, small peptide sequences with hydroxamic acid zinc-binding groups [85–87]. The main approach pursued for the identification of such inhibitors was the substrate-based design of peptide derivatives based on the residue sequence near the LF cleavage site. The peptidic structures were designed to parallel the natural MAPKK substrate, with a hydroxamate grouping to effectively chelate the catalytic Zn^{2+}. Montecucco and coworkers devised the first series of highly active peptide hydroxamates [85], one of which was a furin as well as a LF inhibitor and may therefore also have blocked toxin uptake into the cell. Cantley and coworkers built upon this approach by screening peptide libraries for suitable LF substrate sequences [86], which resulted in the key findings that aromatic residues were preferred at the substrate P1′ position, and that the S1′ subsite on the receptor was most likely hydrophobic. The Cantley group also found that the dipeptide hydroxamate GM6001 (Ilomastat) was active against LF with micromolar inhibition constants [86, 87]. GM6001 was originally developed as an MMP inhibitor, and exhibited broad-spectrum MMP inhibition in the nanomolar range, with highest potency against MMP-8 (human neutrophil collagenase) at 0.1 nM. Ilomastat was subsequently cocrystallized with LF by Liddington et al. (1PWU.pdb [86]), which yielded significant insight into LF substrate binding. The GM6001 leucine mimetic moiety was found to bind to the LF S1′ subsite, while the aromatic Trp sidechain was located in the S2′ region [87]. The weaker activity of GM6001 against LF compared to collagenases is attributable to the relatively small P1′ leucine mimetic, which fits the narrower collagenase and gelatinase hydrophobic subsites but only partly fills the deep and somewhat wider LF S1′ area.

Some of these early peptidic LF inhibitors were active against LF in vitro and in cell-based assays; their development helped to establish the structural nature of the hydrophobic S1′ site and its importance for LF inhibition, although they offered little information on the S1–S2 area, with which they generally did not engage. Interestingly, these peptidic compounds contained functionalities that bound to the S2′ area which had previously been overlooked in LF inhibitor discovery efforts, but which has more recently been investigated by Ambrose and coworkers [3–5]. But peptide-based small molecules have significant drawbacks with respect to their use as probe and/or drug compounds, most notably poor oral availability/bioavailability, and none of these early attempts showed promise as a potential therapeutic. To overcome this hindrance, Shoop and coworkers at Merck undertook

a wide-ranging LF inhibitor development program, in which they conducted a high-throughput screen of in-house compound collections with metal-coordinating groups [88, 89]. This screen resulted in an important lead compound with nonselective but potent MMP inhibition and low micromolar activity against LF. Xiong and coworkers subsequently synthesized and evaluated ~500 analogs around that lead structure, and the most active compound identified from that study, the hydroxamic acid compound MK-702/LF-1B [89, 90], exhibited $IC_{50} = 54$ nM in a FRET assay and $IC_{50} = 210$ nM in a macrophage cytotoxicity assay. This compound has been extensively tested in animal model and pharmacological studies, and the researchers have found that a combination of MK-702/LF-1B with ciprofloxacin significantly increases the survival rate of *B. anthracis*-infected mice and rabbits [89]. Structural data of the MK-702/LF-1B complex (1YQY.pdb) shows that the disubstituted phenyl fits well into the hydrophobic S1′ subpocket, while the sulfonyl grouping engages in rather weak hydrogen bonds with the backbone nitrogens of Lys656 and Val675. A chief contributor to MK-702/LF-1B's high affinity may be the tetrahydropyran ring, which targets the flexible S1–S2 region and incorporates a polar oxygen for compatibility with the solvent-exposed end of the S2 area. Challenges rapidly arose with this compound, however, as it is not particularly selective for LF among relevant zinc metalloproteinases; it is active on the micromolar scale against a range of MMPs, which is not surprising given that it incorporates the sulfonyl and hydroxamate functionalities common to potent MMP inhibitors. Hydroxamic acid is an excellent zinc-binding group and is a prominent feature of the most potent in vitro LF inhibitors discovered to date; but hydroxamates usually exhibit cross reactivity with MMPs and other zinc-binding enzymes, and present significant pharmacokinetic liabilities including metabolic instability [91, 92]. Hydroxamic acids are often subject to glucuronidation and sulfation, are poorly absorbed in vivo, and are prone to hydrolysis, resulting in potentially mutagenic hydroxylamines [91]. MK-702/LF-1B was for this and related reasons not pursued as a therapeutic. Much attention has subsequently been focused on the development of zinc metalloproteinase (including LF) inhibitors that incorporate non-hydroxamate ZBGs.

In recent years many attempts have been made [93–108] to identify novel scaffolds for LF inhibition without a hydroxamic acid ZBG. Many of these studies incorporated small- to medium-scale high-throughput screens using FRET assays. The myriad scaffolds investigated include cationic polyamines [95], aminoglycosides [99], pyrazolones [96], ECGC and related polyphenolics [106], tetracyclines [100], and alpha-defensins [107]. The majority of these compounds exhibit LF activity in the micromolar range and are overall not as potent as the Merck hydroxamates. However, the Pellecchia group at the Burnham Institute has made progress toward the development of a potent non-hydroxamate LF inhibitor based on a rhodanine scaffold [93, 94, 101, 108], and has designed a lead series largely in the low micromolar range, with four compounds demonstrating in vitro IC_{50} values between 190 and 320 nM, and demonstrating good selectivity against relevant Zn metalloproteases. These structures display unique binding modes, whereby the rhodanine ring engages in Zn chelation via the thiazolidinedione sulfur atom, and

the rest of the molecule binds at the S1–S2 subpocket [93, 94]. The Pellecchia group has also conducted extensive molecular modeling studies involving 3D-QSAR and pharmacophore mapping of their rhodanine analogs to analyze SAR and optimize affinity, and has performed preliminary in vitro ADME/Tox profiling to assess druglike properties of these new compounds. While these compounds exhibit good selectivity against relevant Zn enzymes and feature non-hydroxamate ZBGs, rhodanines unfortunately present problems as therapeutics, as they can generate singlet oxygen and be photo-activated to become covalent modifiers. To the author's knowledge, no rhodanine is currently in the clinic or moving toward clinical development.

The Ambrose (Amin) group has carried out extensive experimental and computational studies to pinpoint novel small-molecule LF inhibitors [3–6, 109, 110]. In 2009, to explore current chemical space as broadly as possible for potential LF active-site lead/probe scaffolds, to investigate LF ligand–receptor interactions, and to select a small, structurally diverse library of previously unevaluated compounds for preliminary experimental assays, those workers formulated an original virtual and experimental screening strategy that was able to identify three non-hydroxamate, previously uninvestigated small molecules with biological activity against the anthrax toxin lethal factor in the low micromolar range, with an overall 12.8% experimental hit rate (five hits out of 39 final prioritized compounds) [3]. As part of this protocol, these researchers screened approximately 35 million non-redundant compounds in silico for potential activity against LF, followed by topomeric (shape-based) searching, docking and scoring, and druglike property filtering. Each of these final three hits demonstrated monodentate zinc coordination as predicted by virtual screening; none exhibited the traditionally preferred bidentate zinc chelation. Hydrophobic Val and Leu residues in the S1′ area, Leu in the S1–S2 region, and uncharged polar residues including Gly and Ser in the S2′ region appeared to play critical roles in ligand binding, as do two His residues which are also Zn chelators. Critically, the researchers were able to confirm that micromolar-level LF inhibition can be achieved by small molecules with non-hydroxamate, monodentate ZBGs, as long as critical hydrophobic interactions with at least two LF subsites (in this case, S1–S2 and S1′) are maintained.

A novel pharmacophore assembly **UM1** [4] was also reported by Ambrose and coworkers, including features representing interactions involving all three subsites of the LF catalytic binding region, to be used as a search tool to identify new potential LF scaffolds. This eight-featured, comprehensive model was constructed from five preliminary models and was validated and optimized by screening all published LF inhibitors with experimental biological activity data – an extremely diverse dataset occupying a variety of distinct regions in chemical space – including an unbiased test set of 68 nanomolar-level LF inhibitors that are not structurally related to the compounds used in pharmacophore construction. The model exhibited strong preference for nanomolar-level LF inhibitors in validation studies when used as a database search tool [4].

More recently, these researchers reported the synthesis, experimental evaluation, modeling, and structural biology for a novel series of sulfonamide hydroxamate LF

inhibitor analogs specifically designed to extend into, and probe chemical preferences of, the LF S2′ subsite, a large, dynamic, solvent-exposed channel that changes configuration in response to various ligands [5]. Crystallographic characterization of multiple ligand–receptor complexes was also carried out, in which three distinct, previously unreported conformations of LF were identified dependent on ligand binding: "open," "tight," and "bioactive" [109, 110]. The research team synthesized a series of these sulfonamide probe analogs to take advantage of the residues that were exposed due to this conformational change, and to probe binding preferences at this subsite. Biological activity and X-ray data revealed a decrease in inhibitory activity exhibited by these new analogs with respect to the parent compound, which likely resulted from the elimination of a key ligand–receptor H-bond to Tyr728. It was, however, found that biological activity can partly be recovered by installing amine-containing substituents on the N-sulfonamide that are capable of interacting with key residues Asp325, Ser326, and Ser327. Possible steric clashes with Tyr728 were found to significantly decrease compound potency. Notably, inhibitor design targeting the S2′ subsite can result in novel scaffolds with a wide variety of substituents that do not induce a significant loss in biological activity; one could therefore take advantage of this accommodating S2′ subsite to modify other scaffolds to improve druglike and related properties.

Ambrose and coworkers have since reported the development and optimization of a variety of SVM-based models from published LF inhibitors with experimental biological activity data, the most optimal of which were able to sharply distinguish between active and inactive compounds [6]. Accuracy and predictivity of these models were assessed internally via tenfold cross-validation and externally by means of test set compounds not incorporated in the original models. A broad selection of molecular descriptors were examined in this study, including subdivided surface areas, rotatable bonds, partial charge distribution, number of reactive functional groups, number of metabolic reactions, solvent-accessible surface area of amide oxygen atoms, and 2D fragments including C–N–O, C–C–C–C–N, H–C*C–F, H–O–C–C–N, and C-N-C-C-O. These fragments helped to elucidate specific hydrogen bonding donor and acceptor and hydrophobic requirements for LF inhibitors. These workers were able to generate an optimized SVM model based on a randomly split training set, yielding a very high cross-validated accuracy of 95.44% for an internal test set of LF inhibitors. This model achieved an accuracy of 94.44% on a heterogeneous external test set, and was able to identify 99.65% of compounds correctly in an external inactive test set, and could be used to quickly identify novel and potentially active LF inhibitor compounds from large datasets [6].

In summary, while the antibody-based Abthrax and Valortim constitute important steps forward in the design of post-exposure anthrax therapeutics, they do not directly counteract the LF hydrolase/metalloprotease activity that is responsible for cytotoxicity and subsequent host death and are therefore not fully effective countermeasures. Since interfering with LF proteolytic activity is the only demonstrably certain path to eliminating the threat of anthrax as a bioterror agent, further

medicinal chemistry work is needed to develop small molecules that deactivate LF, demonstrate favorable pharmacokinetic properties, and are feasible as drugs.

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Inhibition and Activity Regulation of Bacterial Collagenases

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Abstract The imminent antimicrobial resistance dilemma requests for drug discovery initiatives outside the box of classical antibiotics strategies, including the identification of anti-virulence targets. Given their critical roles in diverse infectious diseases, bacterial collagenases constitute one such class of anti-virulence targets. Here we review the essential catalytic elements of bacterial collagenases, including the zinc-coordinating residues, as well as their typical domain organization with relevance to collagenolysis. We further present the structural basis for the substrate specificities, both towards linear and triple-helical peptides. These enzymatic properties shape the structural framework for the discovery and development of competitive, active site-directed inhibitors. While currently available compounds bind the catalytic zinc, alternative interaction possibilities at the active site promise an improved specificity towards other metalloproteases. We finally outline inhibition opportunities that result from exploiting collagenase exosites.

Keywords Exosite interaction, Gluzincin, Infectious diseases, Multi-domain organization, Zinc proteases, Zinc-chelation

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1 Introduction

We are on the eve of a post-antibiotic era. Multidrug resistances in bacterial pathogens are spreading worldwide. It is projected by the EU commission that by 2050 ten million people will die per year due to antimicrobial resistances [1]. We need new antimicrobial agents to complement and replace existing antibiotic treatments. Classical antibiotic therapies target the vitals of a pathogen. They aim for essential components of the bacterial cell cycle (i.e., cell wall synthesis, protein synthesis, DNA replication, and folate coenzyme synthesis), in order to stop the growth of a pathogen and kill it [2]. This antibiotic "license to kill" has caused a high selective pressure among the targeted bacteria and has facilitated the development and spread of resistances. One approach to escape this vicious circle in antimicrobial treatment focuses on the development of anti-virulence agents rather than antibiotics. In contrast to antibiotics, anti-virulence drugs want to disarm, not kill the pathogen. Interference with virulence targets the pathogen's ability to enter, disseminate, replicate, and persist in a host [3].

Bacterial virulence factors include toxins, adhesins, secretion systems, and exoenzymes. They all help to establish and maintain an infection. Among the exoenzymes, we find bacterial proteases as crucial players in bacterial pathogenesis. Bacterial proteases are key factors in many processes: tissue invasion and pathogen spread within the tissue, the disruption of the physiological homeostasis at the site of infection, the activation and dissemination of bacterial toxins, the acquisition of nutrients for proliferation, and the evasion of the host immune system [4–8].

A major physical and biochemical barrier that pathogens encounter in humans and animals upon infection is the extracellular matrix (ECM). The ECM is composed of proteoglycans and fibrous proteins. It encloses and mechanically supports tissues. Its most abundant building block is collagen (up to 90%). Self-assembled collagen fibrils, fibers, and networks are essential for the maintenance of tissue shape and integrity [9, 10]. The hallmark feature of collagen is the collagen triple-helix, which is the major constituent of all collagen assemblies. This tightly packed polyproline type II helix is perpetuated by triplet repeats of Gly-X-Y, in which the X and Y positions are mostly occupied by proline (28%) and hydroxyproline (38%). Gly-Pro-Hyp represents the most frequent triplet (10.5%) in collagen [11]. Befitting their critical physiological role, collagens are – due to their unique triple-helix structure – highly resistant to proteolysis. Even the promiscuous proteases of the digestive system cannot process natively folded triple-helical collagen [12, 13]. Only a small number of highly regulated endogenous collagenases can remodel collagen in humans (e.g., MMP-1, -2, -8, -13, -14) [14, 15].

Given the abundance of collagens in animals, it appears astonishing that only very few microbes have evolved collagenolytic enzymes to capitalize on collagen as a carbon and energy source. The scarcity of collagenolytic microbes may reflect the complexity and evolutionary burden to develop and control a collagenolytic machinery. Yet, some pathogens have evolved means to tackle the collagenous stronghold in mammals, in order to facilitate their spread in the host. They pursue one of two strategies, either they high-jack the host collagenases for their purposes, or they secrete a collagenase of their own. An elaborate example for the first approach to enhance virulence is given by *Porphyromonas gingivalis*. *Porphyromonas gingivalis*, the leading cause of periodontitis, upregulates collagenolytic MMP activity at multiple levels, by upregulation of mRNA levels, proteolytic activation of MMPs, and downregulation of endogenous MMP inhibitors [16]. The strategy of producing collagenolytic exoenzymes is employed by a small number of bacterial species, most notably in the genera of *Clostridium*, *Bacillus*, and *Vibrio*. They have evolved secretory collagenases [17], which are the focus of this article. But also some non-bacterial pathogens secrete collagenases, e.g., the extracellular protozoan parasite *Entamoeba histolytica*, a major cause of amoebiasis in humans and primates. The virulence of its pathogenic strains was shown to clearly correlate with its secreted collagenolytic activity [18–20].

2 Bacterial Collagenases and Their Role in Disease

Many of the bacterial species found to encode a collagenase are important human pathogens (Table 1). These collagenolytic microbes were identified based on an Interpro database search using the signature domain architecture of the collagenase unit (see Sect. 4). Among the clostridia, there are such well-known pathogens as *C. botulinum*, *C. tetani*, *C. perfringens*, *C. sordellii*, *C. novyi*, *C. septicum*, and *C. histolyticum*, to name but a few.

Yet intriguingly, although the significance of proteolytic exoenzymes as virulence factors is well-established and collagenases are regularly implicated and discussed as virulence factors [27, 54–57], it comes as a surprise that the detailed role of bacterial collagenases in host physiology and bacterial pathogenesis has hardly been addressed. The current situation may reflect an underlying strategy to identify a broad spectrum anti-virulence strategy, and collagenases might be considered too narrow and neglected as a pharmaceutical target.

In 1967, Rippon and Peck performed a small-scale study to investigate the effect of collagenase secretion on the virulence of the human pathogen *Actinomadura madurae*, a causative agent of actinomycosis. This infection is typically acquired after trauma, surgery, or infection, and causes painful abscesses and draining sinus tracts [44]. Infection of mice by intraperitoneal injection with collagenase-deficient strains led to a delayed onset and extent of symptoms (nodule formation, skin loss) and significantly increased survival rates; while infection with a mutant strain with

Table 1 Collagenase-secreting human pathogens and associated infectious diseases

Family	Species	Disease
Clostridium	*C. botulinum*	Foodborne botulism, wound botulism, infant botulism [21][a]
	C. tetani	Tetanus [21, 22]
	C. perfringens	Food poisoning, diarrhea, enteritis necroticans, necrotizing pneumonia, myonecrosis, gangrenous cholecystitis, bacterial keratitis, septic arthritis [21, 23–28]
	C. difficile[b]	Pseudomembranous colitis, antibiotic-associated, and nosocomial diarrhea [29–31]
	C. sordellii	Myonecrosis, obstetric/gynecological-associated infections [21, 32]
	C. novyi	Myonecrosis [21][a]
	C. septicum	Myonecrosis [21, 23][a]
	C. histolyticum	Myonecrosis [21, 33]
Bacillus	*B. anthracis*	Anthrax [34, 35][a]
	B. cereus	Food poisoning, wound infections, bacteremia, central nervous system infections, endophthalmitis, pneumonia, gas gangrene-like cutaneous infections [35–38][a]
	B. cytotoxicus sp. nov.	Food poisoning [39][a]
Spirochaetes	*L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilli*, *L. kirschneri*, *L. alexanderi*	Leptospirosis [40–42][a]
Actinobacteria	*Actinomadura* (*Streptomyces*) *madurae*	Actinomycosis [43–45]
Vibrio	*V. alginolyticus*	Wound and ear infections, primary septicemia, otitis media, cholecystitis, meningitis [46–48]
	V. parahaemolyticus	Gastroenteritis, wound infections, primary septicemia [46, 47, 49]
	V. cholerae	Cholera [50]
	V. vulnificus	Food poisoning, gastroenteritis, wound infections, septicemia [46, 51, 52]
	V. mimicus	Gastroenteritis, ear infections [46, 47]

[a]Supplemented by data from BLAST analysis within the UniProt database [53]
[b]Peptidase family U32 (UniProt: Q18B88)

excess collagenase production led to an earlier onset of symptoms and was lethal [45].

C. difficile infections are a major cause of infectious diarrhea and other severe intestinal diseases in hospitalized patients. As established more recently, it also

affects the general population, which lack the typical risk factors associated with *C. difficile* infections such as prolonged antibiotic exposure [58]. In 1990, Seddon et al. demonstrated a correlation between collagenase activity and strain virulence of toxigenic *C. difficile* strains in a hamster model for antibiotic-associated colitis. Poorly virulent strains (after oral infection) displayed no or weak collagenase activity, while the highly virulent ones showed the highest collagenase activity. The authors suggested that the higher rate of tissue damage inflicted by the hydrolytic enzymes and the concomitant release of nutrients such as hydroxyproline might help to establish the *C. difficile* infection [30, 59].

Similarly, it would be of great interest to establish the role of the bacterial collagenase in *C. perfringens*-induced gastrointestinal and wound infections. In developed countries, food-borne *C. perfringens* infections typically cause short-term, non-severe food poisoning with diarrhea and abdominal cramps. Yet, in severely protein-malnutritioned children it can lead to enteritis necroticans, a life-threatening infection of the jejunum and ileum [60]. However, the role of collagenase in *C. perfringens*-mediated acute gastrointestinal infections in humans has, to the best of our knowledge, not been addressed. Studies in broilers suffering from necrotic enteritis suggest the involvement of bacterial collagenases in the initial stages of the infection, in which the ECM of the enteric mucosa is disrupted [61, 62]. While acute infections have not been studied so far, Pruteanu et al. found evidence for the implication of *C. perfringens* in inflammatory bowel diseases. This group of disorders, including Crohn's disease and ulcerative colitis, is a chronic, relapsing inflammatory disorder of the gastrointestinal tract characterized by ulcerations of the bowel. Its pathogenesis is complex and involves various genetic, environmental, and immunological factors [63, 64]. Amongst others, the proteolytic activity arising from the disrupted gut microbiota found in patients is thought to contribute to the development of the disease [65]. This process might be associated with the collagenase ColA from *C. perfringens*, as initial findings suggest [66].

Among the clostridia, *C. perfringens* is the major cause of clostridial myonecrosis (gas gangrene), a fast-spreading, potentially life-threatening infection of soft tissue and muscles. It predominantly arises after traumas and surgeries, and is difficult to manage, as it requires aggressive surgical interventions to remove the necrotizing tissue [67, 68]. Initial symptoms of clostridial myonecrosis are excessive pain and marked oedema formation at the site of infection, which then spread upon further tissue invasion by the bacteria. As shown by Legat et al. edema formation could be induced in a dose dependent manner within 20 min by the injection of proteolytically active *C. histolyticum* collagenase into rat paws and was accompanied by plasma protein extravasation; by contrast, heat-inactivated collagenase could not induce oedema or plasma protein extravasation [69, 70]. Edema are excessive accumulations of interstitial fluids in tissues and impair normal tissue function, as the diffusion-limited supply with oxygen and nutrients of the tissue is disrupted [71]. These hypoxic conditions could favor the establishment and replication of the anaerobic pathogen at the site of infection. In contrast to that, a study by Awad et al. in a mouse myonecrosis model could find no correlation between

collagenase activity and the virulence of *C. perfringens*. Collagenase-deficient strains of *C. perfringens* showed no altered virulence compared to wildtype strains. No differences in the swelling phenotype and histology were observed after intramuscular injection of the inoculum of wildtype and mutant strains [72]. Thus, further studies are needed to elucidate the role of the bacterial collagenase in *C. perfringens*-mediated pathogenesis.

While the significance of the collagenase in *C. perfringens* infections is still controversial, it is a known important virulence factor in *Leptospira interrogans*-induced leptospirosis [42]. Leptospirosis, a zoonotic disease, is transmitted via contact with urine or urine-contaminated surfaces or liquids and can display a wide range of symptoms, from mild to life-threatening [40]. Its pathogenesis is not well understood. Yet, in hamsters infected via intraperitoneal injection it was shown that invasiveness and virulence decreased clearly when collagenase-deficient strains were used instead of wildtype strains using cellular and animal models [42].

Also several pathogenic Vibrio species secrete collagenases. Similar to the clostridial collagenases, these collagenases have been implicated in pathogenesis, but their role has not been studied yet [47]. Intriguingly, it was shown recently that *Vibrio cholerae* harbors a type II secretion system-dependent collagenase VhcC. However, its role in transmission and host colonization is still unclear [50].

In sum, the presented findings form a robust basis for the correlation of collagenolytic activity and pathogenicity caused by a broad spectrum of microbes. At the same time, there is a considerable lack in our understanding of the mechanistic and causal role of bacterial collagenases in pathogenesis and their distinct significance in the individual infection environments, e.g., in traumatized tissue or the gastrointestinal tract. The discovery of specific inhibitors represents one avenue to address these important questions.

3 Catalytic Class of Bacterial Collagenases

To date, most known bacterial collagenases belong to the metallopeptidase family M9, comprising subfamily M9A (*Vibrio* type) and M9B (*Clostridium* type) of the MEROPS database [17]. They are multi-domain zinc metalloproteases that harbor the canonical zinc-binding HEXXH motif in the active site, a hallmark feature of the zincin family. The catalytic zinc ion is coordinated by the two histidines of the motif. The glutamate acts as the general acid/base, which binds and polarizes the catalytic water. The third zinc-binding ligand, a glutamate, is located 28 to 30 aa downstream of the HEXXH motif and is provided by the *glutamate helix* [73–75]. The two zinc liganding histidines are harbored by the *active-site helix* and the complementing third ligand by the *glutamate helix*. This zinc-coordination pattern is also suggested for the Vibrio-type collagenases based on sequence comparison [76, 77]. Thus, both Vibrio- and Clostridium-type collagenases are thought to belong to the gluzincin family of zinc metallopeptidases. A water molecule acts

as fourth zinc ligand. Upon binding of the peptide substrate, the zinc-coordination sphere is further increased, with the zinc serving as the oxyanion hole to the carbonyl oxygen of the scissile peptide bond and thus preparing the nucleophilic attack by the zinc-coordinated water. The subsequent rupture of the peptide bond is assisted by the general acid, releasing the newly formed product termini [78].

Reports about non-metallopeptidase collagenases that are "true collagenases" by definition, i.e., they can cleave native triple-helical collagen under physiological conditions [27], are scarce (e.g., prtC collagenase from *Porphyromonas gingivalis* [79]) or partial, as the identification and characterization of the collagenolytic protein is missing (e.g., in *C. difficile* [30, 80]). These instances are currently assembled in the peptidase family U32, as their catalytic types are unknown [17].

The best studied bacterial collagenases are from the family *Clostridium*; to be specific, the collagenases ColG and ColH from *C. histolyticum* (e.g., 8–16) distantly followed by collagenases in *Bacillus* spp. (e.g., [35, 37]), *Vibrio* spp. [81–85], *Spirochaetes* spp. [42], and *Actinobacteria* spp. [45, 86]. Thus, this review will focus on clostridial collagenases in the following sections.

4 Domain Architecture of Bacterial Collagenases

Clostridial collagenases are calcium-dependent modular proteins [22, 74, 87, 88] (Fig. 1, M9B). At the N-terminus, the mature proteins harbor a collagenase unit of ~78 kD, in which the catalytic zinc ion is coordinated by the two histidines of the HEXXH motif and a downstream glutamate [73, 74]. The collagenase unit is composed of the activator and peptidase domains, and represents the minimal collagenolytic catalytic entity of the enzyme [74]. At the C-terminus, a varying composition of two to three accessory domains can be found, consisting of zero to two polycystic-kidney disease-like domain(s) (PKD) and one or two collagen-binding domains (CBD) (each ~10 kD), which are thought to be involved in collagen swelling and binding to fibrillar collagen [74, 88, 90–92]. As found in culture filtrates, the full-length collagenases can undergo further proteolytic processing which results in various C-terminally truncated isoenzymes [74, 84, 87, 88, 93, 94]. This processing was also shown for VhcC from *V. cholerae* [50].

Crystal structure data on Vibrio collagenases are missing till today. However, based on the comparison and analysis of their sequence, domain and activity, we can deduce that the characteristic domain architecture of both Vibrio- and Clostridium-type collagenases of the M9A and M9B subfamilies, respectively, is formed by the N-terminally located activator domain (peptidase M9N domain) and the thermolysin-like peptidase domain (peptidase M9 domain), in short the collagenase unit [74, 95] (Fig. 1). This is in perfect agreement with the fact that both domains were shown to be essential for collagenolysis in ColG [74] as well as ColH and ColT (Schönauer E, unpublished data). Duarte et al. suggested based on analysis of the PFAM database the presence of 12 unique domain architectures for bacterial collagenases. The majority satisfy the collagenolytic double-domain architecture.

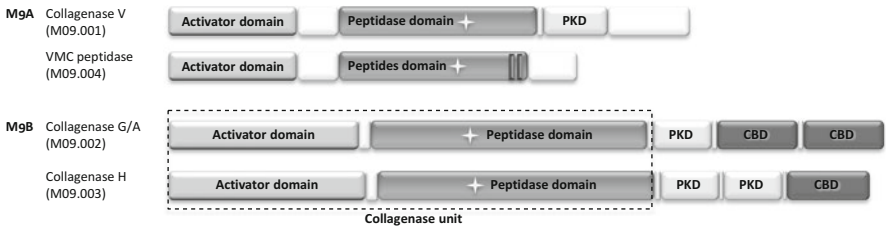


Fig. 1 Domain architecture of the bacterial collagenases of peptidase family M9 exemplified by its holotypes collagenase V from *V. alginolyticus* (Uniprot P43154), VMC peptidase from *V. mimicus* (Uniprot O67990), ColG (Uniprot Q9X721) and ColH (Q46085) from *C. histolyticum* (adapted from [17]). Schematic representation with functional annotation. The zinc-binding HEXXH motif (*asterisk*) is indicated. The *double bar* in the VMC peptidase designates the collagen-binding FAXWXXT motif [89]

Four of the twelve groups, however, appear to violate the postulated signature architecture composed of activator domain and peptidase domain. Yet, a closer examination reveals that three of the remaining groups are in need of revision: One group is founded on an obsolete protein entry (UniProt: Q4MHU9). Two more groups are actually based on the very same protein, i.e., the protein (UniProt: Q81DA6) was erroneously split into two. Moreover, this protein does not harbor a HEXXH-motif. Thus, it is most likely proteolytically inactive. The final remaining architecture is based on the example of ColAH from *Aeromonas piscicola AH3* (Uniprot: A0KFM4) [76]. This enzyme might indeed represent a new architecture, as it harbors no sequential homologue to the activator domain N-terminal of its peptidase domain. Instead, it has a 614-aa long N-terminal extension and no C-terminal accessory domains. This enzyme was shown to degrade gelatin, i.e., heat-denatured collagen, using the cell-free supernatant of AH3 strains. Its collagenolytic activity was assayed using the peptidic substrate FALGPA. However, as shown by Eckhard et al., the peptidolytic and collagenolytic activity are harbored by different protein domains: The peptidase domain of ColG alone is able to degrade FALPGA, but cannot cleave triple-helical collagen. Only the concerted action of activator and peptidase domains enables collagenolysis [74]. Thus, evidence for the collagenolytic activity of ColAH is missing, as it was only assayed with a short peptidic substrate. Intriguingly, the authors showed by Far-Western blotting that a 100 kDa protein, corresponding well to the gelatinolytic protein found in the supernatant, bound to collagen type I. Consequently, they suggest the presence of a novel collagen-binding motif N-terminal of the peptidase domain in ColAH (termed RPT1 and RPT2), which is distinct from the established CBD in clostridial collagenases. Yet, no mutational studies were performed to confirm this hypothesis [76]. Thus, further experiments are needed to clarify the functional role of the RPT motifs and demonstrate the collagenolytic activity of ColAH.

The C-terminal CBD belongs to the family of bacterial prepeptidase C-terminal domains (PPC) and is a ~115 aa large module. Based on studies of the CBD of ColG and ColH, it was shown that the CBDs specifically bind native collagen and that this

binding is enhanced in the presence of calcium [90, 96]. They recognize the triple-helical conformation of collagen and are required for the recognition of insoluble collagen fibers in clostridial collagenases [90, 91, 96]. The CBDs can bind to a broad spectrum of insoluble collagen types in vitro and in vivo [97]. Thus, various types of native collagen can be recruited to the clostridial collagenases through their CBD.

Unlike for the CBD, the role of the PKD in bacterial collagenases is less clear. The PKD is composed of 80–90 amino acids. It was originally identified in the human polycystic-kidney disease protein, polycystin-1, a cell surface protein involved in protein–protein, and protein–carbohydrate interactions [98, 99]. In bacterial collagenases, it is thought to function as an interdomain alignment platform between the collagenase unit and the CBD. Both the CBD and the PKD share an N-terminal calcium switch that stabilizes the interdomain alignment and thus, the overall stability of the collagenases [100, 101]. Its role in collagenolysis is controversial. While the two PKDs of ColH alone or in tandem were shown to be unable to bind to insoluble collagen [91], the PKD of the bacterial collagenolytic serine protease deseasin MCP-01 bound to insoluble collagen. In this case it was even shown to facilitate collagenolysis by swelling collagen fibers, thereby exposing the collagen triple helices, but not unwinding them [92, 102].

Remarkably, there is no evidence for a zymogen state in microbial collagenases, contrasting their human analogues, e.g., MMP-1, -2, -8, -13, -14 [14, 15]. This striking difference between mammalian and microbial collagenases can probably be explained by the scarcity of endogenous substrates in the latter organisms. Therefore, there is less need for activity regulation in single celled organisms.

5 Substrate Specificity of Clostridial Collagenases

Vertebrate collagenases process collagen with strict substrate specificity, cleaving it only at well-defined recognition sites, and with distinct preferences for different fibrillar collagen types. MMP-1, -8, -13, and -14, for example, utilize a shared single cleavage site to cut interstitial collagen into ¾ and ¼ length fragments, cleaving after a glycine in P1 [14, 103]. The preferred substrate of MMP-1 is type III collagen, MMP-8 favors type I collagen, and MMP-13 prefers to process type II collagen as substrate [104–106]. These initial cleavages by the MMPs compromise the “protease-protective” unique triple-helical fold of native collagen and allow the subsequent degradation of the fragments by downstream gelatinases and other proteases.

In stark contrast to that, clostridial collagenases can process collagen triple helices at multiple sites. They possess endo- and tricarboxypeptidase activities, and can decompose collagen completely into small peptides [107, 108]. It was shown for ColG and ColH that they do not exhibit a distinct preference for any interstitial collagen type, whether in the form of soluble tropocollagen or as insoluble collagen fibrils [109]. This broad substrate specificity seems to present

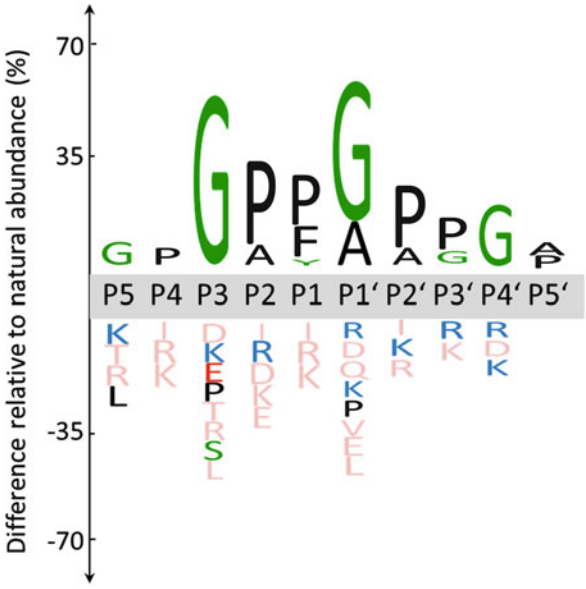


Fig. 2 PICS specificity profiles for ColG from *C. histolyticum*. Cleavage site specificity is shown as iceLogo [110] showing percent difference compared to natural amino acid abundance. Significantly over-represented residues are shown above the axis, under-represented ones beneath the axis. Non-identified amino acids are indicated in pink (adapted from [111])

an inherent feature of the clostridial collagenases. It is encoded in both the peptidase domain and the C-terminal collagen-recruitment domains. Identified by mass spectrometry-based PICS cleavage site specificity assays, the specificity profiles for the peptidase domains of ColG and ColH from *C. histolyticum*, and of ColT from *C. tetani*, all revealed a collagen-like specificity pattern in the proteome-derived substrate library. They displayed a strong preference for glycine in P3 and P1′, and a clear preference for proline in P2 and P2′ (Fig. 2). Thus, the substrate specificity for collagen-like sequences is already imprinted in the active sites of the peptidase domains [111]. Moreover, it was shown for the CBDs of ColG that they exhibit a broad substrate spectrum, binding to insoluble type I, II, III, and IV collagens [97]. This shows that both substrate recruitment and hydrolysis in clostridial collagenases are optimized for fast and promiscuous processing of collagen. This allows saprophytic clostridia, unrivaled by collagenase-deficient species, to utilize collagen directly as source of carbon, while collagenolytic MMPs act as fine-tuned and tightly regulated scalpels in endogenous collagen remodeling [111].

Due to their broad specificity for diverse collagen substrates, ColG and ColH have found widespread application in industry (e.g., food and leather industry [112–115]), in life-sciences research (e.g., [21, 22]), and in clinics [116–121] (for further details on biotechnological application, see [76]).

6 Inhibitors Are Substrate Mimetics

A major step in our understanding of clostridial collagenolysis was achieved by the pivotal crystal structure of the collagenase unit that was only published in 2011 [74]. Since then, only a single publication about novel inhibitors of clostridial collagenases (ChCI) has been published. These were discovered in extracts from the plant *Viola yedoensis* (see below) [122], and represent an example of drug discovery for bacterial collagenase inhibitors from biological sources [123, 124]. Thus, to the best of our knowledge, no inhibitors based on structure-guided design have been published yet.

All to date published inhibitors have been developed as substrate analogues based on known substrate preferences and/or have been patterned after inhibitors for other metalloproteases such as thermolysin or MMPs [125–133]. Importantly, the targets of these efforts have almost exclusively been ColG and ColH from *C. histolyticum*, as these enzymes are easily commercially available and well described. These commercial preparations were derived from culture filtrates and contained a mixture of ColG, ColH, and of various C-terminally truncated derivatives. Thus, the reported inhibition constants, typically assayed with the peptidic substrate FALGPA or the Pz-peptide [134, 135], reflect a kind of averaged inhibition of both collagenases.

The synthetic ChCI follow the classical architecture of metalloprotease inhibitors. They consist of (1) a "backbone" that mimics the natural substrates and provides the donors for subsite interactions, and (2) a zinc-binding group (ZBG) that chelates the catalytic zinc ion. Thereby, the water molecule is expelled from the coordination sphere, rendering the enzyme inactive [136, 137]. Various ZBG have been employed. The majority of them are monodentate zinc ligands such as ketones, aldehydes, thiols, phosphoric amides, phosphonic amides, and carboxylates groups [125–133, 136]. Yet, the bidentate chelator hydroxamate and its derivatives predominate, reflecting their wide-spread popularity in the field of metalloprotease-inhibitor development (Fig. 3). This popularity arose from their high inhibition potency. A comparison between inhibitors with a constant backbone, but divergent ZBGs showed the highest inhibition potency for hydroxamates followed by reverse hydroxamates. Carboxylates were, for example, 100- to 2000-fold less potent than hydroxamates [138].

Claudiu Supuran and colleagues developed a large series of potent inhibitors of vertebrate and clostridial collagenases using hydroxamate as ZBG [128–132, 139–142]. Particularly, sulfonylated amino acid hydroxamates but also the structurally related arylsulfonylureido and arylureido derivatives showed potent ChC inhibition with inhibition constants in the low nanomolar range (Table 2). These groups are thought to mimic the P1 residue of substrates. In combination with alkyl/arylsulfonamido hydroxamates, perfluoroalkylsulfonyl, perfluorophenylsulfonyl, 3-trifluoromethylphenylsulfonyl, 3-chloro-4-nitro-phenylsulfonyl, 3-/4-protected-amino-phenyl-sulfonyl; 3-/4-carboxy-phenylsulfonyl moieties proved to be best in the presumed P1′ position. The inhibition profile of these compounds, as

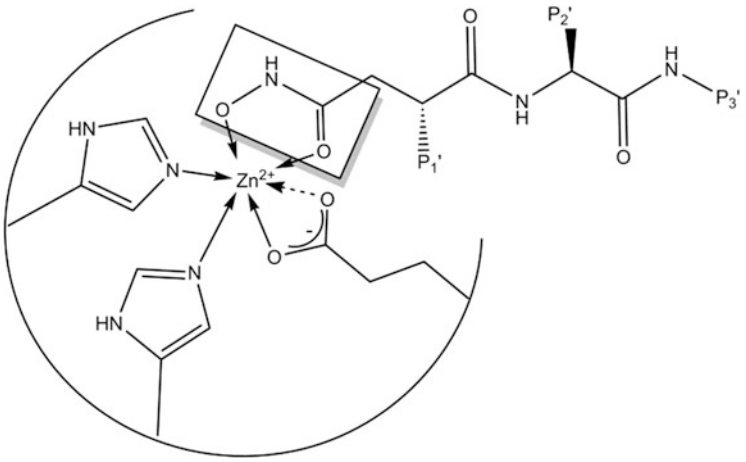


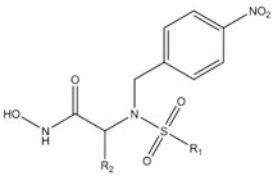
Fig. 3 Bidentate chelation of the catalytic zinc ion by a hydroxamate inhibitor in the active site of a gluzincin. The zinc can be coordinated by five (trigonal bipyramidal coordination) or six (octahedral coordination) ligands, depending on whether both carboxylate oxygens of the glutamate participate in the coordination. The binding mode is modelled based on the inhibitor-complex crystal structures of *C. clostridium* neurotoxin A light chain (PDB code: 3QIY) and thermolysin (PDB code: 4tln, 5tln, 7tln), respectively. The hydroxamate moiety is boxed

exemplified in Table 2, showed a high potency against all tested collagenases. Yet, it also illustrated the problematic lack of selectivity of ChCI between vertebrate and clostridial collagenases, but also among the MMPs. In MMPs, the hydrophobic S1′ pocket represents a crucial determinant for the development of selective inhibitors, as it represents a well-defined P1′-binding site. Based on the S1′ subsite, the MMPs are typically divided into deep, intermediate, and shallow S1′ pocket groups (e.g., deep: MMP-3, -12, and -14; intermediate: MMP-2, -8, and -9; and shallow: MMP-1, and -7) [145]. Based on their inhibition findings, Supuran et al. suggested that the S1′ site in ChC is similar to the shallow pocket of MMP-1 [128–132, 139–143].

Next to their lack in selectivity towards ChC, a second problem of hydroxamate-based ChCI is their poor pharmacokinetics and toxicity imparted by the hydroxamate group [137, 144]. FDA approved hydroxamate-based inhibitors are rare [137]. Therefore, research in metalloprotease inhibitors has turned to different ZBGs and inhibitor design strategies, most evident in the field of MMP inhibitors. Aided by high-resolution enzyme-inhibitor structures, inhibitor design could move on to (1) the identification of novel ZBGs, (2) non-zinc-chelating inhibitors, and (3) suicide inhibitors [146].

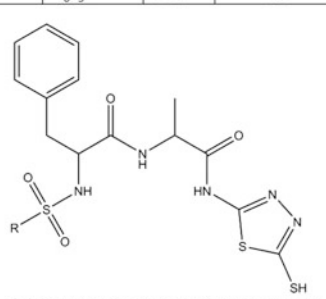
In case of ChCI, thiadiazoles were investigated as alternative ZBG to the hydroxamates [132, 143, 144]. Based on 5-amino-2-mercapto-1,3,4-thiadazole, a series of compounds were generated which showed inhibitory activities in the low micromolar range. Of those, sulfonylureido derivatives proved to be best compared to sulfonamides (Table 2). Yet, again no significant selectivity towards ChC over

Table 2 Inhibition of ChC and MMPs with sulfonylated hydroxamates and thiadiazoles

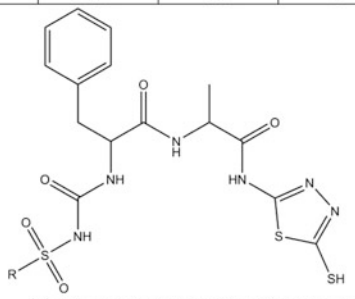


Sulfonylated hydroxamate

			K_i (nM)				
Compound	R_1	R_2	MMP-1[a]	MMP-2[a]	MMP-8[a]	MMP-9[a]	ChC[b]
H1	*n*-C_8F_{17}	H	79	0.9	1.3	1.3	8
H2	C_6F_5	H	3.0	0.7	0.1	0.6	5
H3	C_6F_5	*i*-Bu	2.5	0.5	0.1	0.5	3



(A) Sulfonamido-substituted thiadiazole



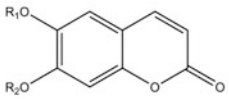
(B) Sulfonylureido-substituted thiadiazole

		K_i (μM)				
Compound	R	MMP-1[a]	MMP-2[a]	MMP-8[a]	MMP-9[a]	ChC[b]
A	C_6H_5	22	18	24	15	15
	4-F-C_6H_4	19	17	18	14	21
B	C_6H_5	19	5	5	4	3
	4-F-C_6H_4	12	2	0.1	0.3	0.2

[a]Spectrophotometric assay with Ac-Pro-Leu-Gly-S-Leu-Leu-Gly-O-Et
[b]Spectrophotometric assay with FALGPA
Adapted from [130, 143, 144]

the tested MMPs could be observed. Inspired by high-resolution data, showing non-primed interactions of a thiadiazole inhibitor with MMP-3 [147, 148], attempts were made to modify inhibitors such as to extend the (presumed) inhibitor-enzyme interface from the S1–S3′ site further to the non-primed site S2 and S3 site. However, these modifications failed to yield more selective ChCI [144].

Intriguingly, Oshima et al. reported a new potent ChCI, scoparone, with an IC50 of 24 nM determined by the Wünsch assay [122]. Scoparone was derived by methylation from esculetin and scopoletin, respectively. The later had been discovered as components with low micromolar inhibitory activity in extracts of *Viola yedoensis* (Fig. 4). Unfortunately, no data is available concerning the effects of these compounds on vertebrate collagenases. Yet, scoparone and scopoletin are recognized bio-active compounds. Scoparone is known for its anti-coagulatory,



Compound	R_1	R_2	IC50 (μM)
Esculetin	H	H	12
Scopoletin	CH_3	H	1.8
Scoparone	CH_3	CH_3	0.024

Fig. 4 Structures and IC50 values of esculetin derivatives [122]

-oxidatory, and -inflammatory effects [149], while scopoletin was identified as an acetylcholinesterase inhibitor [150]. Irrespective of these additional activities, these compounds might represent interesting hit compounds, although their inhibition mode towards ChC is unclear. A potential mode of inhibition would be by chelation of the active-site zinc. Coumarins are utilized as fluorophores in Zn^{2+} sensors. For this purpose, the fluorophore coumarin is usually conjugated to a metal-binding group that acts as chelating moiety. Yet, such a classical ZBG is missing in esculetin, scopoletin, and scoparone. Therefore, the mode of zinc chelation might be atypical in this case, but is still possible. The participation of the lactone oxygen of coumarin in zinc chelation was, for example, suggested in the case of a DPA-substituted coumarin [151]. Deciphering the binding mode of these organic compounds to ChC might provide valuable lessons for the optimization of ChCI.

In sum, a series of highly potent, yet broad spectrum inhibitors of clostridial and vertebrate collagenases were developed, which all target the active site located in the metallopeptidase domain of these enzymes. The development of more selective inhibitors against ChC was hampered by the lack of high-resolution information about clostridial collagenases. Based on the recently published crystal structures of the collagenase unit of ColG and of the peptidase domains of ColG, ColH, and ColT [74, 75], detailed information about the active-site topology is now available and novel structural determinants of activity regulation have been disclosed.

7 The Active Site of Clostridial Collagenases

Both ChC and MMP belong to the zincin family of metallopeptidases. Thus, they share a number of central characteristics in the peptidase domain. Both peptidase domains are segmented horizontally by the active-site cleft into an upper N-terminal and a lower C-terminal subdomains, to which the substrate can bind from the left (non-primed side) to the right (primed side) when viewed in standard orientation [152]. Central components of the N-terminal subdomain (NSD) are the *active-site helix*, and the mixed five-stranded β-sheet. Located in the active-site helix is the zinc-binding consensus sequence HEXXH, which provides the two zinc-coordinating histidines and the general acid/base glutamate. The lowermost β-strand of the mixed β-sheet forms the upper rim of the active-site cleft, the *edge strand*. The edge strand interacts in an antiparallel manner with the substrate predominantly on the non-primed side [74, 153, 154]. The remainder determinants of the active site differ among ChC and MMPs. In MMPs, the zinc-coordination

sphere is completed by a histidine as third proteinaceous ligand that is situated 6 residues downstream of the HEXXH motif in the active-site helix. The sphere is further lined at the bottom by a 1,4-β-turn with a strictly conserved methionine, which forms a hydrophobic basement to the catalytic zinc [155, 156]. In contrast to that, in clostridial collagenases the third zinc ligand is a glutamate residue that is provided by the *glutamate helix* of the C-terminal subdomain (CSD). The ~30 aa long insertion between HEXXH motif and the liganding glutamate shapes the non-primed side of the active-site cleft and forms a calcium-binding site crucial for enzymatic activity, see below. The glutamate helix moreover provides a strictly conserved alanine residue, which is structurally equivalent to the aforementioned methionine in MMPs. It forms the basement of the zinc-coordination sphere in clostridial collagenases [74, 75, 153].

A comparison of the active-site clefts of ColG and MMP-1 as representative examples illustrates the subtle differences between the active sites of the two enzyme families (Fig. 5a–f) [111]. In ChC, the active-site cleft carves a wide and prolonged groove covering nearly the entire face of the catalytic domain [74, 75]. In contrast to that, the active-site cleft in MMPs appears shallow and less well-defined at the non-primed side, while concluding on the primed side in a very narrow and pronounced closing shaped by the S1′ pocket (Fig. 5e, f) [158, 159]. This pronounced difference in active-site cleft topology particularly on the primed side strikes when looking, for example, at the volumes occupied by the clefts here: In ColG, the cavity of the primed side has 649.8 $Å^3$, while in MMP-1 it is considerably smaller with 283.9 $Å^3$ (calculated with the CASTp server [160] and pdb codes: 4are, 1hfc).

Intriguingly, the most prominent difference between ChC and MMPs is in the substrate recognition site S1′. As mentioned, all MMPs harbor a distinct hydrophobic S1′ pocket carved into the CDS, which represents a crucial determinant for substrate binding in MMPs [159]. The S1′ pocket typically can accommodate a leucine or isoleucine as P1′ residue. Even though MMP-1 has one of the shallowest S1′ pockets among the MMPs [145], in comparison to ColG it strikes as a deep and well-defined pocket. In ChC, the S1′ site is formed by a double Gly motif in the NSD, located directly adjacent to the edge strand. This motif forms a secondary oxyanion pocket and aligns via its backbone amides the carbonyl oxygen of preferably small P1′ residues, such as glycine or alanine, to the upper rim of the active site [74]. Binding to the S2′ pocket is thought to be crucially mediated by the aromatic Phe515, which is part of a wall segment that demarcates the active-site cleft on the primed side of clostridial collagenases [111].

In ChC, binding to the non-primed side (S3 to S1) of the wide active-site cleft is facilitated by the edge strand. The extended/pronounced pockets S3 to S1 can accommodate even rigid residues such as proline and hydroxyproline that cannot adopt an ideal extended β-strand conformation for enzyme binding due to their reduced backbone flexibility [74, 111]. This is reflected by the substrate preference of ChC for proline in P2, and, to a minor extent, also in P1 (Fig. 2) [111], whereas in MMPs, small amino acids such as glycine are preferred in position P1 [159]. In ChC, a hydrophobic bulge in the S1 site forces the substrate to adopt a bent

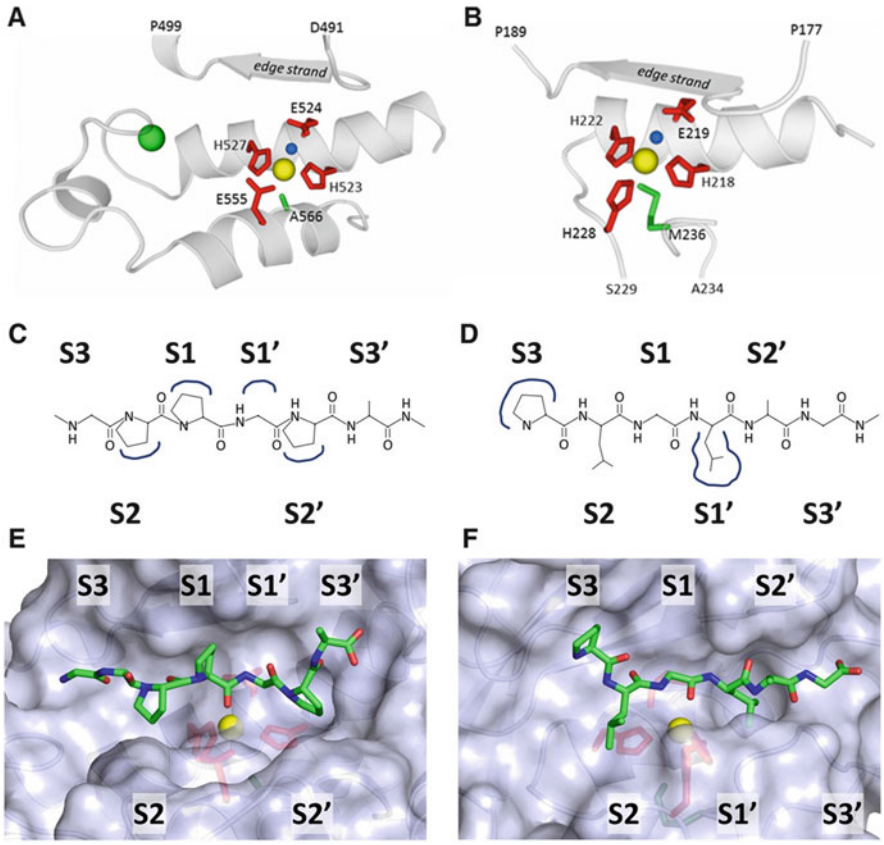


Fig. 5 Comparison of ColG (*left*) and human MMP-1 (*right*). (**a**, **b**) Ribbon representation of the unliganded active-site clefts, depicting the active-site helix, the edge strand, the HEXXH motif (*red sticks*), and the hydrophobic basement (*green stick*). The active-site zinc (*yellow sphere*) is tetrahedrally coordinated by three proteinaceous residues and a water molecule (*blue sphere*). The calcium ion is indicated (*green sphere*) and was modelled after the crystal structure of the peptidase domain of ColT (pdb: 4ar9), as the crystallization condition for the collagenase unit of ColG contained >125 mM citrate, which chelated the calcium ion and consequently the calcium site was occupied by a water molecule rather than by a calcium ion [75]. (**c**–**f**) Substrate binding to the active-site clefts of two hexapeptides (ColG: Gly-Pro-Pro-Gly(P1′)-Pro-Ala; MMP-1: Pro-Leu-Gly-Leu(P1′)-Ala-Gly) determined by active-site docking: (**c**, **d**) Schematic representation, (**e**, **f**) Surface representation of the liganded active-site clefts (adapted from [111]). Molecular figures were created with PyMOL [157]

conformation upon binding, which irrespective of their rigidity can be adopted by proline and hydroxyproline [74, 111]. The strain imposed on the substrate by this distorted conformation is counter-balanced by the successive glycine residue in P1′ that is held in place by the secondary oxyanion pocket. As a consequence, the substrate recognition sites S2′ and S3′ are horizontally mirrored in ChC when compared to MMPs (Fig. 5c–f). Thus, the secondary oxyanion pocket in S1′ and

the hydrophobic bulge in S1 are major determinants of substrate recognition in ChC [111].

8 Exosites

Interestingly, it was recently discovered that clostridial collagenases harbor a calcium-binding site in close proximity to the active site. It is located on the left rim of the active-site cleft, near the substrate recognition site S5 and only 15 Å away from the catalytic zinc ion. The calcium-binding site in ColG is formed by two water molecules, the backbone oxygens of Ala531, Val535, and Gly537, which are provided by the ~30 aa insertion between the active-site helix and the glutamate helix, and by the side chain of the conserved Glu498, downstream of the edge strand. The octahedrally coordinated calcium ties the NSD to the CSD and thereby stabilizes the zinc-coordination sphere (Fig. 5a) [75].

Both ions, calcium and zinc, are essential for the catalytic activity of clostridial collagenases. This was shown for the full-length enzymes and the isolated peptidase domains [75, 87]. The mechanism of this dual metal dependence is not fully understood yet. Nonetheless, the calcium-binding site represents a new and, compared to MMPs, unique target for inhibitors of clostridial collagenases, which might enable the development of highly selective ChCI.

The recent findings on ColG have also disclosed other target sites for inhibitors next to the active-site cleft on the peptidase domain of ChC [74]. The crystal structure of the catalytic core of ColG revealed that the collagenase unit, which is composed of the activator domain and the peptidase domain connected via a short glycine-rich linker, adopts a saddle-shaped architecture (Fig. 6). Intriguingly, it was shown that the peptidase domain is capable of cleaving the collagenase-specific peptidic substrate *N*-(3-[2-furyl]acryloyl)-L-leucyl-glycyl-L-prolyl-L-alanine, while it fails to degrade triple-helical collagen. However, in concert with the activator domain, the peptidase domain is able to unwind and cleave native collagen in vitro, without the need of its accessory C-terminal domains. Accordingly, the authors proposed a two-step model of bacterial collagenolysis, in which the concerted opening and closing of the collagenase unit prime collagen triple helices for cleavage [74]. Although the detailed mechanistic interplay between the activator domain, linker and the peptidase domain during collagenolysis is not fully understood yet, targeting the activator domain and the linker region might represent new avenues for ChCI development.

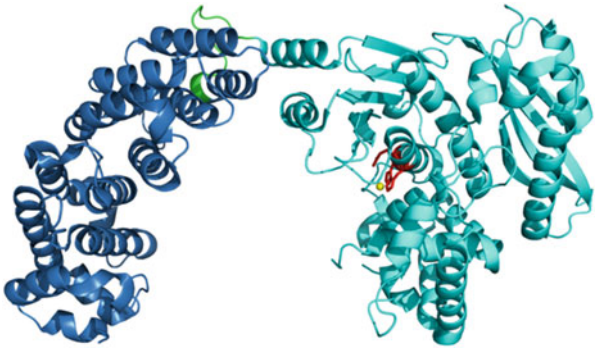


Fig. 6 Quaternary architecture of the collagenase unit of ColG. Ribbon representation of the collagenase unit of ColG (PDB: 2y6i) composed of the activator domain (*dark blue*) and the peptidase domain (*cyan*). The catalytic zinc ion (*yellow*) and the catalytic residues (*red*) are shown in ball-and-stick representation. The linker region is highlighted (*green*). Molecular figures were created with PyMOL [157]

9 Conclusion

The development of potent clostridial collagenase inhibitors can now exploit structural and mechanistic analyses of interesting hit compounds. Comparison of such data with available structure–activity relationship data in vertebrate MMPs will further benefit our understanding of both enzyme classes, and assist the development of ChCI with high specificity. Along the same line, structure-guided screening of new chemical entities will allow to target the clostridial collagenase activity away from the catalytic zinc, e.g., the calcium site on the left rim of the active-site cleft or the glycine hinge connecting and controlling the concerted unwinding and processing of collagens by the activator and peptidase domains.

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Fungal Carbonic Anhydrases and Their Inhibition

Ronny Lehneck and Stefanie Pöggeler

Abstract In fungi, carbonic anhydrases of the β class are widely distributed. These metalloenzymes play important roles in growth, development, virulence, and survival of fungi. Fungal pathogens are the causative agents of various diseases in plants, animals, and in humans. The number of drugs specifically targeting these pathogens such as components of the fungal plasma membrane, the cell wall, or fungal-specific biosynthetic pathways is limited. Recent investigations have therefore focused on the function and inhibition of fungal β-carbonic anhydrases as new antifungal targets and will be described in this review.

Keywords Antifungal drugs, Carbonic anhydrases, Fungi, Inhibitors, β class

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1 Introduction

Carbonic anhydrases (CAs) are metalloenzymes that catalyze the interconversion of carbon dioxide and water to bicarbonate and protons. These metalloenzymes are present in organisms from all three domains of life and can be divided into five classes (α, β, γ, δ, and ζ) that are unrelated in amino acid sequence and structure. Fungi possess multiple CAs of the α and β classes, whereas fungal-like organisms such as the oomycete *Phytophthora infestans* also encode CAs of the γ class [1, 2].

In all organisms, CAs contribute to a broad range of important biological processes by providing high amounts of CO_2 or HCO_3^- to anaplerotic or biosynthetic reactions [3–5]. They are involved in the carbon concentrating mechanism in cyanobacteria, in the biosynthesis of fatty acids, amino acids, and DNA, and have been implicated in proliferation, survival, and differentiation of many pathogenic organisms, both inside and outside their respective hosts [6–10].

For long time CAs were believed to be exclusively Zn^{2+}-dependent metalloenzymes, but in 2000, the first functional CA with cadmium at the active site was reported from the marine diatom *Thalassiosira weissflogii* [11]. The Cd^{2+} ion is typically incorporated under conditions of zinc limitation and fully supports CA activity. The anaerobic methane producing bacteria *Methanosarcina thermophila* contains two CAs (Cam and CamH), that contain ferrous iron at their active site when heterologously purified from *Escherichia coli* under anaerobic conditions [12–14]. The CA activity of the iron enzyme is threefold higher than that of the zinc enzyme purified in the presence of oxygen. The ferrous iron is rapidly oxidized to the ferric form when the enzyme is exposed to ambient air conditions, and this is accompanied by a loss of CA activity. Furthermore, a variety of other divalent metal ions (Co^{2+}, Mn^{2+}, Ni^{2+}, and Cu^{2+}) can replace zinc in the active site of bacterial and mammalian CAs in-vitro without loss of activity [15–17].

Despite the importance of CAs for many cellular processes in normal aerobic conditions, some prokaryotes and the ascomycetous fungus *Sordaria macrospora* can survive without any CA-encoding genes [18, 19]. These microorganisms are adapted to environmental niches where CO_2 is available at high concentrations. In ambient air, their growth depends on HCO_3^- or CO_2 produced by other organisms, or in case of *S. macrospora*, on a switch from growth at the air/medium interface to submerged growth, which provides access to CO_2-enriched niches containing sufficient HCO_3^- (see also below) [19, 20]. Genomic and phylogenetic analyses

of CA-deficient prokaryotes revealed that genes coding for CAs have been lost during evolution [21, 22]. Hence, the lack of CA genes might explain why many of these prokaryotic microorganisms cannot be cultivated under normal CO_2 conditions. Several experiments have shown that CAs are essential for growth in ambient aerobic conditions, therefore, most prokaryotes and all eukaryotes studied so far encode at least one CA. The crucial role of CAs in microorganisms has been demonstrated by gene-deletion studies in bacteria including *E. coli* [23], *Ralstonia eutropha* [24], *Haemophilus influenzae* [25], and *Corynebacterium glutamicum* [26] and in fungi including *Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Aspergillus nidulans* [6, 27–29]. These studies all showed that CA activity is essential for microbial growth in ambient air. Bacterial or fungal CA deletion mutants, with the exception of *S. macrospora*, grow only at elevated CO_2 concentrations (5%), when provided with HCO_3^-, or when co-cultured with other bacteria [19, 30].

Because of their mode of action, CAs are also proposed as biocatalysts for industrial applications such as CO_2 capture and sequestration, generation of biofuels, and cheap CO_2 production for industrial purposes [31]. Attention has increased in recent years due to the need to reduce and ultimately reverse the release of the anthropogenic CO_2 into the atmosphere. Current techniques to extract CO_2 from air or industrial fumes are very expensive and require harsh chemical conditions and elevated temperatures [32]. The utilization of CAs as biocatalysts offers a very attractive alternative approach that is renewable, selective, and relatively inexpensive [33]. The most promising industrial results to date were achieved with mammalian α-CAs that can be easily overproduced in *E. coli* and that have fast kinetic parameters [34, 35].

Since their discovery, CAs have become of pharmacological interest, since their activity has not only been connected with a broad range of human diseases, but they are also virulence factors in bacterial and fungal pathogens [36]. Intensive research has identified some potent CA inhibitory agents (CAI) including anions, sulfonamides, and dithiocarbamates (DTCs) [37, 38]. CAIs have been used to treat human diseases such as glaucoma, convulsion, obesity, and especially cancer [39]. To explore the possible application of CAIs as therapeutic substances in bacterial human diseases, several in-vivo studies have already been initiated [40]. The mode of action of the majority of CAIs is well understood and typically involves the direct interaction between the deprotonated inhibitor with the metal ion at the active site [41]. CAs can also be inactivated by other classes of inhibitors that don't bind the active metal site directly; polyamines bind the zinc-coordinated water/hydroxide ion and coumarins bind to and block the active site entrance [42–44].

Structure-based drug discovery is a promising, emerging field that could lead to the development of efficient and selective anti-CA agents [45]. CAs from various microbial pathogens or of mammalian origin have been crystallized in complex with known or putative inhibitors, which have revealed details of enzyme-inhibitor interactions at the molecular level. This knowledge can be used to design novel anti-CA drugs and enhance the efficiency and selectivity of existing anti-CA agents [46–49].

In recent decades, fungal pathogens have been identified as the causative agents of numerous diseases in plants and animals, and they are a danger to immunocompromised patients [50, 51]. However, the number of drugs specifically targeting fungi such as components of the fungal plasma membrane, the cell wall, or biosynthetic pathways is extremely limited. Moreover, pathogenic fungal species are developing resistance to many of the antifungals currently in clinical use. Therefore, recent investigations have focused on the function and inhibition of fungal β-CAs that are not present in animals and hence represent a potentially selective antifungal target [52–55].

In this review we have collated our current knowledge on fungal CAs and their potential inhibitors.

2 Fungal Carbonic Anhydrases

In the large fungal kingdom, carbonic anhydrases are widely distributed. Hemiascomycetous and basidiomycetous yeasts encode one or two β-CAs, while most filamentous ascomycetes encode multiple β-CAs, with some also possessing genes encoding α-class enzymes [1]. Gene-duplication and gene-loss events during evolution likely explain this high multiplicity. In filamentous ascomycetes, a gene encoding a plant-type β-CA was duplicated, resulting in two closely related isoforms differing in the presence or absence of an N-terminal mitochondrial target sequence [1].

Fungal CAs play an eminent role in growth, virulence, and survival, as well as the production of mycotoxins [56–58]. The pathogenic yeasts *Candida albicans*, *Candida glabrata*, and *Malassezia globosa* and baker's yeast *Saccharomyces cerevisiae* encode only a single plant-type β-CA enzyme, and the growth of the corresponding CA-deletion mutants is, similar to prokaryotic CA-deletion strains, inhibited in ambient aerobic conditions [27, 28, 59, 60]. In contrast, human pathogenic basidiomycetous yeasts such as *Cryptococcus neoformans* and *Cryptococcus gattii* have two β-class CA-encoding genes (*can1* and *can2*), although only the deletion of *can2* inhibits growth under regular CO_2 conditions [6, 61]. As shown for the prokaryotic CA-deletion strains, the high CO_2-requiring phenotype can be restored either by incubation at 5% CO_2, or by addition of the end products (e.g., arginine or fatty acids) of CO_2 and HCO_3^--dependent biosynthetic pathways [6, 62].

The levels of *S. cerevisiae*, *C. albicans*, and *C. glabrata* β-CA gene transcripts are regulated by CO_2 concentration such that under normal CO_2 conditions expression of CA genes is up-regulated, but expression is drastically reduced at elevated CO_2 concentration [27, 59, 63]. Moreover, a low CO_2 concentration induces filamentous growth and promotes a switch from a white to an opaque form that facilitates mating in the human pathogenic yeast *C. albicans*. In addition, it has been shown that *C. albicans* depends on CA activity for pathogenesis on the skin of its host during epithelial invasion [28]. Likewise in *C. neoformans*, bicarbonate

produced by Can2 is required for later mating steps, including production of potentially infectious spores. The in-vivo proliferation and virulence at high CO_2 levels in the host were not affected in the *C. neoformans* and *C. albicans* CA mutants [6, 28]. It was only recently uncovered exactly how fungi sense CO_2 concentration and regulate CA expression. In *C. albicans* and *C. neoformans* a bicarbonate-dependent adenylyl cyclase, homologous to prokaryotic adenylyl cyclases, is activated by bicarbonate [28, 64–66]. These enzymes produce the important second messenger cAMP and are essential for pathogenicity-related morphogenesis such as capsule synthesis in *C. neoformans* or filamentation in *C. albicans*. A *C. albicans* mutant without a functional adenylyl cyclase is not able to grow filamentously and loses its pathogenicity [28]. Surprisingly, the expression of CA genes appears not to be regulated by adenylyl cyclases [67]. Rather, in *C. albicans*, the transcription factor Rca1p was defined as the first direct CO_2 regulator of CA genes in yeast. Rca1p activates CA-gene expression at low CO_2 concentrations, independent of the adenylyl cyclase, and also appears to repress virulence-associated genes, confirming the existence of an additional cAMP-independent CO_2 signaling pathway [67]. Orthologs of Rca1p were also identified in the yeasts *S. cerevisiae* (Cst6p) and *C. glabrata* (CgRca1p) [59]. In the transcription factor deletion mutants of these organisms, CA gene expression was no longer induced in ambient air. Furthermore, the transcription factor Cst6p in *S. cerevisiae* was found to control CA gene expression through a conserved TGACGTCA motif in the promoter of the yeast CA gene *nce103* [67, 68]. An identical sequence was also identified in the promoter of the *C. glabrata* CA gene, but the sequence is not present in the *C. albicans nce103* promoter [59]. Deletion of the TGACGTCA motif in the promoter of *S. cerevisiae* and *C. glabrata* CA genes led to the loss induction of CA gene expression in ambient aerobic conditions (0.04% CO_2).

Similar to the basidiomycete *C. neoformans*, a major and minor β-CA were also discovered in filamentous ascomycetes [8, 29]. *Aspergillus nidulans* has two β-CA class genes (*canA* and *canB*) and the human pathogen *A. fumigatus* possesses four β-CA genes (*cafA*, *cafB*, *cafC*, and *cafD*) [69]. Inspection of the genomes of other members of the genus Aspergillus revealed that some species such as *A. niger*, *A. flavus*, *A. oryzae*, and *A. terreus* encode β-CAs in addition to one or two α-CA enzymes [10]. In *A. fumigatus*, *cafA* and *cafB* are constitutively and strongly expressed, while *cafC* and *cafD* are weakly expressed and are induced by low CO_2 concentrations. In *A. nidulans*, only deletion of *canB* inhibits growth in ambient aerobic conditions, whereas the growth of single-deletion mutant stains of *A. fumigatus* is not inhibited in the presence of ambient CO_2 levels. In *A. fumigatus*, only the ΔcafAΔcafB double mutant is unable to grow in regular CO_2 (0.004%) conditions [29]. Furthermore, deletion of CA genes affects conidiation in *A. fumigatus*, in accordance with studies on *S. cerevisiae* in which CA Nce103p seems to be involved in spore formation [70]. The virulence of single and double CA mutants of *A. fumigatus* is not affected in a low-dose murine infection model, similar to comparable CA mutants of *C. albicans* and *C. neoformans* [29]. While both β-CA genes of *A. nidulans* rescue a CA-deficient

S. cerevisiae mutant (Δnce103), only the *A. fumigatus cafB* gene complements the yeast deletion strain [29]. As with *A. fumigatus*, four CA genes were identified in the genome of *S. macrospora* and were designated as *cas1 – 4* [1, 2, 8]. The β-CA genes *cas1* and *cas2* share high sequence identity and encode enzymes with features characteristic of the plant-like subclass of β-CAs [1]. The β-CA CAS3 belongs to the cab-like subclass, while *cas4* encodes an α-class enzyme. CAS1 and CAS3 are cytoplasmic enzymes, while CAS2 is located to the mitochondria and CAS4 is secreted into the extracellular medium [8, 19, 56].

The functions of the three β-CA genes *cas1-3* and the α-CA gene *cas4* in sexual development have been studied in detail [8, 19]. Comparison of single-deletion mutants of *S. macrospora* β-CA genes with the wild-type (wt) strain revealed that only the Δcas2 strain exhibited significantly reduced vegetative growth and a slower ascospore germination rate, and all single mutant strains grew equally well in ambient air [8]. Double-deletion strains Δcas1/3 and Δcas2/3 also grow and form fruiting bodies in a way that is comparable to the wt strain in normal CO_2 concentrations. However, Δcas1/2 has a drastically reduced vegetative growth rate under these conditions, and produces sterile fruiting bodies only after an elevated incubation time. Sexual development and vegetative growth are fully restored in this double mutant by elevating CO_2 to 5%, but this does not rescue the reduced ascospore germination rate [8]. As mentioned above, *S. macrospora* is the only fungus described to date that can maintain growth in ambient air without any CA-encoding genes [19]. Interestingly, the quadruple mutant (Δcas1/2/3/4) exhibits a distinctive growth phenotype at 0.04% CO_2 on solid medium in which hyphae penetrate the agar instead of growing on the medium surface [19]. This vegetative growth defect is completely restored by the addition of 30 mg/L fatty acids to the culture medium or by incubation in 5% CO_2, but the ability to produce mature fruiting bodies and ascospores is not restored [19].

β-class CAs are present in many pathogenic fungi but absent in animals including humans, and research on these enzymes has intensified in recent years. Since the growth and pathogenicity of most fungal species is dependent on CA activity, selective inhibitors could be promising antifungal agents that have little or no side-effects resulting from interference with mammalian α-class CAs [53, 71, 72].

3 Inhibition of Carbonic Anhydrase Nce103p from *Saccharomyces cerevisiae*

The β-CA Nce103p of *S. cerevisiae* was the first fungal β-CA to be characterized [27, 73, 74], and an N-terminally truncated version of the enzyme was comprehensively studied at both structural and functional levels [74–76]. Nce103p has a dimeric type-I architecture with a typical β-CA fold consisting of an N-terminal arm composed of one or more α-helices and a central β-sheet build from four parallel strands in a β2-β1-β3-β4 arrangement followed by a C-terminal extension

[76, 77]. The N-terminal arm was shown to be important for activity but not for dimerization [76]. The enzyme is highly active (k_{cat} of 9.4×10^5 s^{-1}, and k_{cat}/K_M of 9.8×10^7 M^{-1} s^{-1}) and is inhibited by anions, sulfonamides, sulfamates, and DTCs [63, 75, 78, 79]. In general, sulfonamides/sulfamates such as acetazolamide, ethoxzolamide, methazolamide, and dorzolamide are used in the clinic, all of which are highly active towards the yeast enzyme and have K_i values in the range of 82.6–133 nM [75]. The inhibitor 4-(2-amino-pyrimidin-4-yl)-benzenesulfonamide has the strongest activity ($K_i = 15.1$ nM) against the *S. cerevisiae* enzyme, and some DTCs displayed low nanomolar inhibitory activity (6.4 and 259 nM) against Nce103p [79]. Inhibition of the yeast enzyme with anions has been explored, and bromide and iodide proved best, with K_i values of 8.7–10.8 μM. Activation studies revealed that L-adrenaline and some piperazines incorporating aminoethyl moieties were the most effective activators of *S. cerevisiae* Nce103p [78].

4 Inhibition of Can2 from the Pathogenic Basidiomycete *Cryptococcus neoformans*

The pathogenic basidiomycete *C. neoformans* has two β-class CA-encoding genes, *can1* and *can2*, the latter of which is essential for survival in ambient aerobic conditions [6]. Can2 has an in vitro activity k_{cat} of 3.9×10^5 s^{-1} and k_{cat}/K_M of $4.3 \times 10^7 M^{-1}$ s^{-1}, and is able to complement a *Candida albicans* CA deletion mutant in vivo [7, 80]. Because of its importance to pathogenicity and survival of *C. neoformans* [64], Can2 structure, activity, and inhibition have been thoroughly investigated. The inhibitory potency of anions, dithiocarbamates, and sulfonamide drugs was examined, as well as aliphatic and aromatic carboxylates and boronic acids [7, 38, 80–84]. The sulfonamide drugs acetazolamide and benzolamide ($K_i = 10.5$ nM and 23 nM, respectively), as well as substituted-phenyl-1H-indole-5-sulfonamides ($K_i = 4.4$–118 nM) all inhibited the enzyme effectively, while cyanide and sulfamic acid ($K_i = 8.22$–13.56 mM) were much weaker inhibitors. All other anions tested displayed more potent inhibition of Can2 [7, 80, 83]. Specifically, C1–C5 aliphatic carboxylates, oxalate, malonate, maleate, malate, pyruvate, lactate, citrate, and some benzoates have been investigated, of which acetate and maleate ($K_i = 7.3$–8.7 μM) were the most potent, whereas formate, valerate, oxalate, citrate, and 2,3,5,6-tetrafluorobenzoate were less effective ($K_i = 42.8$–88.6 μM). Likewise, propionate, butyrate, malonate, L-malate, pyruvate, L-lactate, and benzoate were weak Can2 inhibitors ($K_i = 225$–1,267 μM) [81]. Of the boronic acids tested, the aromatic 4-phenylsubstituted- and 2-naphthylboronic acids were the best Can2 inhibitors, with inhibition constants of 8.5–11.5 μM. In contrast, arylalkenyl and aryalkylboronic acids only weakly inhibited the *C. neoformans* enzyme, with K_i values of 428–3,040 μM [82]. A number of compounds from a series of

25 branched aliphatic and aromatic carboxylates had micromolar inhibition constants for Can2, and two (4-hydroxy- and 4-methoxy-benzoate) exhibited low nanomolar activity against the enzyme ($K_i = 9.5–9.9$ nM). Methyl esters, hydroxamates, hydrazides, and carboxamides of some of these derivatives were also effective inhibitors. *N*-mono- and *N*,*N*-disubstituted dithiocarbamates are another inhibitor class that is active against Can2, with inhibitor activity between the subnanomolar and micromolar range, depending on the substituents on the dithiocarbamate zinc-binding group nitrogen atom [38]. Finally, *N*,*N*-dihexyl-DTC and Schiff base sulfonamides also inhibit Can2 in the subnanomolar range, and are among the most potent CAIs for this fungal enzyme [38, 72].

Amino acids and amines have also been tested against Can2, and the *C. neoformans* enzyme was only slightly activated by L-/D-His, L-D-Trp or L-Tyr, with activation constants in the range of 28.7–47.2 μM, while L-Trp was the best Can2 activator [85].

5 Inhibition of β-CAs from the Ascomycetous Yeasts: *Candida albicans and Candida glabrata*

The enzyme CaNce103, from the opportunistic pathogen *C. albicans*, is highly active, with a k_{cat} of 8.0×10^5 s^{-1} and a k_{cat}/K_M of 9.7×10^7 M^{-1} s^{-1} [7]. This enzyme facilitates the growth of *C. albicans* under ambient air and is linked to pathogenicity [65, 86]. CaNce103 is strongly inhibited by cyanide and carbonate ($K_i = 10–11$ μM), and weakly inhibited by sulfate, phenylboronic, and phenyl arsonic acid ($K_i = 14.15–30.85$ mM) [7]. Compared with *C. neoformans* Can2, CaNce103 is more susceptible to inhibition with carboxylates such as maleate, benzoate, butyrate, and malonate ($K_i = 8.6–26.9$ μM). L-Malate, pyruvate, and valerate are less efficient CaNce103 inhibitors ($K_i = 87.7–94.0$ μM), while the remaining carboxylates are relatively efficient inhibitors (K_i in the range of 35.1–61.6 μM) [81]. The inhibition profile of CaNce103 with the 4-phenylsubstituted- and 2-naphthylboronic acids was quite similar to that of Can2, with K_i values in the range of 7.8–42.3 μM. However, the arylalkenyl and aryalkylboronic acids were weaker inhibitors ($K_i = 412–5210$ μM) [82]. Inhibition of the *C. albicans* enzyme was also investigated using a library of sulfonamides and one sulfamate, and CaNce103 was inhibited by these compounds, with K_i values in the range of 132 nM-7.6 μM. The most potent CaNce103 sulfonamide inhibitors were acetazolamide, methazolamide, bromosulfanilamide, and 4-hydroxymethylbenzenesulfonamide ($K_i < 500$ nM). In addition, CaNce103 was also highly sensitive to inhibition with DTCs [38], with K_i values in the range of 4.2–962 nM. The most effective inhibitors of CaNce103 ($K_i = 5.3–7.5$ nM) included aromatic, aliphatic, and heterocyclic moieties. In contrast, several derivatives incorporating aliphatic, arylalkyl, hetaryl, and amino acyl moieties were slightly less effective ($K_i = 37.3–61.3$ nM). The weakest inhibitors were simple derivatives incorporating methyl and ethyl groups ($K_i = 950–962$ nM) [38].

Similar to *C. neoformans* Can2, *C. albicans* β-CA is activated by amino acids such as L/D-His, L-D-Trp, and L-Tyr, with K_a values in the range of 19.5–46 μM, and amines such as histamine, dopamine, 2-aminoethyl-piperazine, and L-adrenaline ($K_a = 13.2$–18.5 μM) are even more effective activators. The best CaNce103 activators identified to date are L- and D-Dopa ($K_a = 0.96$–2.5 μM) [85].

The pathogenic yeast *Candida glabrata* differs from *C. albicans* by being haploid, and has the ability to grow as a facultative anaerobe. Furthermore, *C. glabrata* exhibits an intrinsic low susceptibility to azole antifungals [87].

The protein encoded by the *NCE103* gene (CgCA) of the pathogenic yeast *C. glabrata* is a β-CA that exhibits significant CO_2 hydratase activity ($k_{cat} = 3.8 \times 10^5$ s^{-1} and $k_{cat}/K_M = 4.8 \times 10^7$ M^{-1} s^{-1}). The enzyme is moderately inhibited by metal poisons (cyanide, azide, cyanate, and thiocyanate; $K_i = 0.60$–1.12 mM) but strongly inhibited by bicarbonate, nitrate, nitrite, and phenylarsonic acid (K_i in the range of 86–98 μM). Other anions exhibited inhibition constants in the low millimolar range, with the exception of bromide and iodide ($K_i = 27$–42 mM). Similar to CaNce103, the *C. glabrata* enzyme is highly sensitive to DTCs, with inhibition constants in the range of 3.9–913 nM, with mono- and di-substituted DTCs exhibiting K_i values < 10 nM [38]. Simple *N*,*N*-dimethyl/diethyl derivatives are the weakest CgCA inhibitors ($K_i = 874$–913 nM), and simple sulfonamides display weak to moderate inhibition activity, whereas acetazolamide and a series of 4-substituted ureido-benzene-sulfonamides, sulfamates, and sulfamides inhibit CgCA with K_i values in the range of 4.1–115 nM [88].

6 Inhibition of the Carbonic Anhydrases MgCA from the Dermatophytic Yeast *Malasseziaglobosa*

Infections of the skin are often caused by lipophilic yeasts of the genus Malassezia, of which *M. globosa* appears to be the most common causative agent of pityriasis versicolor [89]. *M. globosa* encodes only a single β-CA (MgCA) that exhibits an appreciable CO_2 hydratase activity, with a k_{cat} of 8.6×10^5 s^{-1} and a k_{cat}/K_M of 6.9×10^7 M^{-1} s^{-1} [90]. With an inhibition constant of 76 μM, the sulfonamide acetazolamide 5-acetamido-1,3,4-thiadiazole-2-sulfonamide that is used in the clinic did not inhibit the *M. globosa* enzyme, and surprisingly, neither did many other normally effective inhibitors. For example, different heterocyclic sulfonamide derivatives showed weak or very weak inhibitory activity, and of the heterocyclic sulfonamides tested, only benzolamide and aminobenzolamide were effective inhibitors [90].

Metal complexing anions such as cyanate, thiocyanate, and cyanide azide are also weak MgCA inhibitors, whereas phenylboronic acid and phenylarsonic acid are more effective [91].

7 Inhibition of CAS1 and CAS2 of the Non-Pathogenic Filamentous Ascomycete *Sordaria macrospora*

The genome of the coprophilous ascomycete *S. macrospora* encodes four CAs; two plant-type β-class enzymes, CAS1 and CAS2, have been analyzed with regard to their protein structure, enzymatic activity, and inhibition [92].

CAS1 is a cytosolic protein composed of 234 amino acid residues with a calculated molecular weight of 25.1 kDa. The truncated *cas2* gene without its N-terminal signal peptide encodes a protein of 225 residues with a calculated molecular weight of 25.9 kDa [92]. Unlike all other structurally characterized fungal β-CAs, *S. macrospora* CAS1 and CAS2 are tetramers. However, the monomeric structure closely resembles that of other β-CAs, with the three core elements (N-terminal arm, α/β core, and a C-terminal extension) present in both proteins [92]. A comprehensive analysis of the structural properties revealed that there is a difference in the active site environment of both proteins. Despite the fact that both enzymes are members of the plant-like subclass of β-CAs, the active center of CAS1 belongs to the type-I subclass of β-CAs while the CAS2 active core resembles the type-II subclass [1, 74, 92].

CAS1 and CAS2 exhibited measurable in-vitro CO_2 hydratase activity (k_{cat}/K_M of CAS1 = 1.30×10^6 M^{-1} s^{-1}; k_{cat}/K_M of CAS2 = 1.21×10^6 M^{-1} s^{-1}) [92]. In addition, CAS1 and CAS2 were only weakly inhibited by the widely used sulfonamide drug acetazolamide, with inhibition constants of 445 nM and 816 nM, respectively. Inhibition by anions was also investigated, as these have been shown to effectively inhibit CA activity in other organism [93]. The majority of the anions tested were ineffective in inhibiting CAS1 and CAS2, and resistance to anionic inhibitors such as SO_x and NO_x that are often present in flue gases makes them good candidates for industrial applications.

Perchlorate and tetrafluoroborate acted as weak inhibitors of CAS1 and CAS2 enzyme activity, similar to other CAs [94]. Nitrite and nitrate anions were also ineffective with inhibition constants over 100 mM, whereas the halogens bromide and chloride inhibited CAS1 with inhibition constants of 9.3 and 9.2 mM, respectively, but inhibited CAS2 much more weakly. Conversely, CAS2 was more strongly inhibited by sulfate ($K_i = 4.8$ mM) than CAS1 ($K_i > 100$ mM). Sulfamide, sulfamate, phenylboronic acid, and phenylarsonic acid proved to be the best anionic inhibitors (K_i of CAS1 = 84–9 μM, K_i of CAS2 = 72–48 μM) [92].

8 Conclusion

Given the emerging problem of drug resistance against antifungal agents currently in clinical use, the prevention of fungal invasive infections will be a challenge in the coming years. Furthermore, the close phylogenetic relatedness between humans and fungi makes it difficult to identify new pharmacological targets for antifungal

drugs, and fungi-specific proteins such as β-CAs are likely to become increasingly important targets for the development of antifungal drugs.

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Protozoan Carbonic Anhydrases

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Abstract Carbonic anhydrases (CAs, EC 4.2.1.1) belonging to the α-, β-, and η-classes are present in many pathogenic protozoa, such as those belonging to the *Trypanosoma*, *Leishmania*, and *Plasmodium* genera. In the last years many such enzymes have been cloned, purified, and extensively characterized. Their inhibition profiles with several classes of CA inhibitors (CAIs) such as the sulfonamides, anions, thiols, hydroxamates, and dithiocarbamates were also investigated. CA inhibition in such protozoa leads to interference with the life cycle of the pathogen, which can be exploited clinically for fighting these widespread infections. However this field is still in its infancy, and these enzymes are attractive yet underexplored drug targets for the management of malaria, Chagas disease, or Leishmaniasis. We also predict that in future years CAs will be characterized in other protozoans, with the possibility to explore alternative drug targets for fighting diseases provoked by them.

Keywords Anions, Carbonic anhydrase, Carbonic anhydrase inhibitors, Chagas disease, Dithiocarbamates, Leishmaniasis, Malaria, Protozoa, Sulfonamides

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1 Introduction

All mammalian hosts are at risk of infection by parasites [1, 2]. Parasites live, damage, and can cause diseases by obtaining necessities of life from the host. Parasites are divided as follows: (a) nonliving parasites, such as prions (unique protein) and viroides (nucleic acids); (b) nonliving/living parasites, such as viruses; (c) prokaryotes, such as bacteria and cyanobacteria; and (d) eukaryotes, such as microscopic fungi, protozoa, and helminths [3, 4]. The host–parasite relationship is the most important factor in determining whether an infection is successful or is cleared up by the host [4, 5]. Several mechanisms are involved in this complex interaction, and various aspects of both the host and the parasite organisms are essential in the response to infection [6–8]. Different types of pathogenic parasites exist: the obligate parasites colonize defined hosts and they may not be found in the normal flora; facultative parasites are members of the normal flora and the infection risk factors are induced by the condition of both host and microbe, whereas the opportunistic parasites are found in the environment, being not pathogenic for healthy people, but taking advantage in case of host disorders, such as immunosuppression, etc. [3, 9].

Parasites express their pathogenicity by means of their virulence, which is a term referring to the degree of their pathogenicity [2, 5, 10]. Hence, the determinants of virulence of a pathogen are any of its genetic, biochemical, or structural features that enable it to cause a disease through its ability to enter a host, evade host defenses, grow in the host environment, counteract host immune responses, assimilate iron or other nutrients from the host, or sense environmental change [11]. All these abilities involve the action of numerous enzymes. Enzymes considered as virulence factors are generally active against host components and contribute to virulence by damaging host tissues [12–16].

Each year there are hundreds of millions of people infected with disease-causing protozoa, particularly in tropical and subtropical regions of the world because humidity and high temperatures provide the necessary conditions for vectors and

protozoans growth [17, 18]. It has been estimated that approximately one million die each year due to protozoan infections, especially malaria. Several of these diseases are neglected ones, either because of their incidence in countries with little purchasing power or their low visibility [19–23]. Leishmaniasis, provoked by protozoa *Leishmania* spp., Chagas disease caused by the protozoan *Trypanosoma cruzi*, toxoplasmosis determined by protozoan *Toxoplasma gondii*, and malaria caused by parasitic protozoans belonging to the genus *Plasmodium*, are neglected yet widely spread protozoa provoked diseases [11, 21, 23, 24].

2 Carbonic Anhydrases

The lack of efficient treatments and acquired resistance to the existing treatments has stimulated efforts to identify new, less toxic, and more effective chemotherapies and novel therapeutic targets against the causative agents of protozoan diseases. Among the new targets studied in the last period, the superfamily of metalloenzymes carbonic anhydrases (CAs, EC 4.2.1.1) led to interesting research related to neglected, protozoan diseases such as those mentioned above.

Carbonic anhydrases (CAs, EC 4.2.1.1) are enzymes present in most organisms all over the phylogenetic tree, and also in many pathogenic species, suggesting a pivotal role of these enzymes in microbial virulence [4, 25–30]. CAs catalyze the reversible hydration of carbon dioxide with the production of bicarbonate and protons [11, 26, 30–36], and were classified into six classes including the α-, β-, γ-, δ-, ζ-, and η-CAs. α-, β-, δ-, and, probably η-CAs use Zn(II) ions at the active site, γ-CAs are Fe(II) enzymes but they are active also with bound Zn(II) or Co (II) ions, whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis [4, 26, 37–43]. The α, β-, γ-, and ζ-CAs have been crystallized, but not δ- and η-CAs. The metal ion from the enzyme active site is coordinated by three His residues in the α-, γ-, and δ-classes, by one His, and two Cys residues in β- and ζ-CAs or by two His and one Gln residues in η-class with the fourth ligand being a water molecule/hydroxide ion acting as nucleophile in the catalytic cycle of the enzyme [4, 26, 38–43].

3 CA Inhibitors

Several classes of CA inhibitors (CAIs) are known to date: (1) the metal ion binders (anion, sulfonamides and their isosteres, dithiocarbamates, xanthates, etc., Fig. 1, panel 1); (2) compounds which anchor to the zinc-coordinated water molecule/hydroxide ion (phenols, polyamines, thioxocoumarins, sulfocoyumarins, etc., Fig. 1, panel 2); (3) compounds occluding the active site entrance, such as coumarins and their isosteres – Fig. 1, panel 3, and (4) compounds binding out of the active site, Fig. 1, panel 4 [11, 31, 44–46].

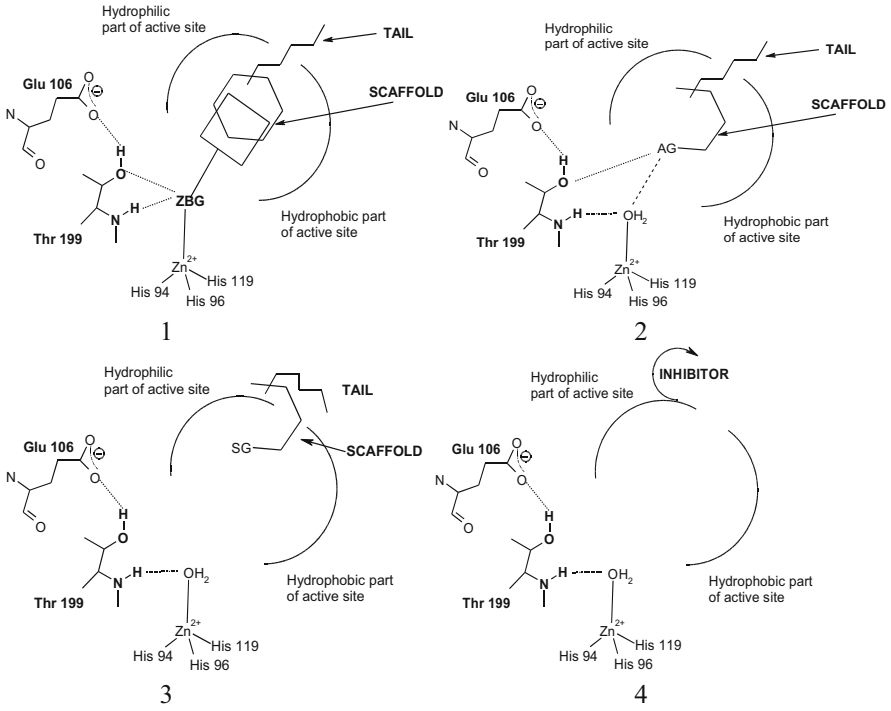

Fig. 1 CA inhibition mechanisms: *Panel 1*: Zinc-binding; *Panel 2*: Anchoring to the metal ion coordinated water; *Panel 3*: Occlusion of the active site entrance; *Panel 4*: Out of the active site binding

Few such compounds apart the anions, sulfonamides and dithiocarbamates were investigated so far for the inhibition of protozoan CAs [37].

3.1 Anions

Anions, such as the inorganic metal-complexing ones or more complicated species such as the carboxylates, are also known to bind to the CAs, but generally with less efficiency compared to the sulfonamides [44, 47, 48]. Anions may bind either the tetrahedral geometry of the metal ion or as trigonal–bipyramidal adducts, as shown for the tetrahedral geometry in Fig. 1, panel 1. Enzymes found in vertebrates, arthropods, corals, fungi, bacteria, diatoms, and Archaea have been investigated for their inhibition with simple inorganic anions. Anion inhibitors are important both for understanding the inhibition/catalytic mechanisms of these enzymes fundamental for many physiologic processes, and for designing novel types of inhibitors which may have clinical applications for the management of a variety of disorders in which CAs are involved [11, 49–55].

3.2 Sulfonamides

The sulfonamides were the first antimicrobial drugs, discovered in 1935 by Domagk, and they paved the way for the antibiotic revolution in medicine [56]. The first sulfonamide showing effective antibacterial activity, Prontosil, was a prodrug, with the real antibacterial agent being sulfanilamide, a compound isosteric/isostructural with pABA. Sulfanilamide is generated by the in vivo reduction of Prontosil. In the years following the discovery of sulfanilamide as a bacteriostatic agent, a range of analogs has entered into clinical use (constituting the so-called sulfa drug class of antibacterials), and many of these compounds are still widely used (Fig. 2) [57]. Sulfonamides, such as the clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, and brinzolamide, bind in a tetrahedral geometry to the Zn(II) ion in deprotonated state (Fig. 1, panel 1), with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II) and an extended network of hydrogen bonds, involving amino acid residues Thr199 and Glu106 (numbering system used for the human CA, isoform I), also participating to the anchoring of the inhibitor molecule to the metal ion [57]. The aromatic/heterocyclic part of the inhibitor interacts with hydrophilic and hydrophobic residues of the cavity.

3.3 Dithiocarbamates

Another class of CAIs recently investigated is constituted by the dithiocarbamates (DTCs) [58–63]. They were discovered by considering the inorganic anion trithiocarbonate (TTC, CS_3^{2-}) as lead compound, as an X-ray crystal structure of this weak inhibitor with the human isoform hCA II was available [64]. DTCs, as TTC coordinate through one sulfur atom to the Zn(II) ion from the enzyme active site, and also interact with the conserved Thr199 amino acid residue. DTCs are micromolar-low nanomolar CAIs against many CA isoforms as their organic scaffold participates in supplementary interactions with the enzyme active site. These compounds were also investigated for the inhibition of some pathogenic CAs such as those of *Mycobacterium tuberculosis, Porphyromonas gingivalis*, etc. [30, 60, 65, 66]. Some DTCs effective against several bacterial CAs are shown in Fig. 3.

4 Protozoan CAs of Therapeutic Interest

4.1 Malaria

Malaria, a mosquito-borne disease of humans and other animal species, is caused by parasitic protozoa species belonging to the genus Plasmodium. Six different

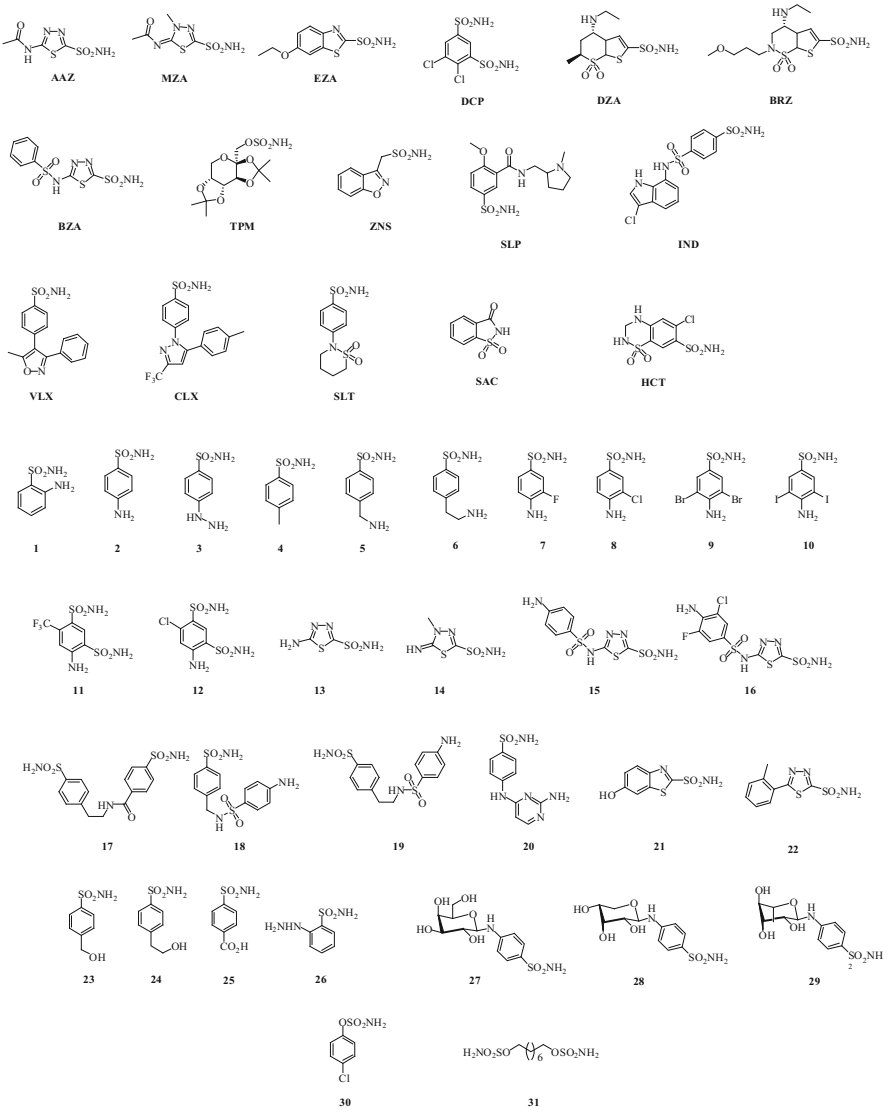

Fig. 2 Sulfonamides investigated as inhibitors of bacterial CA

Plasmodium species infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and the zoonotic *Plasmodium knowlesi* [67, 68]. Globally malaria afflicts more than 200 million people and kills about 600,000 annually, mainly young children in sub-Saharan Africa, with most deaths caused by *P. falciparum* infection. Malaria parasites follow a complex lifecycle that involves an intermediate host such as humans and the definitive host, the mosquito

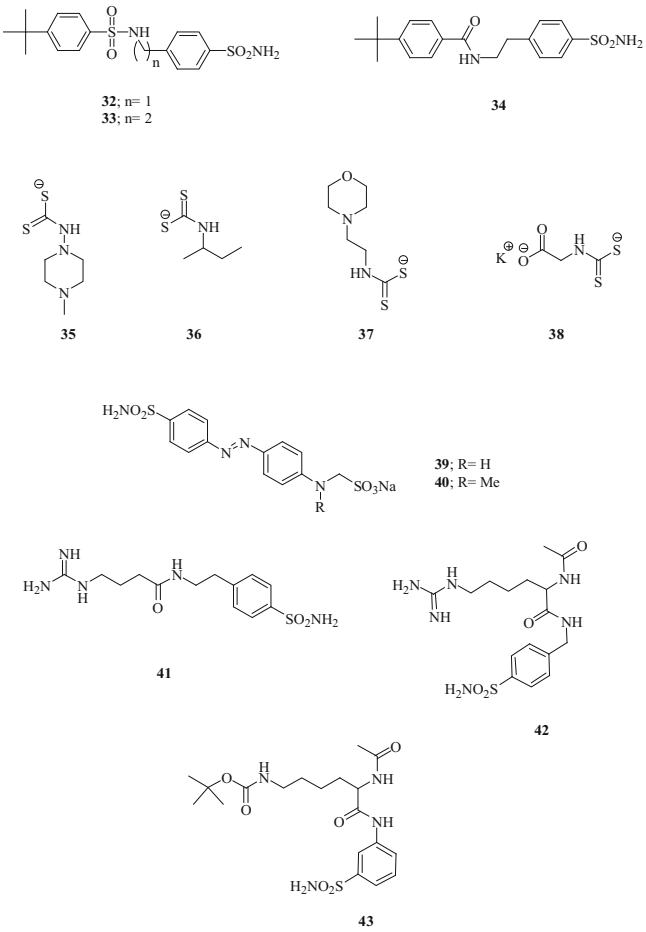

Fig. 3 Sulfonamides and dithiocarbamates **32–43** showing selective bacterial CA inhibitory properties

vector [26, 43, 69–74]. Following injection of sporozoite stage parasites from an infected female Anophelene mosquito into a human host, Plasmodium parasites move to the liver and invade hepatocytes where they replicate to form merozoites that are ultimately released into the blood circulation [69]. Plasmodium merozoites can then invade erythrocytes and undergo cycles of asexual replication within these cells, resulting in the clinical symptoms of malaria [69]. During this part of the lifecycle, sexual stage gametocytes can also form, and when taken up by a feeding female Anophelene mosquito, can undergo sexual reproduction in the mid-gut of the mosquito. This ultimately results in the completion of the life cycle through formation of sporozoites that can then be transferred to another individual by the mosquito vector during a blood meal [75, 76].

4.1.1 Plasmodia CAs: Identification of a New Class of CAs, the η-CA

Few protozoan parasites have been investigated for the presence and druggability of CAs [71, 77–79]. Pathogenic protozoa, such as *Plasmodium* spp., *T. cruzii*, and *Leishmania* spp., encode α- β- or, a new class of CAs, the η-CAs. The causative agent of human malaria, *P. falciparum*, was one of the first protozoa to be investigated for the presence of CAs [77]. The open reading frame of the malarial CA enzyme (*P. falciparum* CA, accession number AAN35994.2, PlasmoDB: PF3D7_1140000) encodes a 600 amino acid polypeptide chain. In 2004, Krungkrai and coworkers cloned a truncated form of *P. falciparum* CA gene (GenBank: AAN35994.2) encoding for an active CA (named PfCA1) with a primary structure of 235 amino acid residues [77]. The metalloenzyme showed a good esterase activity with 4-nitrophenylacetate as a substrate and was inhibited by known sulfonamide CA inhibitors (CAIs). The authors observed that the highly conserved α-CA active site residues, responsible for binding of the substrate and for catalysis, were present also in PfCA1 and considered thus the Plasmodium enzyme belonging to the α-CA class. Subsequent, it was showed that different *Plasmodium* spp. encoded CAs, all considered to belong to the α-class, and that primary sulfonamides inhibited in vitro and in vivo the growth of Plasmodium parasites [71, 77]. Recently our groups reanalyzed and realigned the amino acid sequence of the truncated PfCA1 with the two human α-CA isoforms, hCA I and II, in order to identify other features of the protozoan enzyme [43]. We observed that to have three histidines aligned with the three zinc-coordinating histidines of the human isoforms, it was necessary to "force" the alignment, introducing in the PfCA1 sequence a five-residues insertion and a six-residues deletion between residues 96–119. Nevertheless, the other residues crucial for the catalytic mechanism of the α-CAs, such as the proton shuttle His64 and one of the gatekeeper residues, Thr199, seemed to be not conserved in the Plasmodium enzyme. The dyad Glu106-Thr199 is highly conserved in all α-CAs investigated so far, being involved in the orientation of the substrate for the nucleophilic attack by the zinc hydroxide species of the enzyme. Thus, the proposed alignment showed important amino acid substitutions that differentiated the sequence of Plasmodium enzyme from those of other α-CAs [43]. Hence, a phylogenetic tree was constructed to better investigate the relationship of the Plasmodia amino acid sequences with CAs from prokaryotic and eukaryotic species belonging to different classes (α-, β-, γ-, δ-, and ζ-CAs). In Fig. 4 has been represented only the branches with α-, δ-, η, and ζ-CAs. The complete phylogenetic tree is showed in the paper published by Del Prete et al. in 2014 [43]. It was observed that Plasmodia CAs clustered in a branch different from that of the a-CAs, although close to it, while they were well separated from the other CA classes (Fig. 4). On the basis of these data, we hypothesized that the Plasmodia CAs were the result of modifications of an ancestral δ-CA gene, which originated a new class of CA that we denominated η-class.

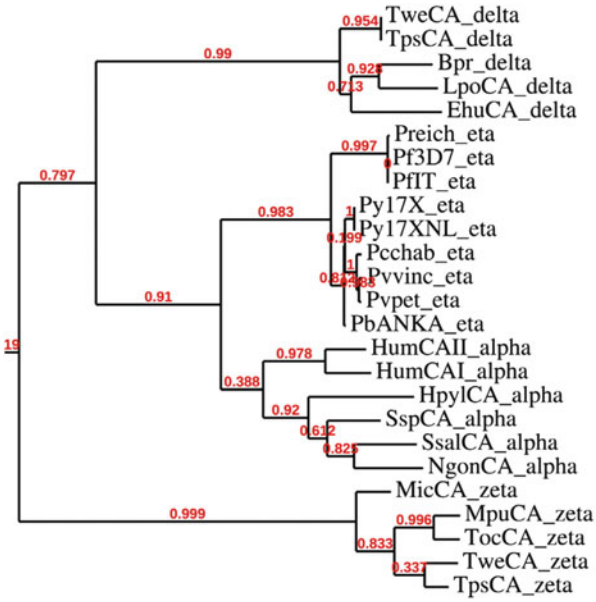

Fig. 4 A particular of the phylogenetic tree leading to the discovery of the η-CA genetic family in Plasmodia. Tree was constructed using amino acid sequences of the α-, δ-, and ζ-CAs from selected prokaryotic and eukaryotic species [43]

4.1.2 η-CA Zinc Coordination Pattern

The metal ion coordination pattern of the η-CA from the malaria producing protozoa *P. falciparum* is unique among all six genetic families encoding for such enzymes, comprising two His and one Gln residues, in addition to the water molecule/hydroxide ion acting as nucleophile in the catalytic cycle (Fig. 5) [40]. Although the η- and α-CAs share many similar features, strongly suggesting the first ones to be evolutionary derived from the last, there are significant differences between the two families to allow some optimism for the drug design of selective inhibitors for the parasite over the host enzymes. However, these studies are still in their initial phase and further work by X-ray crystallography should validate the model proposed in order to detect inhibitors with high affinity and selectivity for the η-CAs over the α-CAs [40].

4.1.3 η-CA Kinetic Constants

The truncated form of the enzyme η-CA, named PfCA1, showed catalytic activity typical of a CA, having the following kinetic properties for the CO_2 hydration reaction to bicarbonate and protons: k_{cat} of 1.4×105 s^{-1} and k_{cat}/K_m of 5.4×10^6 $M^{-1} \times s^{-1}$ [40, 43, 70, 71, 77].

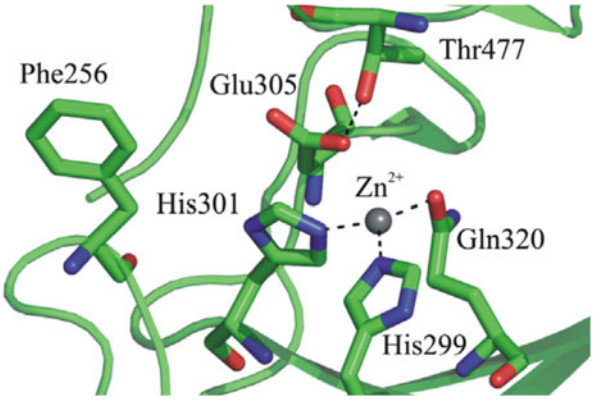

Fig. 5 Representation of the η-CA active site obtained by homology modeling. The detail shows the coordination of the zinc ion. His299, His301, and Gln 320 form the catalytic triad [40]

4.1.4 η-CA Inhibition Studies

Our groups reported an inhibition study of the η-CA from *P. falciparum* against a panel of sulfonamides and one sulfamate compound, some of which are clinically used. The strongest inhibitors identified were ethoxzolamide and sulthiame, with K_I of 131–132 nM, followed by acetazolamide, methazolamide, and hydrochlorothiazide (K_I of 153–198 nM) [70, 71, 77]. Brinzolamide, topiramate, zonisamide, indisulam, valdecoxib, and celecoxib also showed significant inhibitory action against η-CA, with K_I ranging from 217 to 308 nM [70, 71, 77]. An interesting observation was that the more efficient η-CA inhibitors are representative of several scaffolds and chemical classes, including benzene sulfonamides, monocyclic/bicyclic heterocyclic sulfonamides, and compounds with a more complex scaffold (i.e., the sugar sulfamate derivative, topiramate, and the coxibs, celecoxib, and valdecoxib). Moreover, four generations of poly(amidoamine) (PAMAM) dendrimers incorporating benzenesulfonamide moieties were investigated as inhibitors of *P. falciparum* η-CA [28].

Acetazolamide is a rather ineffective inhibitor for this enzyme (K_I of 170 nM) and many other simple sulfonamides investigated earlier showed the same activity. To our surprise, dendrimers **G0–G3** investigated here showed a potent inhibition of this enzyme, with K_I in the range of 47.8–85.5 nM. The best inhibitors were **G2** and **G1**, whereas the activity was slightly worse for **G0** and **G3** (Fig. 6) [28]. However we should mention that the human isoforms hCA I and II were much more sensitive to inhibition by the dendrimers incorporating sulfonamide moieties compared to the pathogenic enzymes included in this study, which may lead to off target effects.

Considering the small number of inhibition studies reported at this moment for the η-CAs, these results demonstrate it is quite probable that effective, low nanomolar inhibitors may be developed. Moreover, some dendrimers investigated showed a better inhibitory power compared to acetazolamide. The main conclusion is that this class of molecules may lead to important developments in the field of anti-infective CA inhibitors. Given that drug resistance has emerged against most antimalarials in clinical use, the discovery of η-CA-specific inhibitors may lead to a

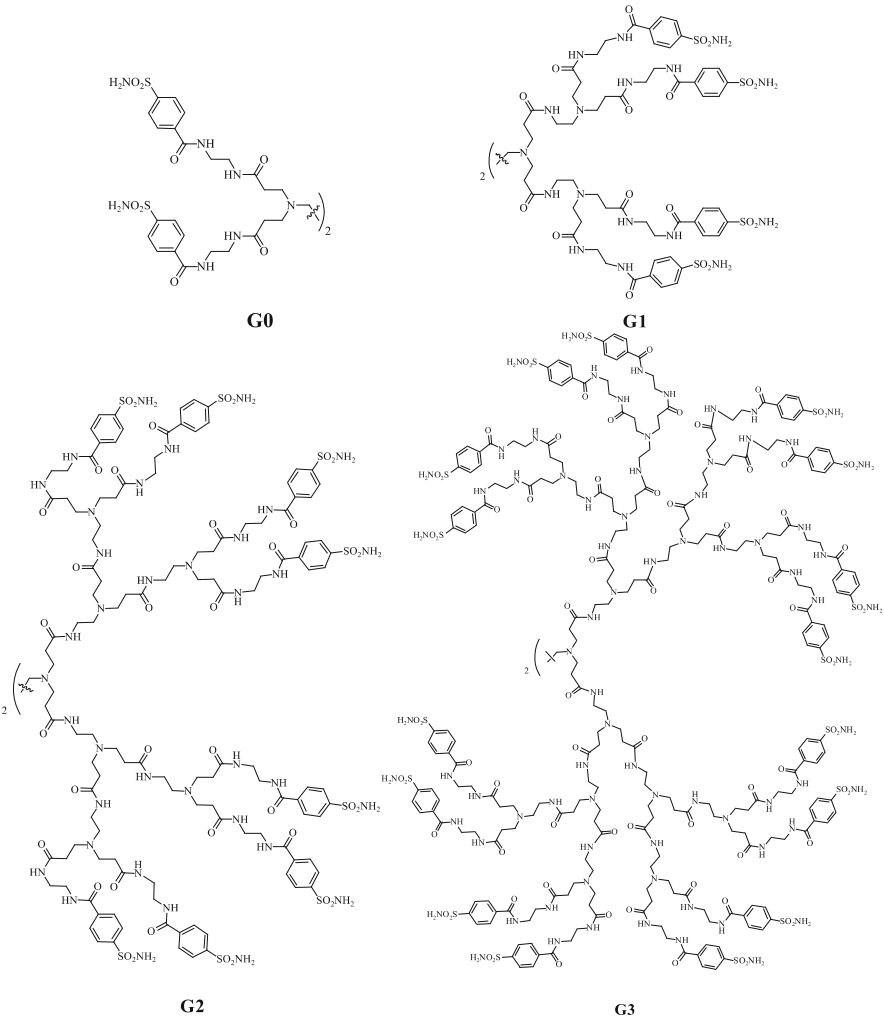

Fig. 6 Chemical structure of sulfonamide-derivatized PAMAM dendrimers **G0–G3**

novel therapeutic approach for malaria once the biology of η-CA has been further investigated in different life cycle stages.

4.1.5 Role of CA in *P. falciparum*

Protozoa, such as *P. falciparum*, utilize purines and pyrimidines for DNA/RNA synthesis during its exponential growth and replication [43–45]. Plasmodia synthesize pyrimidines de novo from HCO_3^-, adenosine-5′-triphosphate (ATP), glutamine (Gln), aspartate (Asp), and 5-phosphoribosyl-1-triphosphate (PRPP). HCO_3^-

is the substrate of the first enzyme involved in the Plasmodia pyrimidine pathway, being generated from CO_2 through the action of η-CA [43–45]. Some benzene sulfonamide derivatives were effective in vitro as *P. falciparum* CAIs and also inhibited the ex vivo growth of the parasite efficiently. One such sulfonamide was also effective as an antimalarial agent in mice infected with *P. berghei*, an animal model of human malaria infection, with efficiency similar to that of chloroquine, a standard clinically used drug [71, 78]. CAIs thus show antimalarial activity because they inhibit the first step of pyrimidine nucleotide biosynthesis in the protozoan parasite, i.e., the CA-mediated carbamoylphosphate biosynthetic pathway [71, 78].

4.2 Leishmaniasis

Leishmaniasis is a vector-borne tropical disease caused by unicellular protozoan organisms from the genus Leishmania. The parasites are transmitted to the mammalian host organism by an infected sand fly [17, 80]. Some Leishmania species, such as *Leishmania major*, remain mainly in the skin, while other species, like *Leishmania donovanichagasi*, infect visceral organs. Leishmaniasis is associated with a wide range of clinical manifestations, from self-healing skin lesions to no less than fatal visceral infections. Visceral leishmaniasis accounts for 20,000–40,000 deaths every year [81, 82].

4.2.1 Leishmania CAs

Leishmaniasis is an infection provoked by protozoans belonging to the genus Leishmania. Among the many species and subspecies of such protozoa, *L. donovanichagasi* causes visceral leishmaniasis [83]. Analysis of the genomes of the species of Leishmania predicted the presence of two CA genes encoding for α- and β-CAs. Our groups identified, cloned, and characterized the β-CA (indicated with the acronym LdcCA) from the unicellular parasitic protozoan *L. donovanichagasi*.

4.2.2 LdcCA Primary Structure

LdcCA has all the amino acid residues involved in catalysis, as is the case for the other members of the **β**-CA family [12, 17]: (1) the three Zn(II) ligands, Cys160, His220, and Cys223 (*Pisum sativum* CA numbering system); the Asp162–Arg164 catalytic dyad, which forms a hydrogen bond network with the water coordinated to the Zn(II) ion, enhancing its nucleophilicity; (3) a cluster of hydrophobic amino acid residues considered to be involved in the binding of the substrate (and inhibitors), including those in positions 151, 179, and 184. Indeed, some of them

are conserved in almost all the enzymes used in the alignment, even if they are from organisms in various life kingdoms [12, 17, 23, 84].

4.2.3 LdcCA Kinetic Constants

This enzyme showed interesting catalytic activity for the CO_2 hydration reaction, with k_{cat} of 9.35×10^5 s^{-1} and k_{cat}/K_m of 5.9×10^7 M^{-1} s^{-1}. LdcCA is a highly effective catalyst for the CO_2 hydration to bicarbonate and protons, being more effective compared to the plant enzyme FbiCA 1 or the bacterial one Cab [12, 17, 23, 84].

4.2.4 LdcCA Inhibition Studies

LdcCA inhibition profile was carried out with a range of aromatic/heterocyclic sulfonamides of types **1–24**, AAZ through HCT, and 1,3,4-thiadiazole-2-thiols. Most of the simple sulfonamides investigated here, such as compounds **1–17**, **19**, DCP, DZA, BRZ, BZA, TPM, ZNS, SLP, IND, VLX, CLX, SLT, and SAC, were weak or ineffective as inhibitors of LdcCA [12, 17, 23, 84]. Indeed, several such sulfonamides (and TPM, the only sulfamate investigated) did not inhibit significantly LdcCA (K_I values >100 μM), whereas the largest majority of these derivatives showed inhibition constants in the range of 136–9,251 nM. It may be observed that they belong to rather heterogeneous classes of aromatic or heterocyclic sulfonamides, so that the structure–activity relationship (SAR) is not straightforward. A better inhibition profile of LdcCA has been observed with the following derivatives: **18**, **20–24**, AAZ, MZA, EZA, and HCT, which had K_I values in the range of 50.2–95.1 nM. Apart from the 4-carboxybenzenesulfonamide **18**, which is a simple and rather compact molecule, compounds **20–24** incorporate an elongated molecule of the arylsulfonylated sulfonamide type. The five-membered heterocyclic sulfonamides AAZ and MZA were also among these effective inhibitors, but they were weaker LdcCA inhibitors compared to the bicyclic EZA and HCT, which were the most potent sulfonamide inhibitors detected here, with K_I values in the range of 50.2–51.5 nM. Interesting to note that the inhibition profile of LdcCA with the investigated sulfonamides is very different from that of the mammalian enzymes hCA I and II. This is a positive feature if one needs to inhibit the parasite and not also the host enzymes. As most of the sulfonamides were not highly effective CAIs against the protozoan enzyme reported here, we decided to investigate thiols as possible LdcCA inhibitors, it being well documented that the mercapto moiety (in ionized, anionic form) may act as a good zinc-binding group (similar to the SO_2NH^-) for obtaining effective CAIs. The 1,3,4-thiadiazole-5-mercapto derivatives **25–34** (Fig. 7) were reported earlier by our group as medium potency hCA I and II inhibitors and were investigated for their interaction with LdcCA.

It may be observed that already the simple semicarbazido derivative is a rather effective LdcCA inhibitor (K_I of 74.1 nM), whereas many of its Schiff's bases of

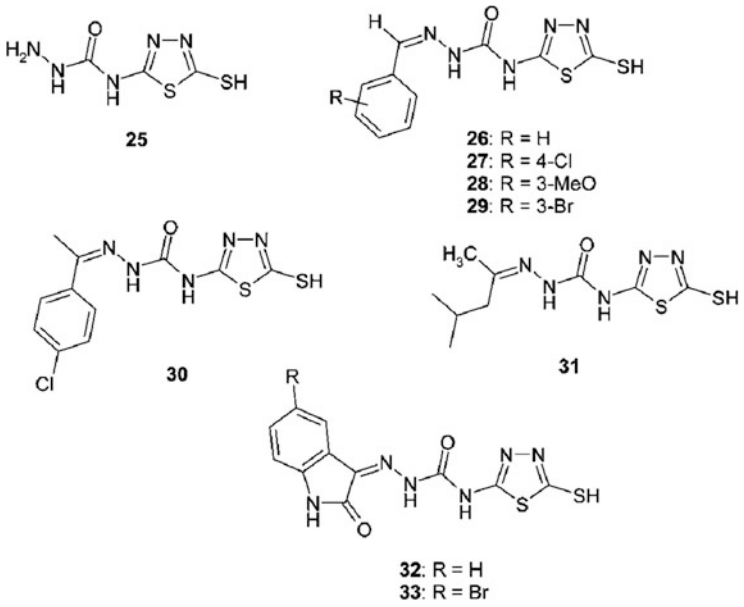

Fig. 7 The 1,3,4-thiadiazole-5-mercapto derivatives **25**–**33**

types **26–30** and **32** are even better inhibitors of the protozoan enzyme, with inhibition constants in the range of 13.4–40.1 nM [84]. A loss of inhibitory activity was observed for compounds **31**, **33**, and **34**, which showed K_I values in the range of 95.3–152 nM. Moreover, some of the investigated thiols efficiently inhibited the in vivo growth of *Leishmania chagasi* and *Leishmania amazonensis* promastigotes, by impairing the flagellar pocket and movement of the parasites and causing their death. Leishmania CA may thus be a potential target for developing antileishmanial drugs with a novel mechanism of action [17].

4.2.5 Role of CA in *Leishmania*

In literature is described a constitutive expression of the two CA transcripts (encoding for the α and β CAs) in *L. major* promastigotes. Moreover, it was detected considerable CA activity in *L. major* cell lysates, confirming the presence of functional CA in *L. major*. Our groups noted that the CA is effectively involved in the development of the morphological state of the protozoa. In fact, some of the investigated thiols efficiently inhibited the in vivo growth of *L. chagasi* and *L. amazonensis* promastigotes, by impairing the flagellar pocket and movement of the parasites, and causing their death. Leishmania CA may thus be a potential target for developing antileishmanial drugs with a novel mechanism of action [12, 17].

4.3 American Trypanosomiasis (Chagas Disease)

American trypanosomiasis, or Chagas disease, is caused by the parasite *T. cruzi*. The infection was described in 1909 by the Brazilian physician Carlos Chagas (1879–1934). About eight million people worldwide are estimated to be infected by *T. cruzi* [19, 21–24]. Furthermore, because of growing population migration, the disease has spread to other continents. Chagas disease is transmitted to humans by the infected feces of blood-sucking triatomine bugs, a vector for the *T. cruzi* parasite; however, other routes of transmission are known, such as consumption of contaminated food and drink, congenital, and blood transfusions. The pathogenesis of the disease can be divided into three phases represented by a short acute phase, a long-lasting latent phase, and a chronic phase appearing in about 30% of the patients. Serious chronic symptoms such as cardiomyopathy and malformations of the intestines (e.g., megaesophagus and megacolon) have been reported. Chagas disease chemotherapy is limited to nifurtimox and benznidazole; both drugs were developed more than 30 years ago. They are predominantly active during the acute phase of the disease. However, they have serious adverse effects because of their high toxicity and low efficacy, especially in the chronic phase.

4.3.1 Trypanosoma CA

Only one α-CA was found in the genome of *T. cruzi*, and a truncated (307 amino acid long) enzyme was thereafter cloned in the Bac-to-Bacbaculovirus expression system [21, 22, 24, 85], leading to the purified enzyme, TcCA.

4.3.2 TcCA Primary Structure Analysis

It has the conserved three His ligands, which coordinate the Zn(II) ion, which as mentioned above is crucial for catalysis. The fourth zinc ligand is a water molecule/hydroxide ion, acting as nucleophile in the catalytic cycle. These residues are His94, 96 and 119 (hCA I numbering system). Furthermore, the other two amino acids important for the catalysis in all α-CAs, i.e., the gate-keeping residues Glu106 and Thr199, are also present in the TcCA sequence. They orientate the substrate for catalysis, and are also involved in the binding of inhibitors through a network of hydrogen bonds involving the COOH and OH moieties of the amino acid residues and the non-protein zinc ligand (i.e., water, hydroxide ion, or a coordinated inhibitor molecule). Surprisingly, the proton shuttle residue His64, which is conserved in most α-CAs investigated to date, was not present in TcCA, being replaced by an Asn residue. His64 assists the rate-determining step of the catalytic cycle in α-CA, transferring a proton from the water coordinated to the Zn(II) ion to the environment, with formation of the zinc hydroxide nucleophilic species of the enzyme, and is thus essential for the high catalytic efficiency of CAs. It has been

hypothesized that some other His residues present in the amino-terminal part of TcCA (e.g., His-27 or His35) may participate in these processes, but this hypothesis was not validated to date.

4.3.3 TcCA Kinetic Constants

TcCA showed a significant catalytic activity for the CO_2 hydration reaction to bicarbonate and protons, with the following kinetic parameters: k_{cat}/K_m of 1.49×10^8 $M^{-1} \times s^{-1}$, which are highly similar to those of hCA II, one of the best catalyst known in nature.

4.3.4 TcCA Inhibition Studies

A large number of aromatic/heterocyclic sulfonamides and some 5-mercapto-1,3,4-thiadiazoles were investigated as TcCA inhibitors [21, 22, 24, 85]. The aromatic sulfonamides were weak inhibitors (K_I of 192 nM to 84 μM) whereas some heterocyclic compounds inhibited the enzyme with K_I in the range of 61.6–93.6 nM. Intrudingly, none of these rather effective in vitro TcCA inhibitors had any effect in vivo on the growth of the pathogen [21]. This is probably due to the fact that sulfonamides are rather poorly penetrating agents through biological membranes, a phenomenon already encountered for the inhibition of pathogenic CAs from nematodes or fungi with such derivatives. For this reason, our groups explored additional CAI, such as the thiols of type **25–33** (Fig. 8) [23, 24]**.** Thiols were the most potent in vitro inhibitors (K_I of 21.1–79.0 nM) and some of them also inhibited the epimastigotes growth of two *T. cruzi* strains in vivo. All these data were promising and proved that TcCA may be considered as an interesting target for developing anti-trypanosomal drugs with a novel mechanism of action.

Again, halogeno/-methoxy-phenacetamido tails were attached to the molecules of aromatic or heterocyclic sulfonamides such as sulfanilamide, 3-halogenosulfanilamides, 4-aminomethyl/ethyl-benzenesulfonamide, or 5-amino-1,3,4-thiadiazole-2-sulfonamide [86, 87]. Unfortunately, as the sulfonamides investigated earlier, these derivatives did not show any ex vivo anti-trypanosomal effects.

A series of 4,5-dihydroisoxazoles incorporating hydroxamate moieties were prepared (Fig. 9), being shown that they act as effective in vitro TcCA inhibitors, with inhibition constants in the range of 39.8–615 nM [21].

One compound (**36i**) was further evaluated in detail ex vivo, and showed promising features as an anti-trypanosomal agent. Excellent values for the inhibition of growth for all three developmental forms of the parasite were observed at low concentrations of this hydroxamate, with in vivo IC_{50} values ranging from 7.0 to <1 μM. The compound had a selectivity index of 6.7 and no cytotoxicity to macrophage cells. Preliminary in vivo data showed that it reduces bloodstream parasites and that all treated mice survived, being more effective than the standard

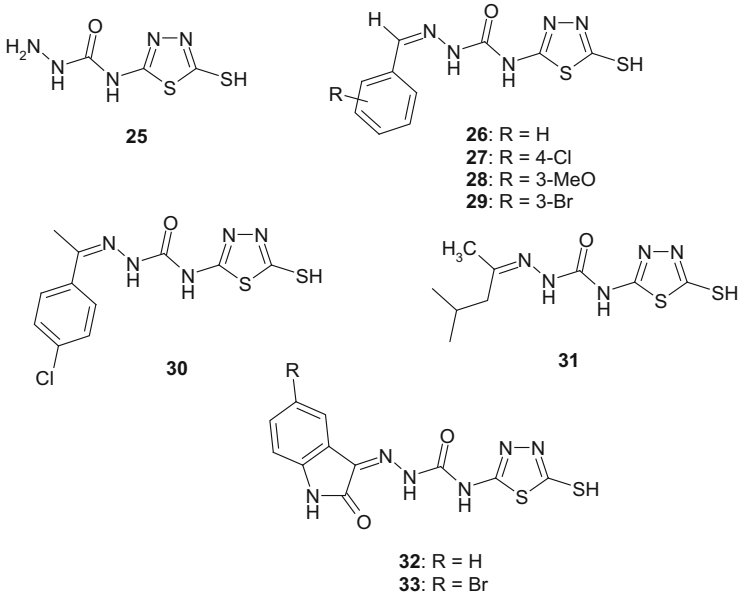

Fig. 8 Thiols investigated as TcCA inhibitors

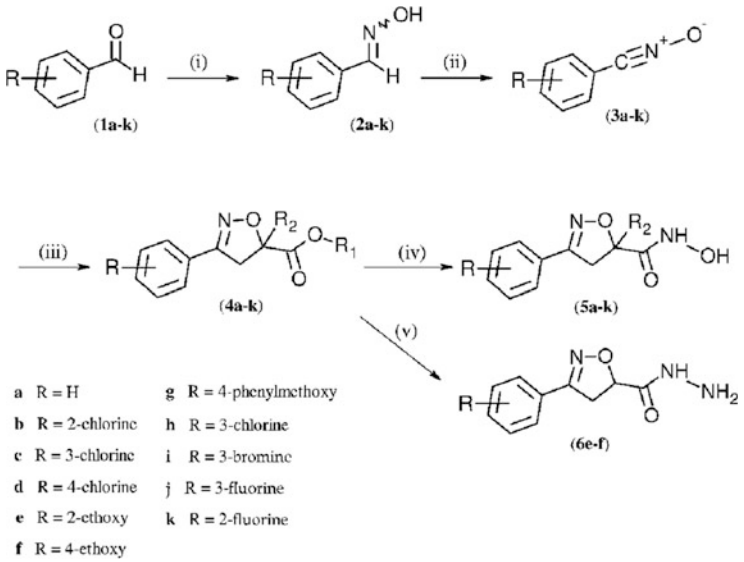

Fig. 9 4,5-Dihydroisoxazoles incorporating hydroxamate moieties obtained as described by Rodrigues et al. [21]

drug benznidazole [21]. Recently, congeners of this compound were further evaluated as anti-Chagas disease agents with interesting results.

Interesting, TcCA was inhibited in the low micromolar range by iodide, cyanate, thiocyanate, hydrogensulfide, and trithiocarbonate (K_I in the range of 44–93 μM), whereas the best inhibitor was diethyldithiocarbamate ($K_I = 5$ μM) [23]. Sulfamide showed an inhibition constant of 120 μM, and sulfamic acid was much less effective (K_I of 10.6 mM). It has been thus hypothesized that the discovery of diethyldithiocarbamate as a low micromolar TcCA inhibitor may be useful to detect leads for developing anti-Trypanosoma agents with a diverse mechanism of action compared to clinically used drugs (benznidazole, nifurtimox) for which significant resistance emerged, but no other dithiocarbamates were investigated to date as CAIs of this enzyme, although this class of compounds was reported recently to possess such an activity [20–24, 28].

4.3.5 Role of CA in *T. cruzi*

Differentiation of *T. cruzi* epimastigotes to metacyclic trypomastigotes occurs in the insect rectum, after adhesion of the epimastigotes to the intestinal wall. It was hypothesized that TcCA is involved in the epimastigotes growth of Trypanosma [20–24, 28]. In fact, our results demonstrated that thiols, considered the most potent in vitro inhibitors of TcCA, were able to inhibit the epimastigotes growth of two *T. cruzi* strains in vivo as described in the section inhibition studies [20–24, 28].

5 Conclusions

The CAs catalyze the reversible hydration of carbon dioxide with the production of bicarbonate and protons, being present in most organisms all over the phylogenetic tree, and also in many pathogenic species, suggesting a pivotal role of these enzymes in microbial or parasite virulence. Leishmaniasis, provoked by protozoa belonging to *Leishmania* spp., Chagas disease caused by the protozoan *T. cruzi*, and malaria caused by parasitic protozoans belonging to the genus *Plasmodium*, are neglected yet widely spread diseases. In all these protozoa, CAs are effectively involved in the development of the morphological state or in their life cycle. For example, CAs are involved in the epimastigotes growth of the protozoa or in the synthesis of pyrimidines de novo through the production of HCO_3^-, which is the substrate of the first enzyme involved in the Plasmodia pyrimidine pathway. Moreover, some CA inhibitors (CAIs), such as sulfonamide derivatives, were effective in vivo inhibitors blocking the growth of the protozoan. One such sulfonamide was also effective as an antimalarial agent in mice infected with *Plasmodium berghei*, an animal model of human malaria infection, with efficiency similar to that of chloroquine, a standard clinically used drug. Here, we have presented an overview of the CAs encoded from the genome of the protozoa aforementioned

describing their feature and the inhibition profiles obtained using known CAIs. This review represents an effort in identifying new, less toxic, more effective chemotherapies and novel therapeutic targets against the causative agents of protozoan diseases, especially for the lack of efficient treatments and acquired resistance to the existing treatments [88, 89].

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Bacterial Carbonic Anhydrases

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Abstract Carbonic anhydrases (CAs, EC 4.2.1.1) are metalloenzymes which catalyze the hydration of carbon dioxide to bicarbonate and protons. Many pathogenic bacteria encode such enzymes belonging to the α-, β-, and/or γ-CA families. In the last decade enzymes from *Neisseria* spp., *Helicobacter pylori*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Brucella* spp., *Streptococcus pneumoniae*, *Salmonella enterica*, *Haemophilus influenzae*, *Legionella pneumophila*, *Vibrio cholerae*, *Porphyromonas gingivalis*, *Streptococcus mutans*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, etc., have been cloned and characterized in detail. For some of these enzymes the X-ray crystal structures were determined, and in vitro/in vivo inhibition studies with various classes of inhibitors (e.g., anions, sulfonamides, and sulfamates) performed. For *Neisseria* spp., *H. pylori*, *B. suis*, and *S. pneumoniae* enzymes it has been possible to evidence inhibition of bacterial growth in vivo when inhibitors were present in the medium. Considering such preliminary results, bacterial CAs represent promising targets for obtaining antibacterials devoid of the resistance problems to the clinically used antibiotics, but further studies are granted for validating most of these enzymes as drug targets.

Keywords Antibiotic, Bacteria, Carbonic anhydrase, Inhibitor, Sulfonamide, Virulence factor

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1 Introduction

Resistance to antibiotics belonging to all pharmacological classes is escalating and represents a worldwide problem [1–10]. Both Gram-negative and Gram-positive bacteria (such as *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Brucella suis*, *Streptococcus pneumoniae*, etc.) no longer respond to many clinically used such drugs belonging to several antibiotic classes [9–12]. Cloning of the genomes of many bacterial pathogens offers, however, the possibility to explore alternative pathways for inhibiting virulence factors or proteins essential for their life cycles [8, 13–17]. Among many such new possible drug targets explored recently, there is a class of enzymes catalyzing a simple but physiologically relevant process, i.e., the hydration carbon dioxide to bicarbonate and protons [18–23]. These metalloenzymes are the carbonic anhydrases (CAs, EC 4.2.1.1). Six genetically distinct CA families are known to date, the α-, β-, γ-, δ-, ζ-, and η-CAs [18, 24–29]. The α-, β-, δ-, and η-CAs use Zn(II) ions at the active site, the γ-CAs are probably Fe(II) enzymes (but they are also active with bound Zn (II) or Co(II) ions), whereas the ζ-class uses Cd(II) or Zn(II) to perform the physiologic reaction catalysis [18, 22, 26, 27]. The 3D fold of the five enzyme classes is very different from each other (a nice example of convergent/divergent evolution [22]), as it is their oligomerization state: α-CAs are normally monomers and rarely dimers; β-CAs are dimers, tetramers, or octamers; γ-CAs are trimers [28], whereas the δ-, ζ-, and η-CAs are probably monomers but in the case of the ζ-CA family, three slightly different active sites are present on the same protein backbone (which is thus a pseudotrimer, at least for the best investigated such enzyme, from the diatom *Thalassiosira weissflogii*) [29]. Many representatives of all these enzyme classes have been crystallized and characterized in detail, except for representatives of the δ- and η-CAs, for which the exact structure is not known to date [22]. The mammalian CAs and their inhibitors/activators have been thoroughly reviewed [22–24] and these enzymes will be not discussed here.

The α-CAs are present in vertebrates, protozoa, algae, and cytoplasm of green plants and in some *Bacteria*; the β-CAs are predominantly found not only in *Bacteria*, algae, and chloroplasts of both mono- and dicotyledons, but also in many fungi and some *Archaea* [28–32]. In bacteria and fungi they are mostly homodimers/tetramers [30]. The γ-CAs were found in *Archaea* and some *Bacteria*,

whereas the δ- and ζ-CAs seem to be present only in marine diatoms [28–32]. In most organisms these enzymes are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes (thoroughly studied in vertebrates), whereas in algae, plants, and some bacteria they play an important role in photosynthesis and biosynthetic reactions. In diatoms δ- and ζ-CAs play a crucial role in carbon dioxide fixation [28, 29].

The classical CA inhibitors (CAIs) are the primary sulfonamides, RSO_2NH_2, which are in clinical use for more than 50 years as diuretics and systemically acting antiglaucoma drugs [33–41]. In fact there are more than 30 clinically used drugs (or agents in clinical development) belonging to the sulfonamide or sulfamate class, which show significant CAI inhibitory activity [18]. However, it has emerged in the last years that sulfonamide/sulfamate CAIs have potential as anticonvulsant, antiobesity, anticancer, antipain, and antiinfective drugs [18, 34–41]. All these drugs target in fact mammalian CAs, of which 16 different isoforms are known so far [18]. For antiinfective applications, obviously the targets are bacterial proteins.

Here we review the current state of the art regarding the bacterial CAs cloned and characterized so far, as well as the in vitro and in vivo inhibition studies of these enzymes, which may reply to this stringent question: are the bacterial CAs future drug targets for obtaining conceptually novel antibiotics?

2 Bacterial Carbonic Anhydrases

Bacteria are ubiquitous microorganisms, with most available niches occupied by some members of this kingdom. In prokaryotes, the existence of genes encoding CAs from at least three classes (α-, β-, and γ-class) suggests that these enzymes play an important role in the prokaryotic physiology (Fig. 1). In Fig. 1 a phylogenetic tree of the various CAs from a series of bacteria is presented (the species, their accession numbers, and acronyms of the various CAs are shown in Table 1). It should be mentioned that not all these CAs were characterized in detail for the moment, but for most of them catalytic and inhibition data are available, as it will be shown shortly in this chapter. The data of Fig. 1 clearly demonstrate that the three genetic families, the α-, β-, and γ-CA classes, are genetically quite distinct between each other, with the various members of the same class clustering always together, even in the case of enzymes from the same organism belonging to a different genetic family (e..g., HpylCAalpha clusters with the other α-CAs and not with HpylCAbeta, etc.). [8].

Bacterial CAs are involved in the transport of CO_2 or HCO_3^-, in supplying CO_2 or HCO_3^- for the biosynthetic reactions (and thus metabolisms); in pH regulation

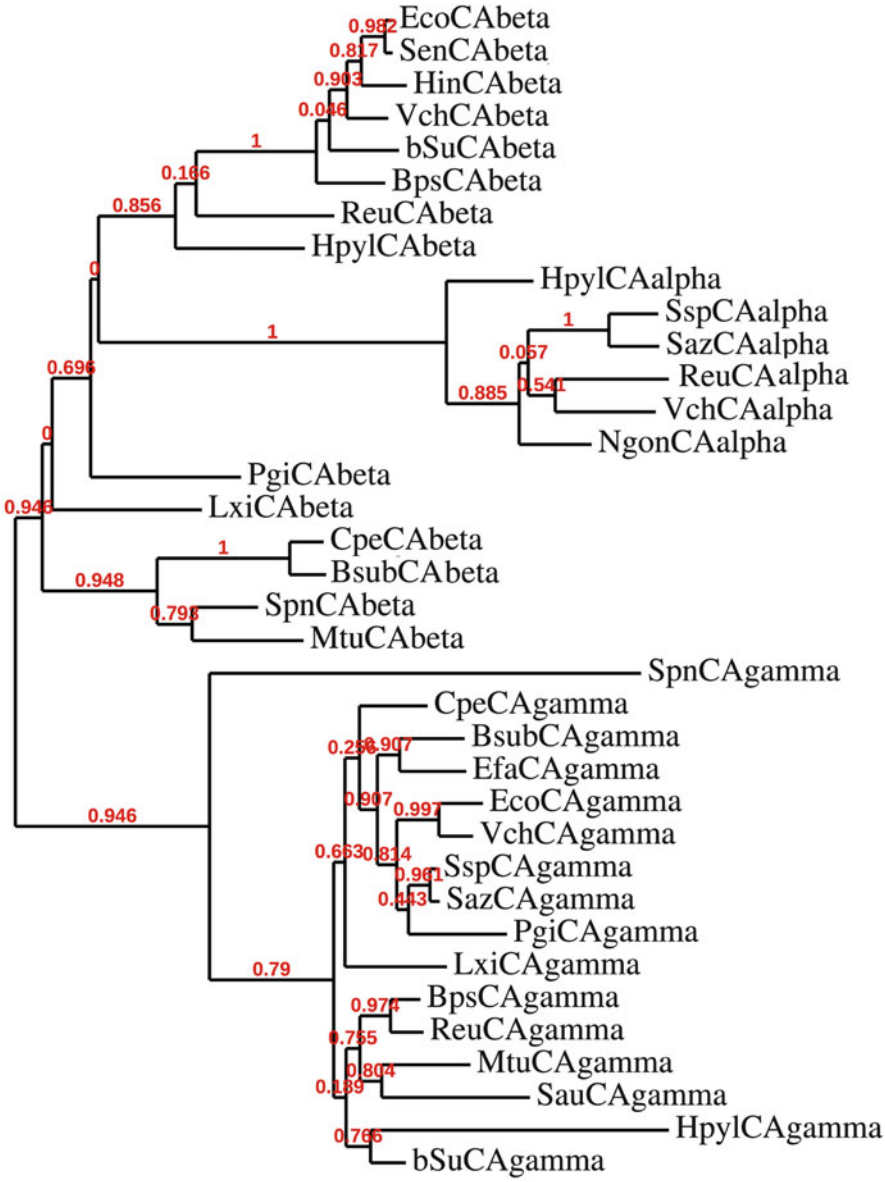

Fig. 1 Phylogenetic analysis of bacterial CAs. The tree was obtained by using all three CA classes identified in the genome of Gram-positive and Gram-negative bacteria (see Table 1 for details) and was carried out using the program PhylML

Table 1 Organisms, CA classes, accession numbers, and cryptonyms of the sequences used in the phylogenetic analysis

Organism	Accession number and CA class			Cryptonym
	α	β	γ	
Neisseria gonorrhoeae	YP_207719.1	–	–	NgonCA
Helicobacter pylori	NP_223829.1	WP_000642968.1	WP_000034119.1	HpyCA
Escherichia coli	–	WP_001709803.1	CDL59494	EcoCA
Haemophilus influenzae	–	NP_439452	–	HinCA
Brucella suis	–	EEY28164.1	NP_698263.1	bSuCA
Salmonella enterica	–	WP_023203629.1	–	SenCA
Vibrio cholerae	AEA79886.1	WP_002051193.1	YP_001218355.1	VchCA
Sulfurihydrogenibium yellowstonense	ACD66216.1	–	WP_007547159.1	SspCA
Sulfurihydrogenibium azorense	ACN99362.1	–	WP_012674376.1	SazCA
Porphyromonas gingivalis	–	WP_021663681.1	WP_012457873.1	PgiCA
Ralstonia eutropha	YP_841915.1	YP_841782.1	YP_725701.1	ReuCA
Burkholderia pseudomallei	–	WP_004550949.1	YP_108862.1	BpsCA
Mycobacterium tuberculosis	–	P64797.1	YP_008227613.1	MtuCA
Clostridium perfringens	–	WP_003471412.1	NP_562567.1	CpeCA
Streptococcus pneumoniae	–	NP_344575.1	YP_816426.1	SpnCA
Bacillus subtilis	–	YP_003867325.1	YP_005558024.1	BsubCA
Leifsonia xyli	–	YP_062554.1	YP_062697.1	LxiCA
Staphylococcus aureus	–	–	EVX10196.1	SauCA
Enterococcus faecalis	–	–	EPH93090.1	EfaCA

and also in cyanate degradation (at least in *E. coli*), as well as in the survival of intracellular pathogens within their host [7, 8].

3 α-Class Bacterial CAs

Table 2 shows the α-CAs cloned and characterized so far from pathogenic bacteria. The first one was an enzyme from *Neisseria gonorrhoeae* [42, 43], although older reports mention a similar CA in *N. sicca* and related species (which have not been cloned so far) [44, 45]. The *N. gonorrhoeae* CA contains 252 amino acid residues and has a molecular mass of 28 kDa, being quite homologous to mammalian CAs [42]. A comparison with the amino acid sequences of human (h) isoforms hCA I and II suggested that the secondary structures are essentially identical in the

Table 2 CAs from pathogenic bacteria cloned and characterized so far, and their inhibition studies with various classes of CAIs

Bacterium	Family	Name	Inhibition study		References
			In vitro	In vivo	
Neisseria gonorrhoeae	α	–	Sulfonamides, anions	Sulfonamides	[42, 43]
Neisseria sicca	α	–	Sulfonamides	Sulfonamides	[44, 45]
Helicobacter pylori	α	hpαCA	Sulfonamides, anions	Sulfonamides	[46–48]
	β	hpβCA	Sulfonamides, anions	Sulfonamides	[49, 50]
Escherichia coli	β	–	NI	NI	[51]
Haemophilus influenzae	β	HICA	Bicarbonate	NI	[52]
Mycobacterium tuberculosis	β	mtCA 1	Sulfonamides, phenols	Phenols	[14, 15, 53]
	β	mtCA 2	Sulfonamides	Phenols	[14, 15, 54–56]
	β	mtCA 3	Sulfonamides	Phenols	[54–57]
Brucella suis	β	bsCA 1	Sulfonamides	Sulfonamides	[58–60]
	β	bsCA 2	Sulfonamides	Sulfonamides	[58–60]
Streptococcus pneumoniae	β	PCA	Sulfonamides, anions	NI	[61]
Salmonella enterica	β	stCA 1	Sulfonamides, anions	NI	[62, 63]
	β	stCA 2	Sulfonamides, anions	NI	[62, 63]
Vibrio cholerae	α	VchCA	VchCA sulfonamide, anions	NI	[64, 65]
	β	VchCAβ	Sulfonamide, anions	NI	[30]
	γ	VchCAγ	Sulfonamide, anions	NI	[66]
Porphyromonas gingivalis	β	PgiCAβ	Sulfonamide, anions	NI	[67–69]
	γ	PgiCAγ	Sulfonamide, anions	NI	[31, 32]
Streptococcus mutans	β	SmuCA	Sulfonamide, anions	NI	[70, 71]
Clostridium perfringens	β	CpeCA	Anions	NI	[72]
Legionella pneumophila	β	LpeCA1	Sulfonamide, anions	NI	[40, 41]
	β	LpeCA2	Sulfonamide, anions	NI	[40, 41]
Enterobacter spp. B13	β	EspCA	Sulfonamides	NI	[73]
Pseudomonas aeruginosa	β	psCA3	Anions	NI	[66]

NI not investigated

bacterial enzyme but several loops are much shorter than in the human isoforms [42]. This has been confirmed thereafter by resolving the X-ray crystal structure of this enzyme [43]. Most of the active-site residues are identical to those found in hCA II, the crucial Zn(II) ion being coordinated by three His residues and a water molecule/hydroxide ion, being placed at a bottom of a rather deep and large active site. The bacterial enzyme showed a high CO_2 hydrase activity, with a k_{cat} of 1.1×10^6 s^{-1} and Km of 20 mM (at pH 9 and 25°C) [42]. The enzyme also showed esterase activity for the hydrolysis of 4-nitrophenyl acetate, similarly to the mammalian isoforms hCA I and II.

One of the best-studied bacterial α-CA is the one from the gastric pathogen provoking ulcer and gastric cancer, *H. pylori*, hpαCA [46–49]. The genome project of *H. pylori* identified in fact two different classes of CAs, with different subcellular localization: a periplasmic α-class CA (hpαCA) [46–49] and a cytoplasmic β-class CA (hpβCA) [50]. These two CAs were shown to be catalytically efficient with almost identical activity to that of the human isoform hCA I, for the CO_2 hydration reaction, and highly inhibited by many sulfonamides/sulfamates, including acetazolamide, ethoxzolamide, topiramate, and sulpiride, all clinically used drugs [48–50]. Furthermore, certain CAIs, such as acetazolamide and methazolamide, were shown to effectively inhibit the bacterial growth in cell cultures [48–50]. Since the efficacy of *H. pylori* eradication therapies currently employed has been decreasing due to drug resistance and side effects of the commonly used drugs, the dual inhibition of α- and/or β-CAs of *H. pylori* could be applied as an alternative therapy in patients with *H. pylori* infection or for the prevention of gastro duodenal diseases provoked by this widespread pathogen [48–50]. Acetazolamide, in fact, was clinically used as an antiulcer drug by Puscas [74], although the rationale for its use was a wrong one, as this scientist considered that inhibition of gastric mucosa CAs (mainly hCA II) led to a decrease of HCl production. In fact the antiulcer effects of this sulfonamide CAI are due to inhibition of H. pylori CAs [75]. The X-ray crystal structure of hpαCA in adduct with acetazolamide **AAZ** and methazolamide **MZA** was also reported [76], opening the way to a structure-based drug design of potent and possibly selective inhibitors for this enzyme.

Recently, an α-CA was identified, cloned, and characterized from *Vibrio cholerae* (denominated VchCA) [64, 65], a Gram-negative bacterium, which is the causative agent of cholera. This bacterium colonizes the upper small intestine where sodium bicarbonate is present at a high concentration. Sodium bicarbonate is an inducer of virulence gene expression. *V. cholerae* utilizes the CA system to accumulate bicarbonate into its cells, suggesting a pivotal role of this metalloenzymes in the microbial virulence [77, 78]. VchCA showed a low esterase activity with 4-nitrophenyl acetate as substrate, and a high activity for the hydration of CO_2 to bicarbonate [64, 65]. The inhibition study with sulfonamides and sulfamates led to the detection of a large number of low nanomolar inhibitors, among which are methazolamide (**MZA**), acetazolamide (**AAZ**), ethoxzolamide (**EZA**), dorzolamide (**DZA**), brinzolamide (**BRZ**), benzolamide (**BZA**), and indisulam (**IND**) (K_i values in the range 0.69−8.1 nM, see Fig. 2). As bicarbonate is a virulence factor of this bacterium and since ethoxzolamide was shown to inhibit

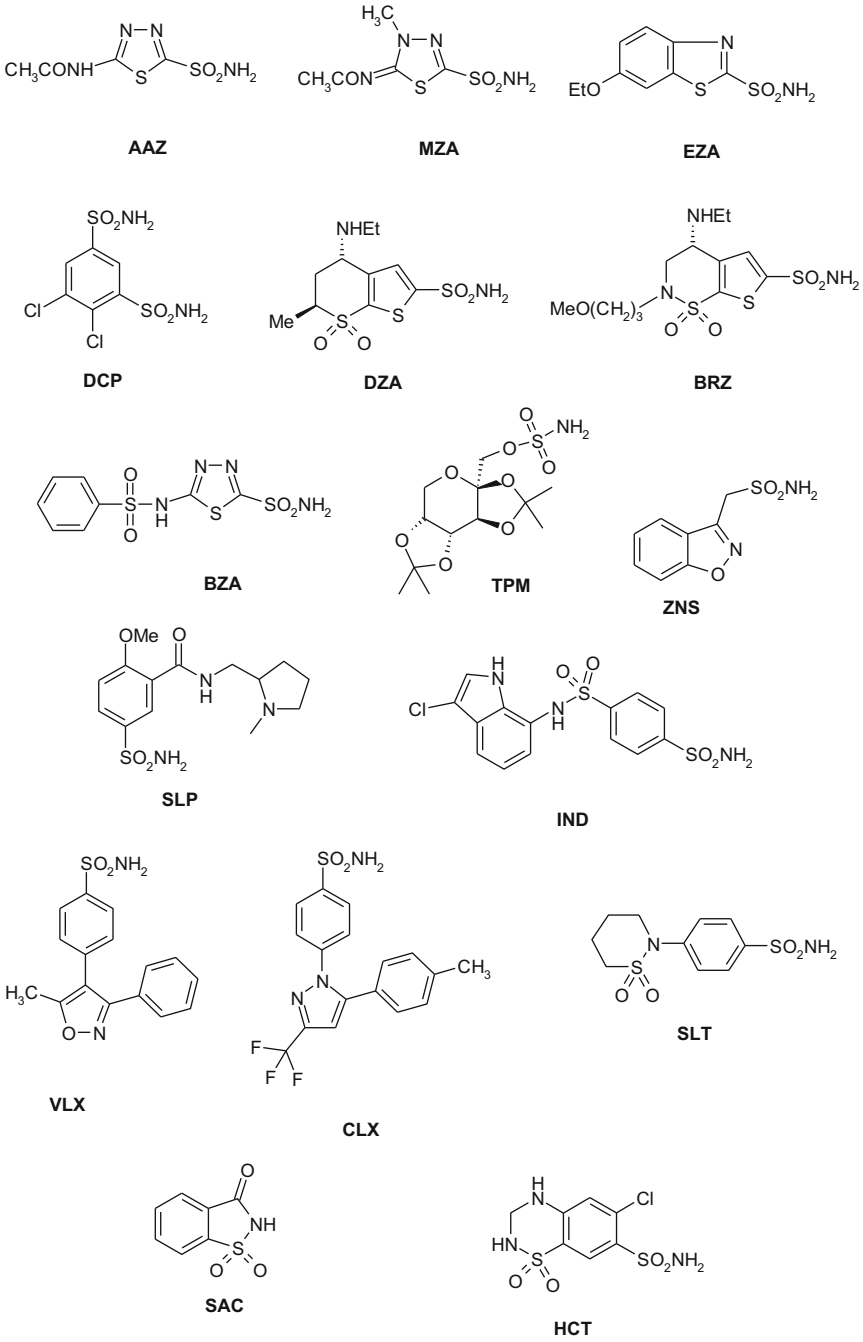

Fig. 2 Some sulfonamide CAIs investigated for their interaction with various bacterial pathogenic CAs (see text for details and Table 3 for inhibition data)

the in vivo virulence, it was proposed that VchCA might be a target for antibiotic development, exploiting a mechanism of action rarely considered until now [64, 65, 79–81].

4 β-Class Bacterial CAs

The β-CA class is the most widespread in bacteria [5–9]. The proof-of-concept study that such an enzyme may be a drug target has been published by Nishimori et al. [50] who cloned and purified the *H. pylori* enzyme (hpβCA), showing that it is highly susceptible to be inhibited by sulfonamides and sulfamates (see discussion above for the in vivo data). Afterwards, a rather large number of other β-CAs were cloned, purified, and characterized from many other pathogenic bacteria (see Tables 1 and 2).

X-ray crystal structures are available for several of these enzyme, such as the *E. coli*, [51] *H. influenzae* [52], two of the three *M. tuberculosis* enzymes [14, 15] one from *S. enterica* (stCA 1) [82] as well as the β-CA from V. cholera, VchCAβ [30]. The 3-D folds of these enzymes are rather conserved (Fig. 3), although some of them are dimers [51, 52, 82] whereas others are tetramers [30]. All crystallized bacterial β-CAs so far are active as dimers or tetramers, with two or four identical active sites [22, 30]. Their shape is that of a rather long channel at the bottom of which the catalytic zinc ion is found, tetrahedrally coordinated by Cys42, Asp44, His98, and Cys101 – Fig. 3 (in the VchCAβ enzyme, which possesses a closed active site at pH values <8.3) [30]. This is indeed the so-called closed active site, since these enzymes are not catalytically active (at pH values <8.3) [14, 15, 30]. However, at pH values >8.3, the "closed active site" is converted to the "open active site" (with gain of catalytic activity), this being associated with a movement of the Asp residue from the catalytic Zn(II) ion, with the concomitant coordination of an incoming water molecule approaching the metal ion. This water molecule (as hydroxide ion) is in fact responsible for the catalytic activity, as for the α-CAs investigated in much greater detail [14, 15, 22, 30].

Many of these enzymes displayed excellent activity for the physiologic CO_2 hydration reaction and were inhibited (sometimes in the low nanomolar range) by sulfonamides and sulfamates, see Table 2 [40, 41, 50, 53–63, 67–73, 83–85]. Their inhibition profiles with anions were also investigated [51, 52, 54–65, 67–72, 77–85]. However, in vivo, it has been possible to observe inhibition of the bacterial growth only for *H. pylori*, *B. suis*, *S. pneumoniae*, and *M. tuberculosis* [50, 53, 59, 63]. For example, in the case of *M. tuberculosis*, although nanomolar and sub-nanomolar sulfonamide in vitro inhibitors were detected [57, 83], no in vivo inhibition of growth has been observed, probably because the highly polar sulfonamides have difficulties to penetrate through the bacterial wall of these pathogens [57]. However, recently, Colina's group showed that a class of phenol derivatives (Fig. 4) possess antimycobacterial activity in vivo [53]. Unlike the sulfonamides, these derivatives (of type **1**–**8**) are more lipophilic and may better penetrate through

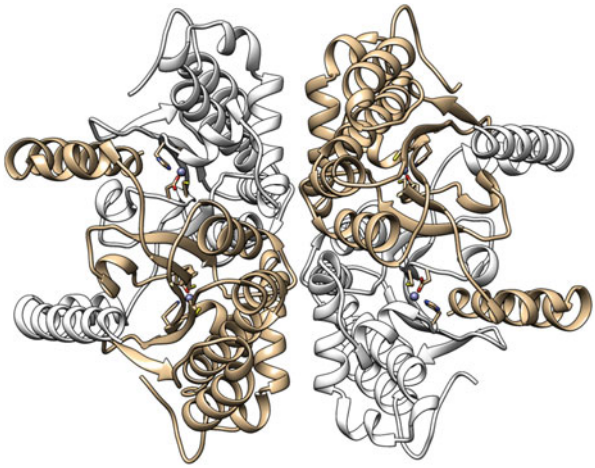

Fig. 3 View of the tetrameric VchCAβ as obtained by X-ray crystallography (PDB file 5CXK1) [30]. The four polypeptide chains are represented as ribbons. The Zn(II) ions (*blue spheres*) and their ligands (Cys42, Asp44, His98, and Cys101) are shown as stick representation. The four active sites are identical and consist of a long channel at the bottom of which is found the Zn(II) ion in a tetrahedral geometry [30]

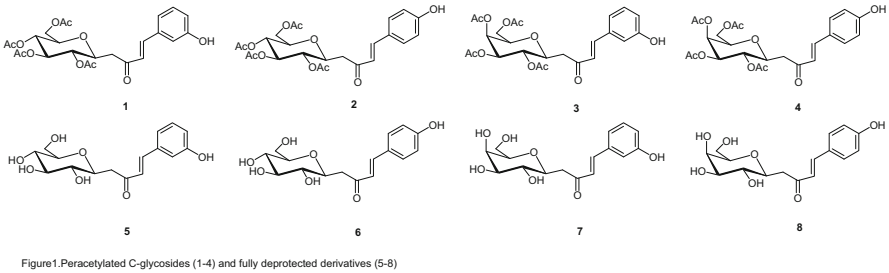

Fig. 4 Phenol derivatives **1–8** showing *M. tuberculosis* growth inhibition in vivo [53]

the bacterial cell walls. Thus, many of these β-CAs are in fact validated drug targets, but much work is warranted in order to detect potent in vitro CAIs that also work in vivo, in order to thoroughly validate these CAs as drug targets.

Table 3 shows the in vitro inhibition data of several of these enzymes with sulfonamide/sulfamates, which represent one of the main classes of CAIs, as mentioned above. Such compounds are clinically used drugs, e.g., acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, dorzolamide **DZA**, brinzolamide **BRZ**, benzolamide **BZA**, topiramate **TPM**, zonisamide **ZNS**, sulpiride **SLP**, indisulam **IND**, celecoxib **CLX**, valdecoxib **VLX**, sulthiame **SLT**, hydrochlorothiazide **HCT**, and saccharin **SAC**, as diuretics, antiepileptics, antiglaucoma, and antiinflammatory agents [18]. It may be observed that most CAs from bacterial pathogenic organisms are inhibited in the micro –

Table 3 In vitro inhibition data of bacterial CAs with sulfonamides and sulfamates, some of which are clinically used drugs (only the enzymes for which these data were reported in the literature are included)

	K_i (μM)								
Compound	hpαCA	hpβCA	mtCA 1	mtCA 2	mtCA 3	bsCA 1	bsCA 2	stCA1	stCA2
AAZ	0.021	0.040	0.481	0.009	0.104	0.063	0.303	0.059	0.084
MZA	0.225	0.176	0.781	0.660	0.562	0.054	0.642	0.134	0.068
EZA	0.193	0.033	1.03	0.027	0.594	0.017	0.420	0.528	0.721
DCP	0.378	0.105	0.872	2.01	0.611	0.058	0.112	0.090	0.095
DZA	4.36	0.073	0.744	0.099	0.137	0.021	0.923	0.445	0.607
BRZ	0.210	0.128	0.839	0.127	0.201	0.026	0.625	0.687	0.412
BZA	0.315	0.054	0.810	0.467	0.338	0.075	0.117	0.085	0.098
TPM	0.172	0.032	0.612	0.474	3.02	0.057	0.099	0.624	0.697
ZNS	0.231	0.254	28.68	0.876	0.208	1.85	0.406	5.43	5.70
SLP	0.204	0.035	2.30	0.266	7.92	0.019	0.084	5.64	8.73
IND	0.413	0.143	0.097	0.717	7.84	0.050	0.130	8.86	6.90
CLX	nt	nt	10.35	0.713	7.76	0.018	0.128	5.83	6.11
VLX	nt	nt	12.97	0.682	7.81	0.019	0.612	6.85	6.58

nt not tested

nanomolar range by many such sulfonamide/sulfamate drugs. It should be mentioned that few rational drug design campaigns have been done so far in order to detect better CAIs targeting bacterial CAs so far, but the preliminary screening results summarized in Table 3 are indeed promising, since a lot of effective led compounds have been detected. It is envisageable that more research in this area may lead to highly effective and bacterial CA selective compounds which may validate these enzymes as antibacterial drug targets.

Recently, in our laboratories was identified in the genome of *P. gingivalis* a β-CA, named PgiCAb [67–69]. The enzyme had a good catalytic activity, with a k_{cat} of 2.8×10^5 s^{-1} and a k_{cat}/K_m of 1.5×10^7 $M^{-1} \times s^{-1}$. PgiCAb was also inhibited by the clinically used sulfonamide acetazolamide, with an inhibition constant of 214 nM [67–69]. Other β-CAs which were cloned and investigated in detail were two enzymes from *Legionella pneumophila*, LpeCA1 and LpeCA2 [40, 41], one from *Clostridium perfringens* [72], CpeCA, one from *Streptococcus mutans*, SmuCA [70, 71], one from *Enterobacter* spp. B13 [73], and another from *Pseudomonas aeruginosa* [66]. All these enzymes are highly effective catalysts for the conversion of CO_2 to bicarbonate and their inhibition profile with some classes of CAIs was also investigated, but no detailed in vivo studies regarding their druggability are available so far.

5 γ-Class Bacterial CAs

Bacteria may be extremely abundant in environments that are hostile to all other forms of life. In the Archaea domain, a γ-CA from *Methanosarcina thermophila* was the first reported by Ferry's group [86]. The presence of both the β and γ-CA classes in thermophilic chemolithoautotrophs suggests that ancient CO_2-fixation pathways depended on this enzyme for efficient CO_2 fixation. X-ray crystal structures of some of these CAs were also determined, and in vitro and in vivo inhibition studies with various classes of inhibitors, such as anions, sulfonamides, and sulfamates, have been reported [28]. The genome of *P. gingivalis* encodes for a β- and a γ-CAs. Recently, our group purified the recombinant γ-CA (named PgiCA) which was shown to possess a significant catalytic activity for the reaction that converts CO_2 to bicarbonate and protons, with a k_{cat} of 4.1×10^5 s^{-1} and a k_{cat}/K_m of 5.4×10^7 $M^{-1} \times s^{-1}$ [31, 32]. We have also investigated its inhibition profile with a range of inorganic anions such as thiocyanate, cyanide, azide, hydrogen sulfide, sulfamate, and trithiocarbonate. The role of CAs as possible virulence factors of *P. gingivalis* is poorly understood at the moment but their good catalytic activity and the fact that they might be inhibited by a large number of compounds might pave the way for finding inhibitors with antibacterial activity that may elucidate these phenomena and lead to novel antibiotics [31, 32]. The genome of *V. cholerae* encodes for CAs belonging to α, β, and γ classes [87]. Recently, the last type of this enzyme, VchCAγ was cloned and characterized [87], comparing it with data obtained for the α- and β-CA enzymes from the same pathogen. VchCAγ

activity ($k_{cat} = 7.39 \times 10^5$ s^{-1}) was significantly higher than the other γ-CAs investigated earlier [30, 31]. The inhibition study with a panel of sulfonamides and one sulfamate led to the detection of a large number of nanomolar VchCAγ inhibitors, including simple aromatic/heterocyclic sulfonamides as well as **EZA**, **DZA**, **BRZ**, **BZA**, **TPM**, **ZNS**, **SLP**, **IND** (K_i in the range of 66.2–95.3 nM). As it was proven that bicarbonate is a virulence factor of this bacterium and since ethoxzolamide was shown to inhibit this virulence in vivo, it has been proposed that VchCA, VchCAβ, and VchCAγ may be targets for antibiotic development, exploiting a mechanism of action rarely considered up until now, i.e., interference with bicarbonate supply as a virulence factor [87].

6 Conclusions

By catalyzing the simple but highly important hydration of carbon dioxide to bicarbonate and protons, bacterial CAs are probably involved in critical steps of the bacterial life cycle, some of which are important for survival, invasion, and pathogenicity. Bacteria encode such enzymes belonging to the α-, β-, and/or γ-CA families, but up to now only the first two classes have been investigated in some detail in different species. Indeed, the α-CAs from *Neisseria* spp. and *H. pylori* as well as the β-class enzymes from *Salmonella enterica*, *Haemophilus influenzae*, *Legionella pneumophila*, *Vibrio cholerae*, *Porphyromonas gingivalis*, *Streptococcus mutans*, *Clostridium perfringens*, *Pseudomonas aeruginosa* have been cloned and characterized. For some of these enzymes the X-ray crystal structures were determined at a high resolution, allowing for a good understanding of the catalytic/inhibition mechanisms and constituting the starting point for rational drug design campaigns. Several in vitro and in vivo inhibition studies with various classes of inhibitors, such as anions, sulfonamides, and sulfamates, have been reported. Efficient in vitro inhibitors have been discovered for many such enzymes, but only for *Neisseria* spp., *H. pylori*, *B. suis*, *S. pneumoniae,* and M. tuberculosis the CA inhibition was reported to lead to inhibition of bacterial growth in vivo. Thus, bacterial CAs represent at this moment very promising targets for obtaining antibacterials devoid of the resistance problems of the clinically used such agents but further studies are needed to validate these and other less investigated enzymes as novel drug targets.

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