CANCER DRUG DISCOVERY AND DEVELOPMENT

Deoxynucleoside Analogs in Cancer Therapy

Edited by

Godefridus J. Peters, PhD

HUMANA PRESS

Deoxynucleoside Analogs in Cancer Therapy

Cancer Drug Discovery and Development

Beverly A. Teicher, Series Editor

Regional Cancer Therapy, edited by *Peter M. Schlag and Ulrike Stein, 2007*

Gene Therapy for Cancer, edited by *Kelly K. Hunt, Stephan A. Vorburger, and Stephen G. Swisher, 2007*

Deoxynucleoside Analogs in Cancer Therapy, edited by *Godefridus J. Peters,* 2006

Cancer Drug Resistance, edited by *Beverly A. Teicher,* 2006

Histone Deacetylases: *Transcriptional Regulation and Other Cellular Functions,* edited by *Eric Verdin,* 2006

Immunotherapy of Cancer, edited by *Mary L. Disis,* 2006

Biomarkers in Breast Cancer: *Molecular Diagnostics for Predicting and Monitoring Therapeutic Effect,* edited by *Giampietro Gasparini and Daniel F. Hayes,* 2006

Protein Tyrosine Kinases: *From Inhibitors to Useful Drugs,* edited by *Doriana Fabbro and Frank McCormick,* 2005

Bone Metastasis: *Experimental and Clinical Therapeutics,* edited by *Gurmit Singh and Shafaat A. Rabbani,* 2005

The Oncogenomics Handbook, edited by *William J. LaRochelle and Richard A. Shimkets,* 2005

Camptothecins in Cancer Therapy, edited by *Thomas G. Burke and Val R. Adams,* 2005

Combination Cancer Therapy: *Modulators and Potentiators,* edited by *Gary K. Schwartz,* 2005

Death Receptors in Cancer Therapy, edited by *Wafik S. El-Deiry,* 2005

Cancer Chemoprevention, **Volume 2:** *Strategies for Cancer Chemoprevention,* edited by *Gary J. Kelloff, Ernest T. Hawk, and Caroline C. Sigman,* 2005

Proteasome Inhibitors in Cancer Therapy, edited by *Julian Adams, 2004*

Nucleic Acid Therapeutics in Cancer, edited by *Alan M. Gewirtz, 2004*

Cancer Chemoprevention, **Volume 1:** *Promising Cancer Chemopreventive Agents,* edited by *Gary J. Kelloff, Ernest T. Hawk, and Caroline C. Sigman,* 2004

DNA Repair in Cancer Therapy, edited by *Lawrence C. Panasci and Moulay A. Alaoui-Jamali,* 2004

Hematopoietic Growth Factors in Oncology: *Basic Science and Clinical Therapeutics,* edited by *George Morstyn, MaryAnn Foote, and Graham J. Lieschke,* 2004

Handbook of Anticancer Pharmacokinetics and Pharmacodynamics, edited by *William D. Figg and Howard L. McLeod,* 2004

Anticancer Drug Development Guide: *Preclinical Screening, Clinical Trials, and Approval, Second Edition,* edited by *Beverly A. Teicher and Paul A. Andrews,* 2004

Handbook of Cancer Vaccines, edited by *Michael A. Morse, Timothy M. Clay, and Kim H. Lyerly,* 2004

Drug Delivery Systems in Cancer Therapy, edited by *Dennis M. Brown, 2003*

Oncogene-Directed Therapies, edited by *Janusz Rak,* 2003

Cell Cycle Inhibitors in Cancer Therapy: *Current Strategies,* edited by *Antonio Giordano and Kenneth J. Soprano,* 2003

Fluoropyrimidines in Cancer Therapy, edited by *Youcef M. Rustum,* 2003

Chemoradiation in Cancer Therapy, edited by *Hak Choy,* 2003

Targets for Cancer Chemotherapy: *Transcription Factors and Other Nuclear Proteins,* edited by *Nicholas B. La Thangue and Lan R. Bandara,* 2002

Tumor Targeting in Cancer Therapy, edited by *Michel Pagé,* 2002

Hormone Therapy in Breast and Prostate Cancer, edited by *V. Craig Jordan and Barrington J. A. Furr,* 2002

Tumor Models in Cancer Research, edited by *Beverly A. Teicher,* 2002

Tumor Suppressor Genes in Human Cancer, edited by *David E. Fisher,* 2001

Matrix Metalloproteinase Inhibitors in Cancer Therapy, edited by *Neil J. Clendeninn and Krzysztof Appelt,* 2001

Farnesyltransferase Inhibitors in Cancer, edited by *Saïd M. Sebti and Andrew D. Hamilton,* 2001

Platinum-Based Drugs in Cancer Therapy, edited by *Lloyd R. Kelland and Nicholas P. Farrell,* 2000

Deoxynucleoside Analogs in Cancer Therapy

Edited by

Godefridus J. Peters, PhD

Professor of Medical Oncology, VU University Medical Center
Amsterdam, The Netherlands

Humana Press
Totowa, New Jersey

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

Cover design by Patricia F. Cleary

This publication is printed on acid-free paper. ∞

ANSI Z39.48-1984 (American National Standards Institute)

Permanence of Paper for Printed Library Materials

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel.: 973-256-1699; Fax: 973-256-8341; E-mail: orders@humanapr.com; or visit our Website: www.humanapress.com

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

eISBN 1-59745-148-7

Library of Congress Cataloging-in-Publication Data

Deoxynucleoside analogs in cancer therapy / edited by Godefridus J. Peters.
p. ; cm. -- (Cancer drug discovery and development)
Includes bibliographical references and index.
ISBN 1-58829-327-0 (alk. paper)
1. Deoxyribonucleotides--Therapeutic use. 2. Antineoplastic agents.
3. Cancer--Chemotherapy. I. Peters, Godefridus Johannes, 1952- . II. Series.
[DNLM: 1. Deoxyribonucleotides--therapeutic use. 2. Antineoplastic Agents--therapeutic use. 3. Neoplasms--drug therapy. QV 185 D418 2006]
RC271.C5D45 2006
616.99'4061--dc22

2005036304

PREFACE

Successful cancer chemotherapy relies heavily on the application of various deoxynucleoside analogs. Since the very beginning of modern cancer chemotherapy, a number of antimetabolites have been introduced into the clinic and subsequently applied widely for the treatment of many malignancies, both solid tumors and hematological disorders. In the latter diseases, cytarabine has been the mainstay of treatment of acute myeloid leukemia. Although many novel compounds were synthesized in the 1980s and 1990s, no real improvement was made. However, novel technology is now capable of elucidating the molecular basis of several inborn errors as well as some specific malignancies. This has enabled the synthesis of several deoxynucleoside analogs that could be applied for specific malignancies, such as pentostatin and subsequently chlorodeoxyadenosine (cladribine) for the treatment of hairy cell leukemia. Already in the early stage of deoxynucleoside analog development, it was recognized that several of these compounds were very effective in the treatment of various viral infections, such as for the treatment of herpes infections. This formed the basis initially for the design of azidothymidine and subsequently many other analogs, which are currently successfully used for the treatment of HIV infections. As a spin-off of these research lines, some compounds not eligible for development as antiviral agents appeared to be very potent anticancer agents. The classical example is gemcitabine, now one of the most widely applied deoxynucleoside analogs, used for the (combination) treatment of non-small cell lung cancer, pancreatic cancer, bladder cancer, and ovarian cancer. The knowledge gained with the development of all of these compounds formed the basis for the design of a number of novel analogs currently being used for the treatment of various malignancies or currently in an advanced stage of development. Interestingly, several of the nucleoside analogs are also targeted directly against cell cycle regulatory proteins as well as other protein kinases.

In this volume of the Cancer Drug Discovery and Development series, the current status of development and application of deoxynucleoside analogs has been summarized. A number of scientists well known in their specific area contributed with authoritative up-to-date reviews of their field. Their contributions were not limited to writing, but also included sound advice on structure and topics that was extremely valuable. *Deoxynucleoside Analogs in Cancer Therapy* is organized into several parts: the first part (Chapters 1–5) deals with general aspects of drug uptake and metabolism, the second deals with a number of specific drugs (Chapters 6–12),

while the last part covers pharmacokinetics, prodrugs, and specific applications such as radiosensitization and the use of deoxynucleoside analogs as tracers.

In order to be taken up by the cell, deoxynucleoside analogs require specific transporters, whereas other transporters can mediate efflux of (monophosphorylated) nucleosides. Novel technology enabled a rapid expansion of this field in the last decade (Chapters 1–5). Subsequent phosphorylation of nucleoside analogs is essential for their action and is mediated by a number of specific and less-specific deoxynucleoside kinases; their characteristics and regulation are summarized in Chapters 2 and 3. The role of nucleotidases in resistance to nucleoside analogs is described in Chapter 4. Specific viral deoxynucleoside kinases were recognized to be very suitable for local activation of otherwise inactive deoxynucleoside analogs. The HSV-specific thymidine kinase was therefore extensively used in early gene therapy studies. More active deoxynucleoside kinases with broad substrate specificity seem very suitable for future gene therapy applications, as described in Chapters 3 and 16.

A chapter on cytarabine—the first real deoxynucleoside analog widely used in the clinic—is essential to a complete book (Chapter 6). Various novel deoxynucleoside analogs have been developed in the last decade; several aspects of their mechanism of action and applications have been described throughout the above-mentioned chapters, while several of these analogs are described extensively in specific chapters on gemcitabine (Chapters 11 and 12), troxacitabine (Chapter 9), clofarabine, which was approved recently for acute leukemia (Chapter 7), and ara-G (Chapter 10). Two additional chapters deal with prodrug design (Chapter 15) or the novel class of L-nucleosides (Chapter 8).

Modern drug development tends to focus on specific targets, thereby neglecting that, in order to be effective, a drug needs to be taken up by the body and transported to the malignant tissues. A proper understanding of pharmacokinetics and pharmacodynamics of deoxynucleoside analogs is indispensable to their administration (Chapter 14). Also, pharmacogenomics, specific genetic properties of a tumor or a subject, determines whether a tumor will be sensitive to a drug and whether a patient will tolerate a drug. Proper knowledge of a patient's pharmacogenomics profile enables individualized treatment, as described in chapters on specific drugs.

Deoxynucleoside analogs can be considered ideal compounds to be combined with other drugs, either with classical cytotoxic agents or with novel so-called targeted agents such as cell cycle-directed compounds, various protein kinase inhibitors, and angiogenesis inhibitors. These applications are also described in specific chapters, while a specific chapter is dedicated to the excellent radiosensitizing properties of deoxynucleoside analogs (Chapter 13). The last chapter focuses on a novel application of a deoxynucleoside analog, the use of 3'-deoxy-3'-fluorothymidine as

an active tracer in PET with the potential to replace fluorodeoxyglucose in specific applications (Chapter 17).

Throughout *Deoxynucleoside Analogs in Cancer Therapy*, the focus is on novel aspects of deoxynucleoside analogs in the clinical context, as well as on unexpected targets of these compounds, such as their specific activity against cell cycle-dependent kinases or oncogenes. Modern targeted cancer chemotherapy aims to be more specific than in the past, but it has now been recognized that inhibition of just one target often enables the cell to find another pathway, bypassing this inhibition. Current knowledge of deoxynucleoside analogs has already led to successful combinations with novel targeted agents that prevent inhibition of one target from being bypassed by simultaneous activation of another. Future research in this field should use this knowledge to design rational combinations aimed at inhibiting various cellular signaling pathways, enhancing apoptotic pathways, or combining inhibition of various targets. *Deoxynucleoside Analogs in Cancer Therapy* has been designed specifically to facilitate such an interaction between various fields.

Godefridus J. Peters, PhD

CONTENTS

Contributors

Vassilios I. Avramis, PhD • *Keck School of Medicine, University of Southern California; Department of Pediatrics, Division of Hematology/Oncology, Childrens Hospital Los Angeles, Los Angeles, CA*

Jan Balzarini, PhD • *Professor, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium*

Andries M. Bergman, MD, PhD • *Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands*

Piet Borst, MD, PhD • *Professor, Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands*

F. Javier Casado, MSc, PhD • *Associate Professor of Biochemistry and Molecular Biology, Department of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain*

Youhoon Chong, PhD • *Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, GA*

Chung K. Chu, PhD • *Distinguished Research Professor, Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, University of Georgia, Athens, GA*

Sophie Curbo, PhD • *Department of Laboratory Medicine, Division of Metabolic Disorders, Karolinska Institutet, Stockholm, Sweden*

Wieteke G. E. Direcks, MSc • *Department of Nuclear Medicine and PET Research, VU University Medical Center, Amsterdam, The Netherlands*

Staffan Eriksson, MD, PhD • *Professor of Medical and Physiological Chemistry, Department of Molecular Biosciences, The Swedish University of Agricultural Sciences, The Biomedical Center, Uppsala, Sweden*

Varsha Gandhi, PhD • *Department of Experimental Therapeutics, The University of Texas, M. D. Anderson Cancer Center, Houston, TX*

Zoran Gojkovic, PhD • *ZGene A/S, Hørsholm, Denmark*

Henriette Gourdeau, PhD • *Associate Director, Pharmacology/Toxicology, Ecopia BioSciences Inc., Ville Saint-Laurent, Québec, Canada*

Giuseppe Gumina, PhD • *Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC*

ISABELLE HUBEEK, PhD • *Department of Pediatric Hematology/Oncology, VU University Medical Center, Amsterdam, The Netherlands*

SALLY ANNE HUNSUCKER, PhD • *Lineberger Comprehensive Cancer Center, Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC*

JACQUES JOLIVET, MD • *Vice-President of Clinical Development, Aegera Therapeutics Inc., Montreal, Québec, Canada*

ANNA KARLSSON, MD, PhD • *Department of Laboratory Medicine, Division of Metabolic Disorders, Karolinska Institutet, Stockholm, Sweden*

GERT-JAN L. KASPERS, MD, PhD • *Department of Pediatric Hematology, VU University Medical Center, Amsterdam, The Netherlands*

JUDITH R. KROEP, MD, PhD • *Department Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands*

ADRIAAN A. LAMMERTSMA, PhD • *Professor, Department of Nuclear Medicine and PET Research, VU University Medical Center, Amsterdam, The Netherlands*

THEODORE S. LAWRENCE, MD, PhD • *Professor, Department of Radiation Oncology, University of Michigan Medical School, Ann Arbor, MI*

ASTRID MEERBACH, PhD • *Institute for Virology and Antiviral Therapy, Friedrich-Schiller-University Jena, Jena, Germany*

CHRIS MEIER, PhD • *Professor, Institute of Organic Chemistry, University of Hamburg, Hamburg, Germany*

BEVERLY S. MITCHELL, MD, PhD • *George E. Becker Professor of Medicine, Comprehensive Cancer Center, Stanford University, Stanford, CA*

CARLA F. M. MOLTHOFF, PhD • *Department of Nuclear Medicine and PET Research, VU University Medical Center, Amsterdam, The Netherlands*

BIRGITTE MUNCH-PETERSEN, DSc • *Professor, Department of Life Sciences and Chemistry, Roskilde University, Roskilde, Denmark*

ROBERT A. NAGOURNEY, MD • *Rational Therapeutics Inc., Malcolm C. Todd Cancer Institute, Long Beach, CA*

GERT J. OSSENKOPPELE, MD, PhD • *Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands*

MARÇAL PASTOR-ANGLADA, MSc, PhD • *Professor of Biochemistry and Molecular Biology, University of Barcelona, Barcelona, Spain*

GODEFRIDUS J. PETERS, PhD • *Professor, Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands*

JURE PIŠKUR, PhD • *Professor, Department of Cell and Organism Biology, Lund University, Lund, Sweden*

WILLIAM PLUNKETT, PhD • *Department of Experimental Therapeutics, The University of Texas, M. D. Anderson Cancer Center, Houston, TX*

DONNA S. SHEWACH, PhD • *Professor, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI*

JOZEF SPYCHALA, PhD • *President, VisiScience Corporation, Chapel Hill, NC; Department of Pharmacology, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC*

MARIA STAUB, MD, PhD, ScD • *Professor of Medical Biochemistry, Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Faculty of Medicine, Semmelweis University, Budapest, Hungary*

PETER WIELINGA, PhD • *National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands*

1 Nucleoside Transport Into Cells

Role of Nucleoside Transporters SLC28 and SLC29 in Cancer Chemotherapy

Marçal Pastor-Anglada, MSc, PhD
and F. Javier Casado, MSc, PhD

CONTENTS

SUMMARY

Nucleosides are taken up into cells by either concentrative nucleoside transporter (CNT; SLC28 gene family) or equilibrative nucleoside transporter (ENT; SLC29 gene family) nucleoside transporters, which differ in their

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

substrate selectivity and their energy requirements. Both nucleoside transporter families have also been involved in the transmembrane transport of nucleoside-derived compounds, many of them currently used in antiviral and antitumoral therapies; hence, there is necessity for good knowledge about the function of these transporters. Some key points in the pharmacological understanding of these transporters are addressed. There is a long list of nucleoside derivatives with clinical relevance that are known to be transported by one or several nucleoside transporters; however, until recently little was known about the structural determinants that allow the molecular recognition of the substrates by their transporters. This will be a key point in the development of rationally designed new drugs. Other aspects of nucleoside transporters are also relevant to their function as drug transporters. On the one hand, several polymorphisms have been described in CNT and ENT proteins that could affect their activity, although thorough functional analysis awaits. On the other hand, tissue distribution of these transporters is not homogeneous among tissues, and their expression can be tightly regulated, thus opening the possibility of over- or underexpression of a particular transporter in transformed cells, as has been reported in several cases. All these properties of nucleoside transporters determine their role in the bioavailability and cell sensitivity to anticancer drugs, and the first studies linking nucleoside transporter function to drug sensitivity and clinical outcome in cancer patients are now reported.

Key Words: Concentrative nucleoside transporter; equilibrative nucleoside transporter; nucleoside uptake; passive diffusion.

1. THE CELLULAR REQUIREMENT FOR NUCLEOSIDE SALVAGE IS CRUCIAL TO NUCLEOSIDE-BASED CHEMOTHERAPY

Nucleosides and nucleotides play a variety of roles in cell physiology, and they can be considered as both nutrients and modulators of cell homeostasis. In addition to their role as nucleic acid precursors, nucleosides and nucleotides are also key determinants of energy metabolism, ligands for purinergic receptors, and transducers of endocrine signals, thereby modulating a wide range of cellular events.

Nucleotide biosynthetic pathways are restricted to selected cell types and are energetically costly. Thus, salvage and nucleoside recycling are common events, probably occurring in cell types, such as hepatocytes, in which endogenous synthesis is high. The pharmacological use of nucleoside derivatives, such as the deoxynucleoside-derived drugs used in cancer chemotherapy, relies on the dependence of various key cellular processes (e.g., deoxyribonucleic acid [DNA] replication, ribonucleic acid [RNA] synthesis, and nucleos[t]ide-dependent signaling) on the extracellular nucleoside supply.

In principle, dependence on extracellular nucleoside supply in highly proliferating cells such as tumor cells would result in a relatively specific effect of nucleoside-derived drugs on neoplastic targets. Nucleoside salvage capacity essentially relies on the enzymatic machinery that controls conversion into other nucleo(s)tides, as well as into their phosphorylated derivatives, which also represent the active form of most nucleoside-based chemotherapeutic agents. Thus, metabolism is a key determinant of prodrug activation and action, which might explain differences in responsiveness. This area is reviewed in other chapters.

However, the first metabolic "bottleneck" is the plasma membrane. Nucleosides and nucleoside derivatives are hydrophilic molecules that show low or even negligible permeability across cell membranes. Thus, to facilitate efficient uptake, specific membrane proteins mediate nucleoside translocation from the extracellular milieu into the cytosol. Consequently, nucleoside-based anticancer drugs also interfere with the naturally occurring substrates by competition for entry pathways.

2. HOW ARE NUCLEOSIDE-DERIVED DRUGS TRANSPORTED INTO CELLS?

2.1. Mechanisms of Nucleoside and Nucleoside-Derived Drug Transport Into Cells

The initial evidence in favor of protein-mediated uptake of nucleosides, and consequently of nucleoside-derived drugs, was obtained decades ago using classical biochemical approaches involving measurements of substrate flux across membranes and accumulation in cells. These kinetic determinations revealed that transport processes were sufficiently heterogeneous to be the result of several distinct membrane proteins, the molecular identification of which would have to wait for some years (for comprehensive reviews of the classical "kinetic" classification and properties of nucleoside transport systems, *see*, among others, refs. *1* and *2*).

Two types of nucleoside transport processes occur in cells: an equilibrative transport mechanism, which shows broad selectivity but relatively low affinity for substrates, and a concentrative, Na^+-dependent, high-affinity transport process, which exhibits some substrate selectivity, as discussed in Section 2.2. Although the former is in principle reversible as long as its driving force is the transmembrane substrate concentration gradient itself, the latter mediates the unidirectional influx of nucleosides, taking advantage of the electrochemical transmembrane sodium gradient irrespective of the nucleoside concentration inside the cell. Despite these considerations, equilibrative uptake can also contribute significantly to the unidirectional uptake of nucleosides and nucleoside-derived drugs whenever tight coupling

between nucleoside transport and subsequent metabolism occurs (i.e., phosphorylation leading to intracellular retention of their corresponding nucleotides). Equilibrative processes have been considered ubiquitous, whereas Na^+-dependent concentrative transport was initially thought to be restricted to selected epithelia.

The long-standing utilization of the kinetic classification currently is under replacement by the more accurate use of gene nomenclature and that of associated proteins. This is the classification that is introduced in this chapter. However, to take advantage of some earlier literature linking nucleoside transporter (NT) function to cell sensitivity to anticancer drugs, we would like to emphasize that, for a specific transport system (the so-called *es* system), a high-specificity ligand (nitrobenzylmercaptopurine ribonucleoside [NBMPR] or nitrobenzylthio inosin [NBTI]) often has been used for quantitative purposes, with the number of high-affinity NBMPR- (NBTI-) binding sites representing an estimation of the *es*-related transporters inserted into the plasma membrane *(2)*.

2.2. The Nucleoside Transporter Proteins Concentrative Nucleoside Transporter (SLC28) and Equilibrative Nucleoside Transporter (SLC29)

The two principal types of nucleoside transport processes, concentrative and equilibrative, are the result of the expression of concentrative nucleoside transporter (CNT) and equilibrative nucleoside transporter (ENT) proteins encoded by genes belonging to the SLC28 and SLC29 families, respectively (Table 1; Figs. 1–4). The first two NT-related complementary DNAs (cDNAs) (rCNT1 and rCNT2) were isolated in the mid-1990s by expression cloning from rat jejunum and liver, respectively *(3,4)*. The human orthologs and other members of the two gene families have since been cloned and functionally characterized *(5–10)*. However, the two most recently identified NT transporters, hENT3 and hENT4, remain to be fully characterized *(11)*.

Three subtypes of human CNT transporters have been cloned: hCNT1, hCNT2 and hCNT3 (Table 1; Figs. 1 and 2). All contain 13 transmembrane domains, and the deduced lengths, from their corresponding cDNAs are 649, 658, and 698 amino acid residues, respectively. They all recognize uridine as a substrate, with apparent affinities ranging from 20 to 80 μ*M*, and are responsible for three well-documented nucleoside transport activities.

Human CNT1 displays a pyrimidine-preferring nucleoside transport activity, whereas hCNT2 is purine preferring. Human CNT3 shows broad substrate selectivity. Apparent K_m values for preferred substrates can be as low as 6 μ*M* for the CNT1-mediated uptake of thymidine *(5,12)* and 4 and 8 μ*M* for inosine and adenosine, respectively, when mediated by CNT2 *(6,13,14)*. Despite the original view that all three CNT proteins can accept

Table 1
Kinetic and Molecular Properties of Concentrative and Equilibrative Nucleoside Transporters

Concentrative systems

Transporter	*Gene*	*Protein length*	*Stoichiometry*	*Substrates (K_m)*
hCNT1	SLC28A1	649	1 Na/1 nucl 2 Na/1 nucl	Uridine (40–60 μ*M*) Thymidine (6 μ*M*) Cytidine (34 μ*M*)
hCNT2	SLC28A2	658	1 Na/1 nucl	Adenosine (8 μ*M*) Guanosine (21 μ*M*) Uridine (80 μ*M*) Inosine (4 μ*M*)
hCNT3	SLC28A3	698	2 Na/1 nucl	Uridine (22 μ*M*) Cytidine (15 μ*M*) Thymidine (21 μ*M*) Adenosine (15 μ*M*) Guanosine (43 μ*M*) Inosine (52 μ*M*)

Equilibrative systems

Transporter	*Gene*	*Protein length*	*Inhibitors* (IC_{50})	*Substrates (K_m)*
hENT1	SLC29A1	456	NBTI (0.4 n*M*) dipyr. (5 n*M*)	Adenosine (40 μ*M*) Guanosine (140 μ*M*) Inosine (170 μ*M*) Uridine (260 μ*M*) Thymidine (300 μ*M*) Cytidine (580 μ*M*)
hENT2	SLC29A2	456	NBTI (2.8 μ*M*) dipyr. (356 n*M*)	Adenosine (100 μ*M*) Inosine (50 μ*M*) Uridine (250 μ*M*) Thymidine (710 μ*M*) Cytidine (5610 μ*M*)

The basic selectivity properties of hCNT1, hCNT2, hCNT3, hENT1, and hENT2 are summarized. Data were taken from a variety of published reports cited in the text. Stoichiometry for hCNT1 is still controversial. For the SLC29 (hENT) protein family, the IC_{50} values of high-affinity inhibitors are also given (dipyr stands for dipyridamol), whereas no specific inhibitors have been yet identified for SLC28 (hCNT) proteins. Although hENT3 and hENT4 have been identified, these proteins have not been well characterized and are not included in this table.

adenosine as a substrate, evidence suggests that adenosine is either an inhibitor of hCNT1 *(15)* or a very poorly transported substrate *(16)*.

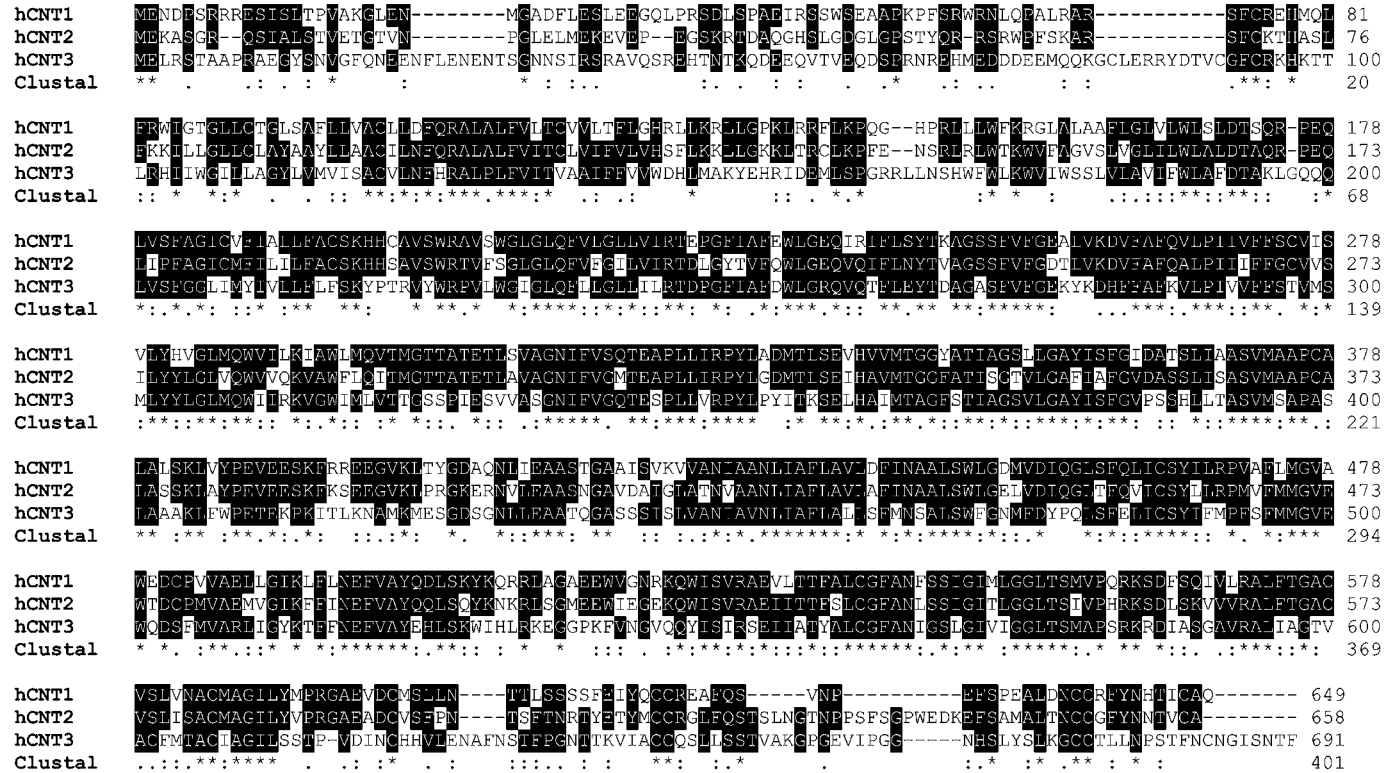


Fig. 1. Homology among the deduced protein sequences of the SLC28 (concentrative nucleoside transporter) family members. The primary structures of SLC28A1 (hCNT1), SLC28A2 (hCNT2), and SLC28A3 (hCNT3) were derived from the available cDNA sequences and compared using *Clustal N* and *Bioedit Sequence Alignment Editor* software. Shaded areas correspond to primary sequences showing a homology threshold of 5%. Symbols in the *Clustal* analysis are as follows: asterisk, identical residues; colon, conserved substitutions, period, semiconserved residue substitutions.

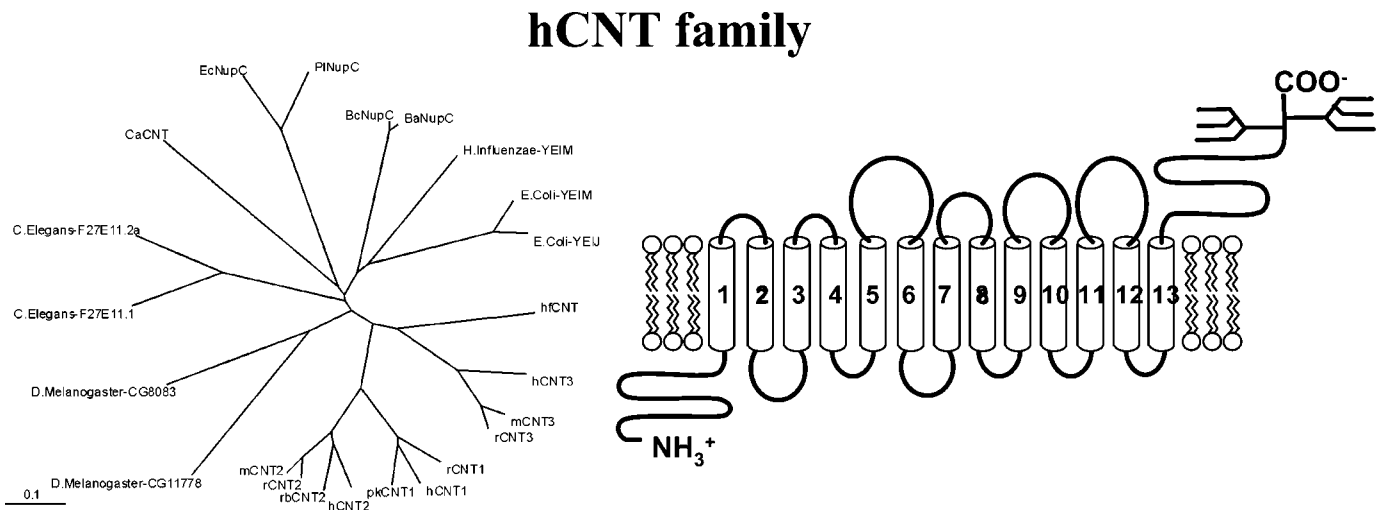


Fig. 2. The concentrative nucleoside transporter (SLC28) family and the suggested topology for the mammalian orthologs. A phylogenetic tree of the CNT family was built using *Tree View* software. Orthologs of the human CNTs are found in many phyla, including bacteria, where they might be coupled to protons instead of sodium ions to mediate nucleoside translocation across the plasma membrane. The suggested topology for the mammalian proteins anticipates 13 transmembrane domains, with the N-terminus tail localized in the cytosol, whereas the glycosylated C-terminus domain is extracellular.

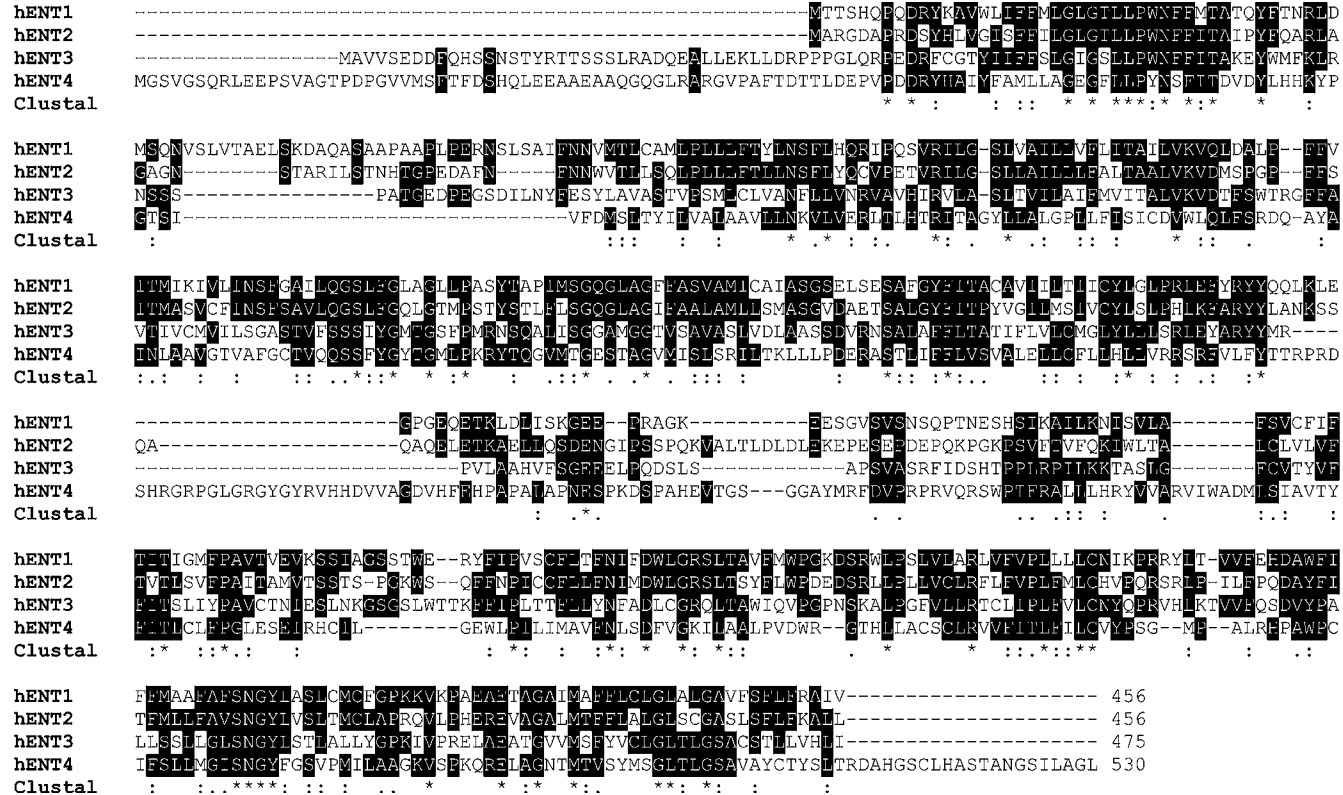


Fig. 3. Homology among the deduced protein sequences of the SLC29 (equilibrative nucleoside transporter) family members. The primary structures of SLC29A1 (hENT1), SLC29A2 (hENT2), hENT3, and hENT4 were derived from the available cDNA sequences and compared using *Clustal N* and *Bioedit Sequence Alignment Editor* software. Shaded areas correspond to primary sequences showing a homology threshold of 5%. Symbols in the *Clustal* analysis are as follows: asterisk, identical residues; colon, conserved substitutions; period, semiconserved residue substitutions.

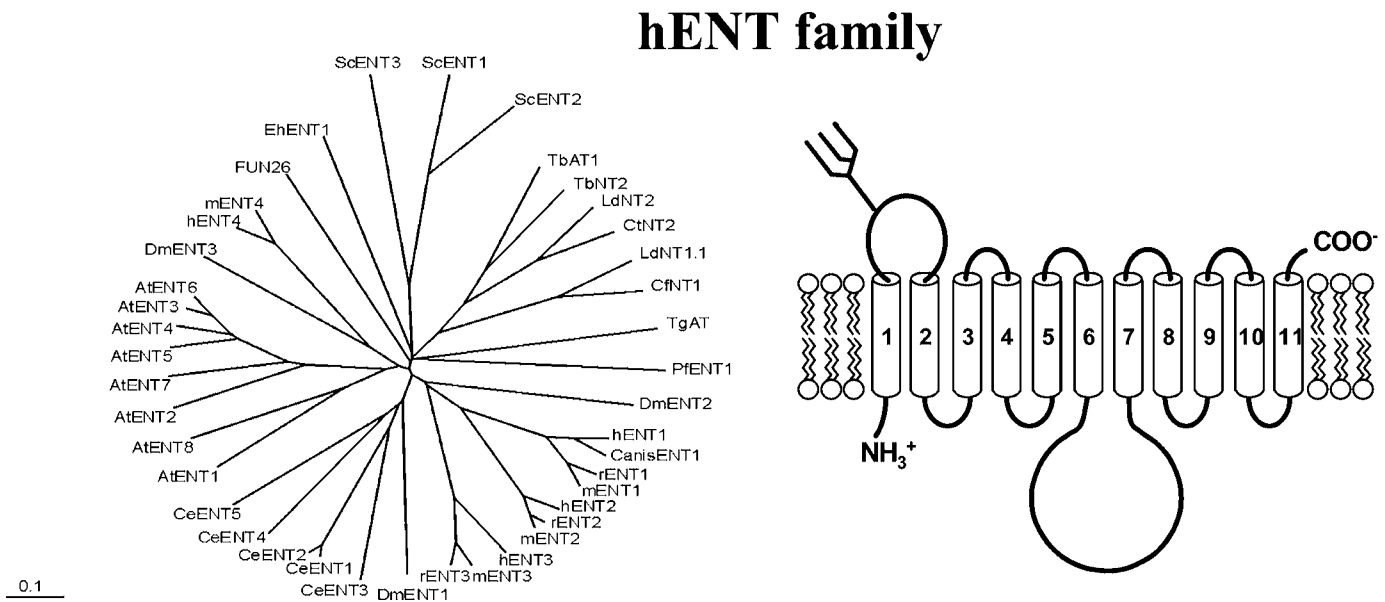


Fig. 4. The equilibrative nucleoside transporter (SLC29) family and the suggested topology for the mammalian orthologs. A phylogenetic tree of the ENT genes was built using *Tree View* software. Orthologs of the human ENTs are also found in many phyla, although, conversely to the CNT protein family, ENT genes are not found in prokaryotes. In some protozoa, ENT-type transporters have been shown to mediate the translocation of nucleosides in an H^+-coupled manner. The topology for the mammalian proteins anticipates 11 transmembrane domains, with the N-terminus tail localized in the cytosol and the C-terminus domain in the extracellular space.

CNT proteins might show different stoichiometry depending on the isoform, a feature that might be associated with their ability to concentrate nucleoside-derived drugs intracellularly and allow efficient phosphorylation. At least theoretically, coupling with either one or two sodium ions would dramatically modify the concentrative capacity of the transporter. Human CNT3 requires two Na^+ ions per translocated nucleoside *(10)*, whereas hCNT2 apparently needs only one *(4)*. The stoichiometry for hCNT1 is still controversial because data suggest that either two *(15)* or one *(16)* sodium ions are required for nucleoside translocation.

Although at least two other CNT-type transport activities have been kinetically identified in human cells (for review, *see* ref. *2*), there is no evidence from the human genome database that other CNT isoforms exist. Thus, either these activities are mediated by other nonrelated proteins or they may be the result of polymorphisms in the three known CNT proteins (*see* below).

Up to four members of the hENT family have been identified *(11)* (Table 1; Figs. 3 and 4). Human ENT proteins span 11 transmembrane domains, the human orthologs are 450 and 452 amino acid residues in length and show a 50% sequence identity between the isoforms hENT1 and hENT2. They mediate the facilitative diffusion (equilibrative) transport of naturally occurring nucleosides with broad selectivity but relatively low affinity when compared to the other family of NTs, CNT. Apparent K_m values for substrates, recognized by hENT1, range from 40 μ*M* for adenosine to 580 μ*M* for cytidine *(17)*.

Although selectivity for nucleosides is similar for hENT1 and hENT2, the latter shows noteworthy differences in substrate specificity, particularly for cytidine, which has an apparent K_m value that, when transported by ENT2, is 10-fold higher than for hENT1 (5610 vs 580 μ*M*) *(17)*. These differences in specificity also predict different roles in nucleoside-derived drug uptake. The hENT1 and hENT2 can be pharmacologically inhibited by NBTI (with 50% inhibitory concentration [IC_{50}] values of 0.4 n*M* and 2800 n*M*, respectively) and dipyridamol (with IC_{50} values of 5 and 356 n*M*, respectively). Thus, the NBTI-sensitive nucleoside transport activity that was characterized long ago, routinely "quantified" by measuring the number of high-affinity NBMPR/NBTI binding sites, is assumed to be associated with ENT1 function. As mentioned, hENT3 and hENT4 are the two most recently cloned NT proteins and have yet to be fully characterized. Thus, their substrate selectivity profile and subcellular localization remain to be determined.

2.3. The Pharmacological Profiles of CNT and ENT Transporters

CNT and ENT are strong candidates to mediate the translocation of most nucleoside derivatives used in anticancer therapies. Although preliminary evidence in favor of common entry pathways for naturally occurring

nucleosides and nucleoside-derived drugs was also obtained by kinetic experimental approaches prior to the cloning era, the ability to express a particular NT protein selectively in a null background has been particularly useful for the determination of pharmacological profiles. Moreover, because CNT proteins mediate nucleoside translocation coupled to Na^+ uptake, it was anticipated, and later demonstrated, that CNT function is indeed associated with Na^+ inward currents that can be measured using electrophysiological approaches. In particular, *Xenopus laevis* oocytes expressing human CNT isoforms can be impaled with electrodes, and using a two-electrode voltage clamp approach, selected nucleoside derivatives can be tested for their ability to generate Na^+ inward currents, which is itself a demonstration of transportability. This approach obviates the need for labeled substrates, which often represent a bottleneck in the elucidation of the drug selectivity of a particular CNT protein.

By combining substrate flux measurements, cross-inhibition studies, and electrophysiology, general profiles for CNTs and ENTs have been obtained. These are summarized in Table 2. Unfortunately, transportability cannot necessarily be inferred from cross-inhibition studies, as discussed above in the particular case of adenosine interaction with hCNT1: Adenosine binds to the transporter with high affinity (K_d 14 μM) but does not translocate *(15)*. Moreover, in many cases, transportability has only been assessed at a single substrate concentration, which does not generate a definitive view of specificity because no affinity constants can be derived from these studies. Some general properties of CNT and ENT pharmacology have been reviewed elsewhere *(18–20)*.

Substrate selectivity in the hCNT gene family is narrower than for hENTs. For instance, gemcitabine is a high-affinity substrate for hCNT1, but it is not recognized by hCNT2 *(21)* and appears to be less effectively taken up by the hCNT3 isoform *(10)*. In contrast, hCNT3 takes up fludarabine with high affinity; hCNT1 does not recognize it, and hCNT2, although inhibited by it, does not take it up. A similar specificity profile seems to apply to cladribine. In any case, it can be assumed that hCNT1 is a high-affinity transporter for fluoropyrimidines *(12,15,21–23)*.

In the case of the ENT protein family, it can be predicted that, as for naturally occurring nucleosides, hENT1 and hENT2 show broad substrate selectivity for nucleoside derivatives, even if apparent K_m values have not been measured for many drugs. Nevertheless, when available (as for gemcitabine), values are much higher than those reported for CNTs *(21)*, as one would expect on the basis of their specificity panel for naturally occurring nucleosides. Moreover, gemcitabine and other nucleoside analogs used in the treatment of lymphoid malignancies also appear to be better substrates for hENT1 than for hENT2 *(19,21,24)*.

Table 2
Pharmacological Properties of Concentrative and Equilibrative Nucleoside Transporters

Concentrative systems		*Equilibrative systems*	
	Drugs as substrates (apparent K_m)		*Drugs as substrates (apparent K_m)*
hCNT1	Gemcitabine + (17 μ*M*) 5′-DFUR (209 μ*M*) Cytarabine + Fludarabine – Cladribine –	hENT1	Gemcitabine + (160 μ*M*) Cytarabine + Fludarabine + Cladribine +
hCNT2	Cladribine – Fludarabine – Gemcitabine –	hENT2	Gemcitabine + (740 μ*M*) Fludarabine + Cladribine + Cytarabine[a] +
hCNT3	Cladribine + Fludarabine + Gemcitabine +		

The interaction of selected nucleoside-derived anticancer drugs with hCNT and hENT proteins has been studied either by testing uptake at a single concentration (+, transported, –, not transported) or by determining kinetics of interaction (apparent K_m values). In this particular case, these values are given. Data came from a variety of reports cited in the text. [a]In some cases, drugs have been tested as inhibitors. This is not by itself proof of transportability, as explained in the text. 5′-DFUR, 5′deoxy-5′fluorouridine.

Overall, available information on the pharmacological profiles of hCNT and hENT proteins reveal substrate selectivity and specificity that is sufficiently different to predict specific drug uptake capabilities in cells according to their expression pattern of NT proteins.

3. HOW ARE NUCLEOSIDES AND NUCLEOSIDE-DERIVED DRUGS RECOGNIZED BY CNT AND ENT PROTEINS?

The structures of CNT and ENT proteins are unknown; consequently, topology models are essentially based on bioinformatic approaches, with limited biochemical information as yet. Although the putative transmembrane domains and residues that might somehow determine substrate recognition and translocation have been identified by combining isoform chimeras and site-directed mutagenesis (for reviews, *see* refs. *11* and *25*), this chapter focuses on the molecular determinants that confer transport

suitability to substrates. This is of interest from a drug delivery and discovery perspective.

Besides the comprehensive analysis of transporter pharmacological profiles by standard biochemical means, as discussed above, the identification of substrate determinants will help to predict transportability on the basis of nucleoside structure.

3.1. Structural Determinants for the Molecular Recognition of NT Substrates

Expression of recombinant hENT1, hENT2, hCNT1, and hCNT3 in *Saccharomyces cerevisiae* has been used to identify binding motifs that enable these transporters to translocate uridine, a naturally occurring nucleoside shared as a substrate by all of them *(26,27)*. Interestingly, a key element for recognition is the sugar moiety, within which the C(3′)-OH appears to be crucial. This may explain why all NTs functionally characterized to date, with the exception of hENT2, are indeed nucleoside and not nucleobase transporters. As indicated above, hENT2 can transport some nucleobases, although the apparent K_m values are much higher than for nucleosides, which is consistent with the view that, at least for binding, the sugar moiety is important.

Human ENT1 and hENT2 form strong interactions with the hydroxyl group in the 3′ position of the sugar *(27)*, whereas substitutions or configurational inversion at this point are barely tolerated by hCNT1 and hCNT3 *(26)*. Nevertheless, these findings do not rule out the possibility of cooperation of other groups, in both the sugar and base moieties, of varying importance depending on the particular NT studied. These observations agree with an extensive bioinformatic analysis that took advantage of previously generated biological data (nucleoside uptake measurements) *(28)*. Moreover, this explains, for instance, why hCNT1, although considered a high-affinity pyrimidine-preferring NT protein, does not interact efficiently with the majority of antiviral nucleosides, such as AZT (zidovudine), d4T (stavudine), ddC (zalcitabine), and 3TC (lamivudine) *(29)*. Although all of these molecules are pyrimidine nucleoside derivatives, they all lack the 3′ hydroxyl group. Thus, these findings strongly support the view that very slight changes in substrate structure provoke a dramatic shift in selectivity; therefore, a nucleoside structure does not by itself imply recognition and efficient translocation by either CNT or ENT proteins.

3.2. Polymorphic NTs

The search for putative single nucleotide polymorphisms (SNPs) and other polymorphic variants in CNT and ENT proteins might be useful in pharmacogenetic terms. As discussed previously, site-directed mutagenesis

has allowed the identification of amino acid residues critical for function, but these occur on highly conserved residues that are unlikely to be mutated in humans. Indeed, until now, no inherited disease associated with a primary defect in NT function has been described. Nevertheless, natural genetic variability may be responsible for slight functional changes that, in principle, might determine drug bioavailability and action.

A comprehensive list of genetic variants of plasma membrane transporters in general, and NTs in particular, is available at www.pharmgkb.org. Although different types of apparent polymorphisms have been detected in a defined ethnically diverse population *(30)*, only a few have been functionally characterized to date. Human ENT1 genetic variants do not seem to affect function significantly *(31)*; thus, differences in hENT1 protein levels might be more important in determining variability in human populations. In contrast, some functionally relevant hCNT1 polymorphisms have been described *(32)*. Surprisingly, two variants, a nonconservative change (Ser546Pro) and a single nucleotide deletion (1153del), appear to be nonfunctional, whereas the Val189Ile polymorphic variant showed a twofold increase in the apparent affinity for gemcitabine when compared to the reference gene. Whether these alterations are really associated with different drug pharmacokinetics remains to be elucidated.

Genetic variants might also explain the occurrence of particular "transport systems" that were characterized kinetically prior to the "cloning era" and for which no specific gene has yet been identified. Interestingly, it has been shown that a single substitution in the hCNT1 gene results in a functional transporter protein with guanosine preference *(33)*, a feature that had been kinetically defined several years ago as a new transport system *(34)*, besides the three well-known systems, now associated with the more abundant variants of hCNT1, hCNT2, and hCNT3.

4. TISSUE DISTRIBUTION OF NT PROTEINS

The understanding of the role of NT proteins in drug pharmacokinetics and drug responsiveness might in fact depend on our knowledge of what we should call the basic pattern of NT expression in normal tissues. Human ENTs, particularly hENT1, may be considered ubiquitous transporters, with significant variability in tissue abundance, as determined previously by NBMPR/NBTI high-affinity binding site measurements (for review, *see* ref. *2*). The lack of appropriate ligands for CNT-type transporters did not allow an accurate estimation of their tissue distribution until they were cloned and suitable molecular tools generated.

However, the original view that CNT protein expression would be restricted to a few epithelia, such as those of the small intestine, kidney, and choroid plexus, has been challenged by the identification of CNT-related

functional activity in cell types showing high endogenous nucleotide biosynthesis, such as rat liver parenchymal cells, which in principle should not rely on salvage pathways *(35–37)*. The generation of the first antibodies against the rat orthologs of CNT1 (rCNT1) and CNT2 (rCNT2) revealed a much broader tissue distribution for both transporter proteins than expected *(38)*. Of the tissues assayed, rCNT1 protein immunoreactivity was detected at high levels in crude homogenates from liver and kidney and, to a lesser extent, in small intestine, heart, lung, brown adipose tissue, and pancreas. CNT2 protein distribution was more homogeneous than that found for CNT1. In addition to the tissues mentioned, CNT2 was also detected in brain and skeletal muscle.

CNTs are also expressed in cells of the immune system. CNT2-related activity has been detected in human B-cells *(39)*, and both transporter proteins are expressed in murine bone marrow macrophages *(40,41)*. The messenger RNA (mRNA) of the most recently cloned member of the SLC28 family, hCNT3, has also been identified in many tissues; it displays a high abundance in mammary gland, pancreas, and bone marrow but is also present, although to a lesser extent, in intestine, liver, lung, placenta, brain, and heart, among other tissues *(10)*.

Taken together, these observations support the view that most tissues and cell types are likely to coexpress more than one single NT protein. The physiological rationale for this coexpression of transporters that, in most cases, show significant overlapping selectivity is still unclear.

5. REGULATED VS CONSTITUTIVE NT EXPRESSION: A BASIS FOR DIFFERENTIAL EXPRESSION OF NTs IN TUMORS?

We have learned that NTs are coexpressed in a variety of cells and tissues, and that they show different substrate selectivity and specificity. Accordingly, their pharmacological profiles do not coincide. The question we want to address now is whether their expression is constitutive or, alternatively, can be regulated. To some extent, the evidence that NTs show different regulatory properties, as discussed here, suggests complementary physiological roles for these membrane proteins and may explain the need for coexpression, at least in highly differentiated cell types. However, from a clinical perspective, regulation of nucleoside transport activity in an isoform-specific manner may offer an opportunity to modulate drug bioavailability. Moreover, evidence of different transporter protein expression patterns in tumors might be important from a therapeutic standpoint, not only because of the variability in drug uptake capabilities, but also because the entry pathway might determine intracellular drug targeting.

Cell types showing high turnover but relying on salvage pathways for nucleoside supply, such as macrophages and enterocytes, coexpress CNTs and ENTs with apparently different physiological roles. In murine bone marrow macrophages, proliferation induced by macrophage colony-stimulating factor is associated with the upregulation of ENT1 activity and mRNA levels. Blocking ENT1 function with NBTI inhibits proliferation and significantly reduces the incorporation of extracellular thymidine into DNA without any effect on uridine incorporation into RNA, thus suggesting some sort of metabolic channeling of extracellular nucleosides *(41)*. Macrophage activation by interferon-γ or promotion of apoptosis by lipopolysaccharide and tumor necrosis factor-α (TNF-α) block proliferation induced by macrophage colony-stimulating factor and upregulation of ENT1, whereas CNT1 and CNT2 show a dramatic increase in expression and activity *(40–42)*. Similarly, in IEC-6 cells, a rat enterocyte cell model, mitogenic agents and epithelial wounding selectively increase ENT1 mRNA levels and activity; induction of differentiation by glucocorticoids upregulates expression of the CNT2 *(43)*. Taken together, these data support the view that ENT1 is a sort of "housekeeping" transporter responsible for constitutive nucleoside supply. In fact, as discussed below, ENT1 expression is often high and is highly retained in tumors. Nevertheless, an ENT1 knockout mouse has been generated, and it is viable *(44)*, thus demonstrating that ENT1 is not crucial for cell survival and suggesting possible redundancy with other ENT members.

Expression of CNT1 and CNT2 is highly dependent on differentiation in human and rat liver parenchymal cells *(45–48)*. Both transporter proteins are upregulated during liver regeneration *(37)*, but CNT1 expression is sensitive to multifunctional cytokines *(49)*, whereas CNT2 is transcriptionally activated by proapoptotic agents such as transforming growth factor-β1 (unpublished observations). CNT1 is highly sensitive to metabolic interference with the endogenous nucleotide pool because pharmacological inhibition of ribonucleotide reductase results in a dramatic increase in CNT1 protein levels *(50)*, whereas CNT2, a high-affinity adenosine transporter, appears instead to be involved in regulating purinergic responses *(51)*. Overall, these data suggest specific roles for CNT1 and CNT2, with the former responsible for fine tuning of nucleoside supply in salvage processes and the latter more likely to modulate adenosine-induced cell signaling.

Leukemia B-cell lines, which express transporters of both CNT and ENT types, respond similarly to the phorbol ester PMA, lipopolysaccharide, and TNF-α treatments by upregulating concentrative transport activity and downregulating equilibrative nucleoside uptake. This is associated with a decrease in hENT1 mRNA levels. However, the effect on CNT-type transporters is sensitive to protein kinase PKC inhibition, whereas the decrease in hENT1-related transport induced by TNF-α is not, suggesting that

different pathways are involved in the response of concentrative and equilibrative transporters to cytokine treatment *(39)*. PMA-induced differentiation of the promyelocytic cell line HL-60 causes downregulation of ENT-type transport systems and the emergence of a Na-dependent, CNT-type transport activity *(52)*. This is likely to be caused, at least partially, by upregulation of the hCNT3 gene *(10)*. The transcription factors involved in this type of regulatory responses are unknown.

Nevertheless, it has been shown that chronic lymphocytic leukemia (CLL) cells display constitutive Ser-727 signal transducer and activator of transcription (STAT) 1 phosphorylation *(53)*. In contrast, normal B-cells phosphorylate STAT1 at this serine residue after treatment with PMA *(53)*. Indeed, phorbol esters cause downregulation of ENT1 activity in B-cell lines *(39)*, a feature that is interesting because the downregulation of ENT1 activity triggered by interferon-γ in murine bone marrow macrophages is STAT1 dependent *(42)*.

Although regulation of NT activity and expression is still poorly understood and has only been studied for particular isoforms in selected cell types, it is already clear that NT expression is not constitutive. Even the ubiquitous, highly abundant, ENT1 protein seems to be regulated at both the transcriptional and posttranscriptional level. CNT expression is highly dependent on differentiation. In fact, we and others have noticed that it is very difficult to obtain good cell models in which to study their biological properties, particularly for CNT1, an isoform with tissue distribution that might be narrower than that of other transporters and with activity that is often lost even in cell types in which CNT1 mRNA is easily detected.

Finally, there is another issue related to NT regulation that might be very important from a clinical perspective: whether nucleoside-derived drugs can by themselves modulate NT activity or expression. As indicated above, inhibition of ribonucleotide reductase by hydroxyurea promotes a dramatic increase in CNT1 protein levels in rat hepatoma cells *(50)*. Thus, given that many nucleoside-derived drugs, such as fluoropyrimidines, strongly interfere with endogenous nucleotide metabolism, it is not surprising that similar responses occur in drug-treated cells. Little is known about this particular aspect of NT regulation, which is briefly summarized below.

6. ROLE OF NTs IN CELL SENSITIVITY TO ANTICANCER DRUGS: FROM DIAGNOSIS TO INDIVIDUALIZED THERAPY?

As discussed above, the molecular cloning of the cDNA encoding membrane proteins, responsible for nucleoside-derived drug uptake into cells, is relatively recent. Thus, the role of nucleoside carriers in controlling drug bioavailability, thereby determining cell sensitivity to anticancer drugs,

has not been appropriately addressed, essentially because of a lack of suitable molecular tools. In particular, no commercial antibodies are currently available, although a few laboratories, including our own, have been able to raise antibodies suitable for immunohistochemical analysis on either frozen or paraffin-embedded tissues.

The putative role of NT function in drug uptake, bioavailability, and cytotoxicity was initially addressed in cell culture models. Some clinical data were also available prior to NT cloning thanks to the ability of the hENT1 protein to be crosslinked by specific inhibitors, such as NBMPR and its derivatives. During the past 2 yr, the first immunohistochemical data have become available. Although there are still very few published studies, we anticipate forthcoming reports in this area that will help to include NT proteins in the complex framework of enzymatic machinery responsible for nucleoside and nucleotide metabolic activation that determines drug action and tumor responsiveness to treatment.

6.1. Analysis of the Role of NTs in Sensitivity to Nucleoside-Derived Anticancer Drugs in Cultured Cell Models

A significant number of studies performed in cell lines have demonstrated that transport processes may be important for drug-induced cytotoxicity and resistance. This idea was already put forward nearly 40 yr ago when analyzing the differential response to 5-fluorouracil (5-FU) of a panel of hepatoma cells showing variable sensitivity to fluoropyrimidines *(54)*. Although cells lacking selected transport functions are resistant to nucleoside-derived analogs *(55–57)*, the pharmacological blockade of ENT-type transport activities might increase sensitivity to nucleoside-derived drugs, presumably by inhibiting efflux pathways *(58–62)*. This apparent discrepancy can be explained by the design of these experiments. Sensitivity is indeed promoted when drugs reach cells prior to the inhibition of transporter function, whereas treatment of cells after exposure to NBTI results in resistance *(63)*. Consistent with this, NBTI is also able to block the acadesine-induced apoptosis of CLL cells, demonstrating that the uptake of acadesine via NBTI-sensitive transporters is required for its apoptotic effect *(64)*.

The role of selected NTs in drug-induced cytotoxicity has been addressed by determining the extent to which heterologous expression of a particular NT protein modifies cell sensitivity to drugs. Thus, hCNT1, when expressed in Chinese hamster ovary cells, induces an increase in cell sensitivity to the cytotoxic action of 5′-deoxy-5′-fluorouridine (5-DFUR), an intermediate of capecitabine metabolism and the immediate precursor of 5-FU *(23)*. In fact, unlike either capecitabine or 5-FU, 5-DFUR is an hCNT1 substrate *(23)*. This sensitization of cells triggered by hCNT1 expression is still retained when endogenous ENTs are blocked using

dipyridamole, thus suggesting that the retention of high-affinity, concentrative nucleoside transport activity might by itself be a determinant of cytotoxicity *(23)*. Similarly, acquisition of hCNT2 function by gene transfer into a T-cell drug-resistant cell line, devoid of nucleoside transport activity, results in increased sensitivity to a variety of halogenated uridine analogs *(65)*. Moreover, slight increases in hCNT1-related function in cells derived from pancreatic adenocarcinomas also induce higher sensitivity to gemcitabine than in their parental cell lines *(66)*.

An important issue in this type of study is the evidence that transporter function, and thereby protein amounts, might be better correlated with cytotoxicity than mRNA levels. The quantification of mRNA for NTs, by real-time reverse transcriptase polymerase chain reaction, in a panel of 50 cell lines did not reveal significant correlations with sensitivity in a variety of anticancer drugs *(67)*. This lack of correspondence between mRNA levels and sensitivity to nucleoside-derived drugs has subsequently been demonstrated to occur in patients with CLL (*see* below), highlighting the need for functional assays, suitable antibodies to NTs, or both.

One additional issue that might help to understand the relationship between transporter function and cell responsiveness to drugs is the possibility that treatment by itself induces changes in transporter protein expression and activity. As explained above, this can be predicted from the recent finding that inhibition of ribonucleotide reductase upregulates CNT1 protein in rat FAO hepatoma cells *(50)*. This kind of interaction was anticipated in the case of hENT1-type transporters when no molecular tools were available, and occurrence at the plasma membrane was indirectly measured by determining high-affinity NBTI-binding sites. When thymidylate synthase activity is blocked in cells, both the number of NBTI-binding sites and the thymidine kinase activity are dramatically upregulated, probably via a common mechanism sensitive to the depletion of deoxythymidine 5′-triphosphate pools *(68,69)*.

Nucleoside-derived drugs can also modulate transporter function by affecting selected signaling pathways. Fludarabine has been shown to deplete STAT1 specifically in normal resting B-cells and in a fludarabine-treated patient with CLL *(70)*, whereas, at least in macrophages, ENT1 activity seems to be posttranscriptionally regulated via STAT1-dependent mechanisms *(42)*. Surprisingly, it has also been reported that an increasing number of protein kinase inhibitors, including rapamycin, inhibitors of cyclin-dependent kinases, mitogen-activated protein (MAP) kinase inhibitors, and others, can interact and even block hENT1-related transport activity *(71)*. This unexpected finding suggests that nucleoside derivatives and protein kinase inhibitors may antagonize each other in some combined therapies.

In summary, evidence obtained from in vitro studies (cultured cell models) clearly suggests that NTs contribute to nucleoside-derived drug

cytotoxicity. In turn, NT function and expression can also be affected by drug treatment itself as well as by other agents used in combined therapies. Nevertheless, key issues are to determine whether these findings can be transferred to clinical settings and whether they will help to understand what roles NT proteins actually play in tumor responsiveness to nucleoside-based therapies.

6.2. Studies Linking NT Function to Drug Sensitivity and Clinical Outcome in Cancer Patients

An intermediate step between cell line models and patient NT profiling is the analysis of the possible correlations among NT mRNA, protein, biological function, and ex vivo cell sensitivity to nucleoside-derived drugs. For obvious reasons, this approach has been possible in lymphoproliferative malignancies, although it is not feasible when dealing with solid tumors, in which functional analysis is difficult, if not impossible to perform, and antibodies are required for a suitable approach to the analysis of NT protein expression.

Human ENT1-type transporter abundance, as measured by high-affinity binding of the fluorescent nucleoside derivative 5′-S-(2-aminoethyl)-N6-(4-nitrobenzyl)-5′-thioadenosine (SAENTA), has been correlated with cytotoxicity of cytarabine (ara-C) in blasts from patients with acute leukemia *(72,73)*. Moreover, a significant correlation between hENT1 mRNA levels and ara-C-induced cytotoxicity has been reported in cells from childhood ALL patients *(74)*. Nevertheless, as discussed above, this type of correlation might not be very common. Indeed, when analyzing the whole panel of NT genes in cells from CLL patients, the mRNAs for hENT1, hENT2, hCNT2, and hCNT3 were easily amplified, whereas no hCNT1 expression was detected *(75)*. No statistical correlations were found between mRNA levels and fludarabine transport or ex vivo cytotoxicity, a finding confirmed by others *(76)*. Interestingly, ex vivo sensitivity to fludarabine showed a strong correlation with fludarabine accumulation *(75)*, which was exclusively mediated by ENT-type transporters *(75,76)*. The hENT2 protein amounts were shown to correlate with fludarabine ex vivo cytotoxicity in these patients *(77)*.

The combined measurement of mRNA levels for hENT1, hCNT3, and a panel of enzymes involved in nucleotide metabolism has allowed patients with CLL to be clustered into two groups with significant differences in the risk of disease progression *(76)*. Surprisingly, this clinical parameter showed statistical correlation with hCNT3 overexpression, although, in agreement with a previous report, no hCNT3-related nucleoside transport was detected *(75)*. Indeed, all hCNT3 protein was located intracellularly *(76)*. In acute

myeloid leukemia, low hENT1 expression has also been associated with decreased disease-free survival *(78)*.

NT profiling in solid tumors is more complex than in lymphoproliferative diseases. The closest approach to NT function here is protein analysis, either by Western blot or immunohistochemistry. This is obviously challenged by the fact that these approaches are only semiquantitative and, most important, by a lack, until recently, of suitable molecular tools (isoform specific anti-NT antibodies).

The first immunohistochemical evidence of selective loss of NT proteins in tumors was obtained in rat models of hepatocarcinogenesis using anti-rCNT1 and anti-rCNT2 polyclonal antibodies that, unfortunately, did not crossreact with the human CNT orthologs *(48)*. Evidence for variable NT expression in solid tumors was later obtained at the mRNA level using commercial tumor RNA arrays *(79)*. Nevertheless, information about NT proteins in human tumors is scarce. hENT1 protein has been analyzed on frozen sections from breast tumors, demonstrating heterogeneity of hENT1 staining in a cohort of 33 patients, 4 of them apparently lacking hENT1 protein expression *(80)*. Immunhistochemical detection of hENT1 protein has been achieved in paraffin-embedded sections of breast tumors *(81)*, and variability in hENT1 protein has also been reported in Reed-Sternberg cells from patients with Hodgkin's disease *(82)*.

A panel of antibodies against hENT1, hENT2, and hCNT1, suitable for immunohistochemistry analysis on paraffin-embedded tissues, has allowed high-throughput analysis of NT protein expression using a tissue array approach *(83)*. Nearly 300 independent gynecological tumors (endometrium, ovary, and cervix) were analyzed. As anticipated from what we know about NT biology, both hENT1 and hENT2 protein expression was highly retained, although a significant number of tumors were hCNT1 negative. Human CNT1 was not detected in 33% and 39% of the ovarian and uterine cervix carcinomas, respectively, whereas only 15% of endometrial carcinomas were hCNT1 negative. In ovarian cancer, the loss of all three transporters was a more common event in the clear cell histological subtype than in the serous, mucinous, and endometrioid histotypes. In cervical tumors, the loss of hCNT1 expression was significantly associated with the adenocarcinoma subtype. Overall, these data suggest that NT expression is variable and is related to the type of gynecological tumor and its specific subtype, with hCNT1 protein loss highly correlated with poor prognosis histotypes *(83)*.

Unfortunately, studies comparing NT protein profiles with clinical parameters relevant to outcome and survival are still scarce. Spratlin et al. *(84)* reported that the absence of hENT1 protein, as determined by immunohistochemistry, is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. Moreover, an unpublished

study of 90 patients with breast cancer *(85)* has shown that the percentage of hCNT1-positive cells correlates positively with reduced long-term survival, with the hCNT1-positive index indicative of poor prognosis. A relative risk of relapse was also associated with high hCNT1-positive indexes. All these patients underwent surgery followed by cyclophosphamide/methotrexate/5-fluorouracil (CMF) chemotherapy, during which they received 5-FU rather than nucleoside-based therapy. Thus, it is tempting to speculate that high expression of hCNT1 is linked to high nucleotide salvage efficiency and consequently might compromise nucleotide metabolism interference triggered by 5-FU.

7. FUTURE PERSPECTIVES

NT proteins are definitely the preferred entry pathways for most nucleoside- based anticancer drugs. Nevertheless, complete pharmacological profiles and molecular determinants of transportability are not yet fully available. Little is known either about the regulatory and physiological properties of these proteins that might be key determinants of drug action. Thus, a major effort should now be put into these particular aspects of NT biology. However, in the meantime, some tools are already available for high-throughput analysis of NT expression in tumors. Unfortunately, as discussed above, there is still little published information regarding the relationships between NT profiles and clinical outcome, but previous evidence supports the view that these membrane proteins might play a significant role in nucleoside-based chemotherapy.

An additional effort should be made toward generating a coordinate view of how transporters, enzymes, and intracellular targets mutually interact to define chemotherapeutic responses. In this context, although it is unlikely that transport processes will become unique biomarkers of nucleotide metabolism, an integrated view might help to identify a discrete number of proteins that, when considered together, prove useful as predictors of therapeutic response.

ACKNOWLEDGMENTS

We thank past and present members of the Regulation of Transport Systems (RST) research laboratory at the University of Barcelona. Special gratitude is also acknowledged to colleagues outside our institution who have made possible fruitful collaborations, particularly in clinical settings. Research at the RST laboratory has been funded by Ministerio de Ciencia y Tecnología, Ministerio de Sanidad y Consumo, Universitat de Barcelona, Generalitat de Catalunya, Fundación Ramón Areces, Fundación La Caixa, and FIPSE. We would also like to thank

Míriam Molina-Arcas and Albert Català-Temprano for critical reading of this chapter. The valuable help of Pedro Cano-Soldado and Sergi Purcet in the design of the figures is also acknowledged. Robin Rycroft helped us in editing this contribution.

REFERENCES

1. Belt JA, Marina NM, Phelps D, Crawford CR. Nucleoside transport in normal and neoplastic cells. Adv Enzyme Regul 1993;33:235–252.
2. Griffith DA, Jarvis SM. Nucleoside and nucleobase transport systems of mammalian cells. Biochim Biophys Acta 1996;1286:153–181.
3. Huang QQ, Yao SY, Ritzel MW, Pateron AR, Cass CE, Young JD. Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transport protein. J Biol Chem 1994;269:17,757–17,760.
4. Che M, Ortiz DF, Arias IM. Primary structure and functional expression of a cDNA encoding the bile canalicular purine-specific Na^+-nucleoside cotransporter. J Biol Chem 1995;270:13,596–13,599.
5. Ritzel MW, Yao SY, Huang MY, Elliott JF, Cass CE, Young JD. Molecular cloning and functional expression of cDNAs encoding a human Na^+-nucleoside cotransporter (hCNT1). Am J Physiol 1997;272:C707–C714.
6. Wang J, Su SF, Dresser MJ, Schaner ME, Washington CB, Giacomini KM. Na^+-dependent purine nucleoside transporter from human kidney: cloning and functional characterization. Am J Physiol 1997;273:F1058–F1065.
7. Griffits M, Beaumont N, Yao SY, et al. Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherpeutic drugs. Nat Med 1997;3:89–93.
8. Griffits M, Yao SY, Adibi F, et al. Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (*ei*) equilibrative nucleoside transporter from human placenta. Biochem J 1997;328:739–743.
9. Crawford CR, Patel DH, Naeve C, Belt JA. Cloning of the human equilibrative, nitrobenzylmercaptopurine riboside (NBMPR)-insensitive nucleoside transporter *ei* by functional expression in a transport-deficient cell line. J Biol Chem 1998; 273:5288–5293.
10. Ritzel MW, Ng AM, Yao SY, et al. Molecular identification and characterization of novel human and mouse concentrative Na^+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). J Biol Chem 2001;276:2914–2927.
11. Baldwin SA, Beal PR, Yao SYM, King AE, Cass CE, Young JD. The equilibrative nucleoside transporter family, SLC29. Plugers Arch Eur J Physiol 2004; 447:735–743.
12. Lostao MP, Mata JF, Larráyoz IM, Inzillo SM, Casado FJ, Pastor-Anglada M. Electrogenic uptake of nucleosides and nucleoside-derived drugs by the human nucleoside transporter 1 (hCNT1) expressed in *Xenopus laevis* oocytes. FEBS Lett 2000;481:137–140.
13. Chandrasena G, Giltay R, Patil SD, Bakken A, Unadkat JD. Functional expression of human intestinal Na^+-dependent and Na^+-independent nucleoside transporters in *Xenopus laevis* oocytes. Biochem Pharmacol 1997;53: 1909–1918.

14. Ritzel MW, Yao SY, Ng AM, Mackey JR, Cass CE, Young JD. Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na^+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. Mol Membr Biol 1998;15:203–211.
15. Larráyoz I, Casado FJ, Pastor-Anglada M, Lostao P. Electrophysiological characterization of the human Na^+/nucleoside cotransporter 1 (hCNT1) and role of adenosine on hCNT1 function. J Biol Chem 2004;8999–9007.
16. Smith KM, Ng AM, Yao SY, et al. Electrophysiological characterization of a recombinant human nucleoside transporter (hCNT1) produced in *Xenopus* oocytes. J Physiol 2004;558:807–823.
17. Ward JL, Sherali A, Mo ZP, Tse CM. Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. ENT2 exhibits a low affinity for guanosine and cytidine, but a high affinity for inosine. J Biol Chem 2000;275:8375–8381.
18. Damaraju VL, Damaraju S, Young JD, et al. Nucleoside anticancer drugs: the role of nucleoside transporters in resistance to cancer chemotherapy. Oncogene 2003; 22:7524–7536.
19. Pastor-Anglada M, Molina-Arcas M, Casado FJ, Bellosillo B, Colomer D, Gil J. Nucleoside transporters in chronic lymphocytic leukaemia. Leukemia 2004;18: 385–393.
20. Kong W, Engel K, Wang J. Mammalian nucleoside transporters. Current Drug Metab 2004;5:63–84.
21. Mackey JR, Yao SY, Smit KM, et al. Gemcitabine transport in *Xenopus* oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. J Natl Cancer Inst 1999;91:1876–1881.
22. Dresser MJ, Gerstin KM, Gray AT, Loo DD, Giacomini KM. Electrophysiological analysis of the substrate selectivity of a sodium-coupled nucleoside transporter (rCNT1) expressed in *Xenopus laevis* oocytes. Drub Metab Dispos 2000;28: 1135–1140.
23. Mata JF, García-Manteiga JM, Lostao MP, et al. Role of the human concentrative nucleoside transporter (hCNT1) in the cytotoxic action of 5′-deoxy-5-fluorouridine, an active intermediate metabolite of capecitabine, a novel oral anticancer drug. Mol Pharmacol 2001;59:1542–1548.
24. Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues and nucleobases in cancer treatment. Lancet Oncol 2002;3:415–424.
25. Gray JH, Owen RP, Giacomini KM. The concentrative nucleoside transporter family, SLC28. Pflugers Arch Eur J Physiol 2004;447:728–734.
26. Zhang J, Visser F, Vickers MF, et al. Uridine binding motifs of human concentrative nucleoside transporters 1 and 3 produced in *Saccharomyces cerevisiae*. Mol Pharmacol 2004;64:1512–1520.
27. Vickers MF, Zhang J, Visser F, et al. Uridine recognition motifs of human equilibrative nucleoside transporters 1 and 2 produced in *Saccharomyces cerevisiae*. Nucleosides Nucleotides Nucleic Acids 2004;23:361–373.
28. Chang C, Swaan PW, Ngo LY, Lum PY, Patil SD, Unadkat JD. Molecular requirements of the human nucleoside transporter hCNT1, hCNT2, and hENT1. Mol Pharmacol 2004;65:558–570.
29. Cano-Soldado P, Larráyoz IM, Molina-Arcas M, et al. Interaction of nucleoside inhibitors of HIV reverse transcriptase (NRT) with the concentrative nucleoside transporter 1 (hCNT1). Antivir Ther 2005;9:993–1002.

30. Leabman MK, Huang CC, DeYoung J, et al. Natural variation in human membrane transporter genes reveals evolutionary and functional constraints. Proc Natl Acad Sci USA 2003;100:5896–5901.
31. Osato DH, Huang CC, Kawamoto M, et al. Functional characterization in yeast of genetic variants in the equilibrative nucleoside transporter, ENT1. Pharmacogenetics 2003;13:297–301.
32. Gray JH, Mangravite LM, Owen RP, et al. Functional and genetic diversity in the concentrative nucleoside transporter, CNT1, in human populations. Mol Pharmacol 2004;65:512–519.
33. Lai Y, Lee EW, Ton CC, Vijay S, Zhang H, Unadkat JD. Conserved residues F316 and G476 in the concentrative nucleoside transporter 1 (hCNT1) impact guanosine sensitivity and membrane expression, respectively. Am J Physiol Cell Physiol 2005; 288:C39–C45.
34. Gutierrez MM, Giacomini KM. Substrate selectivity, potential sensitivity and stoichiometry of nucleoside transport in brush border membrane vesicles from human kidney. Biochim Biophys Acta 1993;1149:202–208.
35. Ruiz-Montasell B, Felipe A, Casado FJ, Pastor-Anglada M. Uridine transport in basolateral plasma membrane vesicles from rat liver. J Membr Biol 1992; 128: 227–233.
36. Mercader J, Gómez-Angelats M, del Santo B, Casado FJ, Felipe A, Pastor-Anglada M. Nucleoside uptake in rat liver parenchymal cells. Biochem J 1996; 317:835–842.
37. Felipe A, Valdés R, del Santo B, Lloberas J, Casado FJ, Pastor-Anglada M. Na-dependent nucleoside transport in liver. Two different isoforms from the same gene family are expressed in liver parenchymal cells. Biochem J 1998;330: 997–1001.
38. Valdés R, Ortega MA, Casado FJ, et al. Nutritional regulation of nucleoside transporter expression in rat small intestine. Gastroenterology 2000;119:1623–1630.
39. Soler C, Felipe A, Mata JF, Casado FJ, Celada A, Pastor-Anglada M. Regulation of nucleoside transport by lipopolysaccharide, phorbol esters, and tumor necrosis factor-α in human B-lymphocytes. J Biol Chem 1998;273:26,939–26,945.
40. Soler C, Valdés R, García-Manteiga J, et al. Lipopolysaccharide-induced apoptosis of macrophages determines the up-regulation of concentrative nucleoside transporters CNT1 and CNT2 through tumor necrosis factor α-dependent and –independent mechanisms. J Biol Chem 2001;276:30,043–30,049.
41. Soler C, García-Manteiga J, Valdes R, et al. Macrophages require different nucleoside transport systems for proliferation and activation. FASEB J 2001;15:1979–1988.
42. Soler C, Felipe A, García-Manteiga J, et al. Interferon-γ regulates nucleoside transport systems in macrophages through signal transduction and activator of transduction factor (STAT1)-dependent and -independent signaling pathways. Biochem J 2003;375:777–783.
43. Aymerich I, Pastor-Anglada M, Casado FJ. Long-term endocrine regulation of nucleoside transporters in rat intestinal epithelial cells. J Gen Physiol 2004;124: 505–512.
44. Choi DS, Cascini MG, Mailliard W, et al. The type I equilibrative nucleoside transporter regulates ethanol intoxication and preference. Nat Neurosci 2004;7:855–861.
45. del Santo B, Valdés R, Mata J, Felipe A, Casado FJ, Pastor-Anglada M. Differential expression and regulation of nucleoside transport systems in rat liver parenchymal and hepatoma cells. Hepatology 1998;28:1504–1511.

46. Pastor-Anglada M, Felipe A, Casado FJ, del Santo B, Mata JF, Valdés R. Nucleoside transporters and liver cell growth. Biochem Cell Biol 1998;76: 771–777.
47. Pastor-Anglada M, Casado FJ, Valdés R, Mata J, García-Manteiga J, Molina M. Complex regulation of nucleoside transporter expression in epithelial and immune system cells. Mol Membr Biol 2001;18:81–85.
48. Dragan Y, Valdes R, Gómez-Angelats M, et al. Selective loss of nucleoside carrier expression in rat hepatocarcinomas. Hepatology 2000;32:239–246.
49. Fernández-Veledo S, Valdés R, Wallenius V, Casado FJ, Pastor-Anglada M. Up-regulation of the high affinity pyrimidine-preferring nucleoside transporer CNT1 by tumor necrosis factor alpha and interleukin-6 in rat liver parenchymal cells. J Hepatol 2004;41:538–544.
50. Valdes R, Casado FJ, Pastor-Anglada M. Cell cycle-dependent regulation of CNT1, a concentrative nucleoside transporter involved in the uptake of cell cycle-dependent nucleoside-derived anticancer drugs. Biochem Biophys Res Commun 2002;296:575–579.
51. Duflot S, Riera B, Fernández-Veledo S, et al. ATP-sensitive K^+ channels regulate the concentrative adenosine transporter CNT2 following activation by A(1) adenosine receptors. Mol Cell Biol 2004;24:2710–2719.
52. Lee CW, Sokoloski JA, Sartorelli AC, Handschumacher RE. Differentiation of HL60 cells by dimethylsulfoxide activates a Na(+)-dependent nucleoside transport system. In Vivo 1994;8:795–801.
53. Frank DA, Mahajan S, Ritz J. B lymphocytes from patients with chronic lymphocytid leukemia contain signal transducer and activator of transcription (STAT) 1 and STAT3 constitutively phosphorylated on serine residues. J Clin Invest 1997;100:3140–3148.
54. Greenberg N, Schumm DE, Webb TE. Uridine kinase activities and pyrimidine nucleoside phosphorylation in fluoropyrimidine sensitive and resistant cell lines of the Novikoff hepatoma. Biochem J 1977;164:379–387.
55. Sobrero AF, Moir RD, Bertino JR, Handschumacher RE. Defective facilitated diffusion of nucleosides, a primary mechanism of resistance to 5-fluoro-2′-deoxyuridine in the HCT-8 human carcinoma line. Cancer Res 1985;45:3155–3160.
56. White JC, Rathmell JP, Capizzi RL. Membrane transport influences de rate of accumulation of cytosine arabinoside in human leukemia cells. J Clin Invest 1987;79:380–387.
57. Mackey JR, Mani RS, Selner M, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 1998;58:4349–4357.
58. Chan TC. Augmentation of 1-β-D-arabinofuranosylcytosine cytotoxicity in human tumor cells by inhibiting drug efflux. Cancer Res 1989;2656–2660.
59. Crawford CR, Ng CY, Noel LD, Belt JA. Nucleoside transport in L1210 murine leukemia cells. Evidence for three transporters. J Biol Chem 265:9732–9736.
60. Crawford CR, Ng CY, Belt JA. Isolation and characterization of an L1210 cell line retaining the sodium-dependent carrier cif as its sole nucleoside transport activity. J Biol Chem 1990;265:13,730–13,734.
61. Alessi-Severini S, Gati WP, Paterson AR. Intracellular pharmacokinetis of 2-chlorodeoxyadenosine in leukemia cells from patients with chronic lymphocytic leukemia. Leukemia 1995;9:1674–1679.
62. Wright AM, Gati WP, Paterson AR. Enhancement of retention and cytotoxicity of 2-chlorodeoxyadenosine in cultured human leukemic lymphoblasts by

nitrobenzylthioinosine, an inhibitor of equilibrative nucleoside transport. Leukemia 2000;14:52–60.
63. Cass CE, King KM, Montano JT, Janowska-Wieczorek A. A comparison of the abilities of nitrobenzylthioinosine, dilazep, and dipyridamole to protect human hematopoietic cells from 7-deazaadenosine (tubercidin). Cancer Res 1992;52: 5879–5886.
64. Campas C, Lopez JM, Santidrian AF, et al. Acadesina activates AMPK and induces apoptosis in B-cell chronic lymphocytic leukemia cells but not in T lymphocytes. Blood 2003;101:3674–3680.
65. Lang TT, Selner M, Young JD, Cass CE. Acquisition of human concentrative nucleoside transporter 2 (hCNT2) activity by gene transfer confers sensitivity to fluoropyrimidine nucleosides in drug-resistant leukemia cells. Mol Pharmacol 2001;60:1143–1152.
66. García-Manteiga J, Molina-Arcas M, Casado FJ, Mazo A, Pastor-Anglada M. Nucleoside transporter profiles in human pancreatic cancer cells. Role of hCNT1 in 2′,2′-difluorodeoxycytidine (gemcitabine) induced cytotoxicity. Clin Cancer Res 2003;9:5000–5008.
67. Lu X, Gong S, Monks A, Zaharevitz D, Moscow JA. Correlation of nucleoside and nucleobase transporter gene expression with antimetabolite drug cytotoxicity. J Exp Ther Oncol 2002;2:200–212.
68. Pressacco J, Wiley JS, Jamieson GP, Erlichman C, Hedley DW. Modulation of the equilibrative nucleoside transporter by inhibitors of DNA synthesis. Br J Cancer 1995;72:939–942.
69. Pressacco J, Mitrovski B, Erlichman C, Hedley DW. Effect of thymidylate synthase inhibition on thymidine kinase activity and nucleoside transporter expression. Cancer Res 1995;55:1505–1508.
70. Frank DA, Mahajan S, Ritz J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. Nat Med 1999;5:444–447.
71. Huang M, Wang Y, Cogut SB, Mitchell BS, Graves LM. Inhibition of nucleoside transport by protein kinase inhibitors. J Pharmacol Exp Ther 2003;304: 753–760.
72. Gati WP, Paterson AR, Belch AR, et al. Es nucleoside transporter content of acute leukemia cells: role in cell sensitivity to cytarabine (araC). Leuk Lymphoma 1998;32:45–54.
73. Wright AM, Paterson AR, Sowa B, Akabutu JJ, Grundy PE, Gati WP. Cytotoxicity of 2-chlorodeoxyadenosine and arabinosylcytosine in leukaemic lymphoblasts from paediatric patients: significance of cellular nucleoside transporter content. Br J Haematol 166:528–537.
74. Stam RW, den Boer ML, Meijerink JP, et al. Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood 2003;101:1270–1276.
75. Molina-Arcas M, Bellosillo B, Casado FJ, et al. Fludarabine uptake mechanisms in B-cell chronic lymphocytic leukemia. Blood 2003;101:2328–2334.
76. Mackey JR, Galmarini CM, Graham KA, et al. Quantitative analysis of nucleoside transporter and metabolism gene expression in chronic lymphocytic leukemia (CLL)-Identification of fludarabine-sensitive and insensitive populations. Blood 2005;105:767–774.
77. Molina-Arcas M, Marcé S, Villamor N, et al. Equilibrative nucleoside transporter-2 (hENT2) protein correlates with ex vivo sensitivity to fludarabine in chronic lymphocytic leukemia (CLL)-cells. Leukemia 2005;19:64–68.

78. Galmarini CM, Thomas X, Calvo F, et al. In vivo mechanisms of resistance to cytarabine in acute myeloid leukemia. Br J Haematol 2002;117:860–868.
79. Pennycooke M, Chaudary N, Shuralyova I, Zhang Y, Coe IR. Differential expression of human nucleoside transporters in normal and tumor tissue. Biochem Biophys Res Commun 2001;280:951–959.
80. Mackey JR, Jennings LL, Clarke ML, et al. Immunohistochemical variation of human equilibrative nucleoside transporter 1 protein in primary breast cancers. Clin Cancer Res 2002;8:110–116.
81. Dabbagh L, Coupland RW, Cass CE, Mackey JR. Immunohistochemical variation of human equilibrative nucleoside transporter 1 protein in primary breast cancers. Clin Cancer Res 2003;9:3213, 3214.
82. Reiman T, Clarke ML, Dabbagh L, et al. Differential expression of human equilibrative nucleoside transporter 1 (hENT1) protein in the Reed-Sternberg cells of Hodgkin's disease. Leuk Lymphoma 2002;1435–1440.
83. Farré X, Guillén-Gómez E, Sánchez L, et al. Expression of the nucleoside-derived drug transporters hCNT1, hENT1 and hENT2 in gynecologic tumors. Int J Cancer 2004;112:959–966.
84. Spratlin J, Sangha R, Glubrecht D, et al. The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. Clin Cancer Res 2004;10:6956–6961.
85. Gloeckner-Hofmann K, Guillén-Gómez E, Schmidtgen C, et al. Expression of the high-affinity fluoropyrimidine-preferring nucleoside tansporter (hCNT1) correlates with decreased disease-free survival in breast cancer. Submitted.

2 The Role of Deoxycytidine Kinase in DNA Synthesis and Nucleoside Analog Activation

Maria Staub, MD, PhD, ScD
and Staffan Eriksson, MD, PhD

CONTENTS

SUMMARY

Deoxycytidine kinase (dCK) is the main enzyme in the salvage of deoxyribonucleosides as a consequence of its broad substrate specificity. dCK is the only enzyme that can supply cells with all four precursors of DNA; is capable of 5′-phosphorylation of the natural substrates deoxycytidine (dCyt), deoxyadenosine, and deoxyguanosine; and can be interconverted into

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

thymine nucleotides. The deoxycytidine triphosphate (dCTP), in addition to DNA, can be utilized for special processes, such as for synthesis of "liponucleotides," which are precursors of membrane phospholipids. The expression of dCK is highest in lymphoid cells/tissues (e.g., such as thymus, spleen, lymph nodes, stimulated peripheral blood mononuclear and bone marrow cells) and in all malignancies of these cells. The cell cycle dependence of the expression of dCK has been a matter of discussion; even higher dCK activity and dCyt metabolism were found in undifferentiated rather than in differentiated human lymphocytes. An enhancement of dCK activity occurred on preincubation of cells with a variety of nucleoside derivatives and nonnucleoside genotoxic agents, such as aphidicolin, etoposide (VP16), taxol, and even the G protein modulator sodium fluoride. γ-Irradiation and ultraviolet (UV) C irradiation also augmented dCK activity in different cells. The decrease of dCK activity was observed with protein phosphatase inhibitors, suggesting a regulatory role for reversible protein phosphorylation in the activation process. Cytosolic Ca^{2+} ion and p53 protein are necessary for the increase of dCK activity in cells after toxic treatments. The reason for the increase of dCK activity after toxic treatment of cells seems to be a compensatory mechanism induced by "metabolic stress" signals; cells need deoxynucleotides to repair damaged DNA. A positive correlation was found between dCK activity and the sensitivity of malignant cells to chemotherapy; thus, dCK has an outstanding importance in human chemotherapy. dCK is often the rate-limiting enzyme in the activation of these analogs. L-2′3′-dideoxy-3′-thiacytidine (lamivudine, 3TC); arabinosylcytosine (Cytosar, ara-C); 2-chlorodeoxyadenosine (cladribine, CdA); and 2′,2′-difluorodeoxycytidine (gemcitabine, dFdC), the first a human immunodeficiency virus drug and the last three valuable anticancer agents, are all substrates for dCK, and they are between 5% and 50% as efficient as dCyt as substrates for the enzyme. dCK prefers nucleoside sugars in the S-conformation (C2′-endo-C3′-exo) because α-2′,3′-dideoxycytidine adopts that conformation preferentially. dCK is composed by two identical polypeptides of 261 amino acids *(54)*, and it shows some significant sequence similarity with the herpes simplex type 1 virus thymidine kinase, as well as about 40% sequence identity to the mitochondrial thymidine kinase 2. In 2003, the structure of dCK in complex with dCyt and ADP-Mg^{2+} was solved.

The activated form of dCK seems to be an altered stable conformation of the enzyme in which the C-terminal is differently exposed to immunoreagents. Further studies are needed to define the molecular mechanism responsible for the activation process, but it is clear that increased understanding in this field may lead to the development of new drug combinations in antitumor or antiviral chemotherapy. With determination of the structure of dCK, it is now possible to define some of the structure function relationships of this enzyme and the related expanding deoxynucleoside kinase enzyme family.

Key Words: Deoxycytidine kinase; deoxyguanosine kinase; deoxynucleoside analogs; deoxynucleoside kinases; deoxynucleosides; thymidine kinases.

1. INTRODUCTION

This chapter mainly concerns the role of deoxycytidine kinase (dCK; EC 2.7.1.74) in cellular nucleotide metabolism and in activation of nucleoside analogs used in chemotherapy. However, Chapter 3 also describes the activation of many of the most important deoxynucleoside analogs by the four cellular deoxynucleoside kinases (dNKs). We therefore focus on the special metabolic function of dCK in relation to metabolism and DNA synthesis; refer to Chapter 3 and to several earlier review articles *(1–4)* for a more comprehensive account of the topic. Here, we summarize the basic facts regarding dCK but focus on the results obtained during recent years.

The adequate maintenance of intracellular deoxyribonucleotide pools to supply the needs of deoxyribonucleic acid (DNA) replication, repair, and recombination is a central issue of nucleotide metabolism, accomplished by the tightly regulated *de novo* and salvage biosynthetic pathways presented in Fig. 1. Purine and pyrimidine ribonucleotides can be synthesized from carbohydrate and amino acid derivatives by the energy consuming *de novo* biosynthetic pathways producing ribonucleoside diphosphates (NDPs), which are reduced by the ribonucleotide reductase (RR) to the corresponding deoxyribonucleotide diphosphates (dNDPs) and converted to deoxyribonucleotidetriphosphates (dNTPs).

The synthesis of deoxythymidine 5′-triphosphate (dTTP) is slightly different from that of the others; deoxyuridine monophosphate (dUMP) is methylated by thymidylate synthase to deoxythymidine monophosphate (dTMP) and then phosphorylated to deoxythymidine triphosphate (dTTP) by kinases. However, the mitochondria and some tissues (i.e., erythrocytes, polymorphonuclear leukocytes, peripheral lymphocytes, and brain) in mammals have reduced capacity for *de novo* synthesis of nucleotides; therefore, they are dependent on exogenous bases or nucleosides (salvage processes) to supply the ribo- and deoxyribonucleotides required for different metabolic processes and nucleic acid synthesis. The main sources of deoxyribonucleotides in the cytosol and in the mitochondrion are presented in Fig. 1.

Nucleosides are transported through the plasma and mitochondrial membranes by different nucleoside transporters and immediately phosphorylated by the cytosolic or mitochondrial dNKs. Two cytosolic dNKs (dCK and TK1) and two mitochondrial dNKs (deoxyguanosine kinase [dGK] and thymidine kinase 2 [TK2]) have been identified in different cells and tissues (Fig. 1).

Among different salvage enzymes in different tissues, dCK is the main enzyme in the salvage of deoxyribonucleosides as a consequence of its broad substrate specificity. The dCK has an outstanding importance in

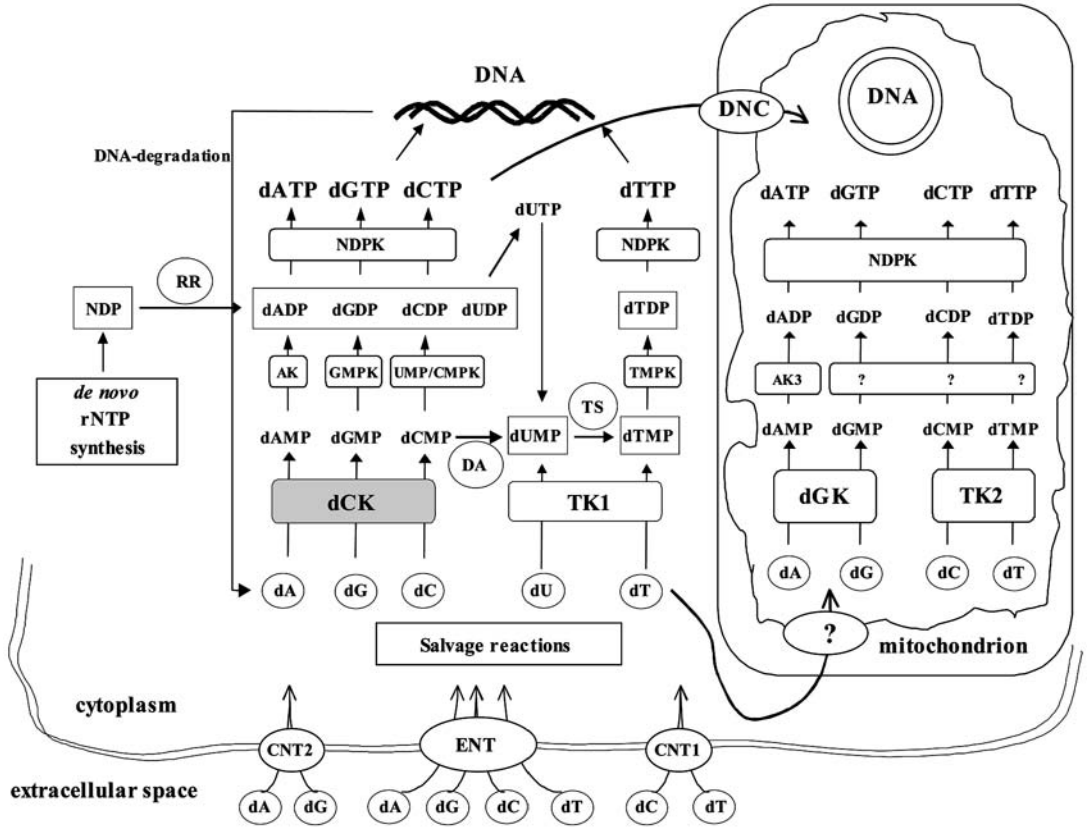


Fig. 1. The sources of deoxyribonucleotides in the cytoplasm and mitochondrion. CNT, concentrative nucleoside transporter (NT); ENT, equilibrative NT; DNC, mitochondrial deoxyribonucleotide carrier; RR, ribonucleotide reductase; DA, dCMP deaminase; TS, thymidylate synthase; AK, adenylate kinase; GMPK, guanylate kinase; UMP/CMPK, TMPK, UMP/CMP kinase and TMP kinase; NDPK, nucleotide diphosphate kinase.

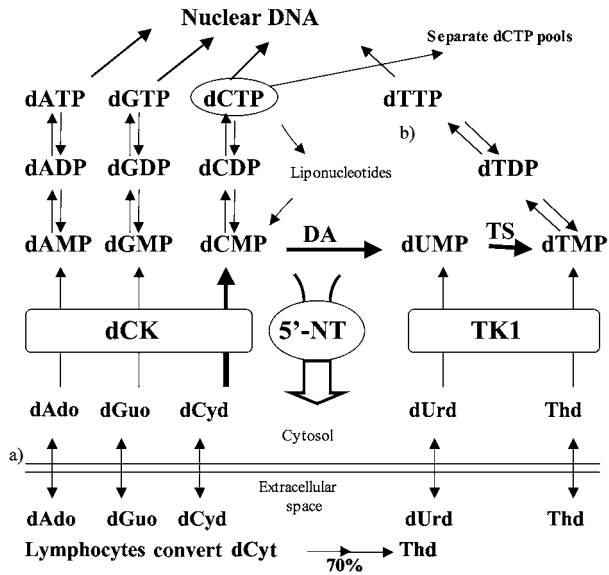


Fig. 2. Salvage of deoxynucleosides into liponucleotides, the separate dCTP pools. dCyt, deoxycytidine; 5′-NT, 5′-nucleotidase.

human therapy because it is also responsible for the activation of many important nucleoside derivatives used in anticancer and antiviral therapy. The enzyme is capable of 5′-phosphorylation of the natural substrates deoxycytidine (dCyt), deoxyadenosine (dAdo), and deoxyguanosine (Fig. 2) and a large number of both pyrimidine and purine analogs *(1–3)*. The physiological phosphate donor for the reaction is most likely both adenosine triphosphate (ATP) and uridine triphosphate (UTP), and the nature of the phosphate donor affects the kinetics of the reaction (*1–4* and references therein). There exists an interconversion pathway between dCyt and deoxythymidine (dThd) nucleotides in the cytosol. The main enzyme in the dCyt-dThd interconversion pathway is the deaminase activity (DA); in the catabolic pathway, it is the nucleotidase activity (5′-NT) presented in Fig. 1.

The metabolic importance of dCK is apparent from the observations that, in some mammalian cells, the major source of dTTP comes also from its product dCMP via dCMP deaminase (dCMP-DA) rather than from deoxyuridine monophosphate via ribonucleotide reduction *(4–6)* as presented in Fig. 2. Approximately 75% of extracellularly added dCyt was converted into dTTP via dCMP-DA and TS in human lymphocytes *(7)*. The importance of the other products, dAMP and dGMP, is less clear because in most situations there is efficient catabolism of purine deoxynucleosides. Only with stable purine analogs or inherited deficiencies of catabolic enzymes is there a significant accumulation of these type of dCK

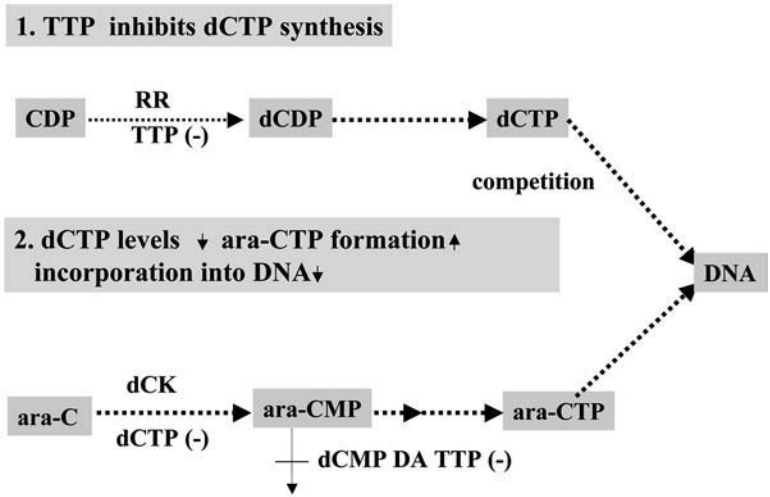


Fig. 3. The regulation of dCTP synthesis and ara-C phosphorylation.

products. The synthesis of dCMP directly by dCK became a central role in supply of all processes utilizing only dCyt for special functions (i.e., for phospholipids) and the other three deoxynucleotides for DNA as seen in Fig. 2.

dCK is the only enzyme that can supply cells with all four precursors of DNA. The final product of the salvage, beside of DNA *(8–10)*, the deoxycytidine triphosphate (dCTP) can also be utilized for special processes, for synthesis of "liponucleotides," precursors of membrane phospholipids *(11–14)*. Treatments of patients with dCyt analogs often resulted in neuropathy as a side effect, which might be the result of altered phospholipid biosynthesis. dCyt is incorporated into dCDP-ethanolamine and dCDP-choline more effectively than cytidine itself, and the anticancer analog arabinosyl-cytosine also participates in these reactions *(11–13)*. The inositol-phospholipid pathway, deoxycytidine diphosphate (dCDP)-diacylglycerol can also incorporate external dCyt, but only in the presence of the amphipathic, neuroleptic drug chlorpromazine *(12,14)*, inhibiting its further metabolism. The incorporation of dCyt into two separate dCTP pools and into membrane phospholipids is presented in Fig. 2.

An important function of dNTP pools has been demonstrated in the activation of origenes during DNA replication *(10)*, and dCK is involved in the overall regulation of the dNTP pools in most cells. The concentrations of dCTP and dTTP pools are regulated at different levels of the metabolism, which then influences the activation processes and effects of nucleoside analogs used in cancer chemotherapy. The inhibition of the dCTP synthesis by the allosteric effect of dTTP occurs at the level of RR, where the reduction of CDP is regulated by dTTP (Fig. 1). There is

	dCK	TK1	TK2	dGK
Resting PBMC	15	<1	20 - 40	20 - 50
Stimulated PBMC	30	100	≈ 100	20 - 50
CEM	30	100	≈ 100	20 - 50
Thymus	100	25	≈ 100	20 - 50
Brain	<1	<1	≈ 100	≈ 100
Muscle	<1	<1	10 - 20	20 - 50
Liver	<1	<1	20 - 50	
Colon	5 - 10	5	50	

Fig. 4. Comparison of dCK activities in different tissues.

another regulation step in the formation of dCTP at the level of dCK; that is, at a high level of dCTP there is no further phosphorylation of dCyt by dCK. However, this regulation will also influence the activation of the antileukemic nucleoside analog(s) arabinosyl-cytosine (ara-C). A high level of dCTP will inhibit the phosphorylation of ara-C, decreasing the effect of the drug on DNA synthesis as presented in Fig. 3.

2. THE EXPRESSION OF dCK IN DIFFERENT CELLS AND TISSUES

The expression of dCK is highest in different lymphoid cells/tissues, such as thymus, spleen, lymph nodes, stimulated blood mononuclear cells, and bone marrow cells and in all malignancies of these cells as presented in Fig. 4 *(1,2,7)*. The dCK levels were intermediate in proliferating epithelial cells (lung, colon, placenta) and in resting peripheral blood mononuclear cells and very low in terminally differentiated tissues such as brain, liver, kidney, and muscle (Fig. 4). The TK1 isoenzyme is a cell cycle-regulated enzyme; its activity is high in all proliferating cell types (Fig. 4). The mitochondrial TK2 and dGK isoenzyme activities are mainly constant in different tissues (Fig. 4).

There is a discrepancy between the relatively high messenger ribonucleic acid (mRNA) levels and the lack of enzyme activity in adult brain extracts *(3)*. However, embryonic sympathetic neuron cultures are inhibited by nucleoside analogs, such as ara-C *(15)* and cladribine (2-chlorodeoxyadenosine, CdA), a dAdo derivative *(16)*, which is an excellent substrate for dCK *(3)*

in vitro. The effect of CdA on neurons could be reversed by dCyt, indicating that phosphorylation by dCK is a necessary step in the nucleoside toxicity observed in embryonic neuron cultures *(16)*. The conclusions from these results might be that, during cell differentiation, there are also fundamental changes in the capacity of the nucleoside metabolic pathways.

The cell cycle dependence of the expression of dCK was a matter of discussion. There was only a slight (two- to threefold) increase in dCK activity in S phases (i.e., large lymphoid cells compared to small, e.g., G1 phase cells) *(1–4)*. Surprisingly, even larger differences were found in dCK activity and dCyt metabolism between undifferentiated and differentiated human tonsillar lymphocytes *(17)* and between cell fractions isolated from germinal centers of lymph nodes, where B lymphocyte differentiation occurs *(18)*. These differences did not correlate with the activities of S-phase marker enzymes (i.e., DNA polymerase-α and TK1), which support the cell cycle-independent synthesis of the dCK enzyme. The differences in dCK activity in lymphomas, different leukemias, and solid tumors seems probably correlated to their differentiation stages, with higher levels of dCK in undifferentiated lymphoid and embryonic neural cells.

The normal differentiation pathway is apparently disturbed in children with Down syndrome (DS, trisomy of chromosome 21), who have a 10- to 20-fold increased risk of developing acute lymphoid leukemia and acute myeloid leukemia (AML) compared to children without DS *(19,20)*. However, DS leukemic lymphoblasts and myeloblasts are more sensitive to ara-C toxicity, leading to longer event-free survival rates after treatment compared to children without DS. This may be related to DS leukemic cells containing two to three times higher dCK levels than other leukemic cells *(20)*.

Comparisons have been done among the levels of dCK mRNA, the level of enzyme activity, and dCK protein using dCK-C-terminal peptide antibodies in different cell lines and transplanted tumors *(21,22)*. There was overall a good correlation between these three parameters, except in some cases. These results may be important for future studies in which the level of dCK in patients could be considered as a parameter to obtain individualized chemotherapy with nucleoside analogs, potentially leading to higher efficacy and reduced side effects.

A positive correlation was found between dCK activity and the sensitivity of malignant cells to chemotherapy *(20–23)*, discussed in Section 7. Resistance to nucleoside analogs has been attributed to impaired dCK function caused by various genetic and epigenetic alterations, as summarized below (e.g., point mutations, exon deletions, and alternatively spliced mRNAs). Antisense oligonucleotides and retroviral introduction of hammerhead ribozymes targeted against dCK mRNA have been used and were reported to result in both decreased dCK activity and sensitivity to cytidine

analogs *(24)*. Furthermore, full sensitivity to ara-C could be restored by viral transfer of the dCK complementary DNA (cDNA) into ara-C-resistant cell lines *(25,26)*.

The gene for human dCK is localized to chromosome band 4q13.3–q21.1 and is a 34-kb single copy gene. The true localization of the dCK protein in cells has been unclear. During biochemical isolation procedures, dCK was found in the cytosol, but the enzyme has a nuclear localization signal sequence in the N-terminal region *(27)*. However, endogenous dCK was found only in the cytosol using immunohistochemistry with a dCK peptide antibody, and nuclear localization of dCK was found only in dCK-overexpressing cells *(28)*. The possible role of such a redistribution process of dCK between the cytosol and nuclei remains to be determined.

3. INCREASE OF dCK ACTIVITY BY TREATMENT OF CELLS WITH GENOTOXIC AGENTS: IMPLICATIONS FOR DNA REPAIR AND APOPTOSIS

The sensitivity of malignant cells to deoxyribonucleoside chemotherapeutic drugs is highly influenced by the cellular dCK levels, so that high dCK activity enhances the efficacy of treatment of the cells with nucleoside derivatives. Pretreatment of resistant cells with bryostatin 1 has been shown to exert an adjuvant effect on the sensitivity of the cells to CdA toxicity *(29)*. The mechanism behind this effect has been studied, and it was shown that a 1- to 2-h pretreatment of human lymphocytes with CdA caused a two- to threefold increase in dCK activity not accompanied by any increase in dCK mRNA or protein levels *(30)*. The TK1 or TK2 isoenzyme activities were not increased by this or other treatments of cells with genotoxic agents, so this phenomenon was specific for dCK. During serial dilutions and affinity chromatography, purification the elevated dCK activity was maintained, indicating that feedback regulation by small molecules or allosteric factors is unlikely *(31)*. On the other hand, incubation with λ protein phosphatase decreased the specific activity of dCK in cell extracts, suggesting that reversible phosphorylation of the enzyme might be involved in this process *(31)*. It has also been reported that protein kinase C in vitro phosphorylates and "activates" recombinant dCK *(32)*, but the increase of dCK activity by this mechanism could not be confirmed in human lymphocytes *(33)*.

CdA is an analog of deoxyadenosine that is not deaminated by adenosine deaminase and has excellent clinical activity against hairy cell leukemia and chronic lymphocytic leukemias of B- and T-cell types. CdA is an excellent substrate for dCK in vitro *(34)* as well as in vivo, and its main product, Cl-dAMP, accumulates in human lymphocytes during short-term incubations, during which the activity of dCK increased two to four times *(35)*. The

same enhancement of dCK activity occurred on preincubation with a variety of nonnucleoside genotoxic agents such as aphidicolin *(35)*, etoposide (VP16) *(36,37)*, taxol, and even the G protein modulator sodium fluoride *(38,39)*. γ-Irradiation *(40)* and UV-C irradiation *(41)* also augmented dCK activity in different lymphoid cells. The decrease of dCK activity was observed with protein phosphatase inhibitors *(40)*, again suggesting a regulatory role for reversible protein phosphorylation in the activation process. The sequence of treatment has also been shown to be important, and a synergistic effect was observed with sequential etoposide and cytarabine therapy combinations *(42,43)*.

The effects of nucleoside-5′-thiosulfate (-5′-TS) derivatives of four deoxy- and two ribonucleosides were measured on the activity of the two main salvage dNKs, dCK and TK1, in primary lymphoid cells. Only thymidine-5′-TS (dThd-5′-TS) enhanced the activity of dCK in cells; none of the other nucleoside-5′-TS derivatives had any effect on either the TK or the dCK activities *(44)*.

The activation of dCK can be achieved not only in lymph node cells but also in thymocytes, spleen cells, and peripheral blood lymphocytes. A marked difference in the extent of dCK activation was found between G- and S-phase enriched subpopulations of lymphocytes. In resting lymphocytes, the extent of stimulation was higher than in S-phase cells *(18)*. The activation of dCK was seen also with treatment of cells with the natural nucleoside dAdo, provided that its deamination was abolished by the addition of the adenosine-deaminase inhibitor deoxycoformycine *(45)*. This could be a contributing factor in adenosine deaminase deficiency, which is associated with a dramatic decrease of T lymphocytes and intracellular accumulation of deoxyadenosine 5′-triphosphate (dATP) *(46)*. An imbalance in the nucleotide pools seems to be a primary reason for DNA damage and inhibition of DNA synthesis and repair.

All the treatments described are toxic for cells, disturbing metabolic processes and leading finally to damage of DNA. DNA damage caused by UV-C or γ-irradiation increased the repair of DNA in parallel with the elevation of dCK activity *(40,41)*, and the dATP concentration was also increased in cells *(46)*, which was shown to be involved in apoptosis *(47)*. The damaged DNA triggered rapid hyperphosphorylation and induction of p53, which promotes either DNA repair or apoptosis *(47,48)*. To explore whether p53 is involved in the activation of dCK on genotoxic stress, pifithrin-α, a recently discovered pharmacological inhibitor of p53, was used *(49)*. Treatment of cells with this inhibitor reduced the activation of dCK *(93)*, suggesting a direct or indirect role for p53 in the enzyme activation process *(93)*.

Unexpectedly, the dCK activity was 22-fold higher in CEM cells transfected with the cystathione-β-synthase cDNA (a gene localized to

chromosome 21 involved in cysteine biosynthesis from homocystein) compared to wild type. However, levels of dCK mRNA and protein were not changed in the transfected cells, similar to toxic treatments of cells. Only the dCK enzyme activity was for as-yet-unknown reasons enhanced by transfection of cells with cystathione-β-synthase cDNA *(20)*. The transfection of cells by a cDNA might disturb metabolic processes similarly to other genotoxic treatments, presented before. The final answer was the increase of dCK activity and the increase of the deoxynucleoside salvage and DNA repair.

The special function of the dCyt salvage in the DNA repair was suggested in an early work in which two dCTP pools were shown in lymphocytes *(8)*; this suggestion was also supported in other cells, where functional compartmentation of dCTP pools was shown *(9)*. Thus, the reason for the increase of dCK activity after toxic treatments of cells seems first to be to supply the repair of DNA.

4. PREVENTION OF dCK ACTIVATION BY dCyt AND DEPLETION OF CYTOSOLIC Ca^{2+} IONS

Among nucleoside-monophosphate analogs, only dThd-5′-TS enhanced the activity of dCK in cells; no other nucleoside-5′-TS derivatives had any effect on either dCK or TK activities *(44)*. The activation process of dCK by dThd-5′-TS and other agents can be prevented by simultaneous addition of dCyd; dThd had no effect on the activation process *(44)*.

The counteracting effect of dCyt and not of dThd, preventing the activation of dCK induced by toxic treatments, was surprising. Similar effects of dCyt were observed in totally different systems; for instance, induction of the glycoprotein hormone αa-subunit and placental alkaline phosphatase by butyrate was also inhibited only by dCyt and not by other nucleosides in HeLa cells *(51)*. The treatment with butyrate could be a similar stress signal for the cells as the toxic treatments listed above. The mechanisms of this “protecting” effect of dCyt against the different “stress” treatments leading to increased dCK activity and DNA repair are still unknown. It might be important to supply all four deoxynucleosides by the action of dCK and by the dCyt–dThd nucleotide interconversion pathway, operating mainly in lymphoid cells *(7)* (Figs. 1 and 2).

Protein phosphorylation is involved in the signaling pathway induced by γ-irradiation in the case of the P53R2 RR *(53)*, and it was suggested that the dCK activation is also dependent on protein phosphorylation *(31,32,52,54)*. BAPTA-AM (1,2-*bis* [2-amino-phenoxy] ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis [acetoxymethyl ester]), a cell-permeable calcium chelator, *(55,56)* selectively inhibited the activation of dCK in a time- and concentration-dependent manner, but extracellular calcium depletion had no effect on dCK activity *(50)*. Denaturing Western blots of extracts from lymphocytes

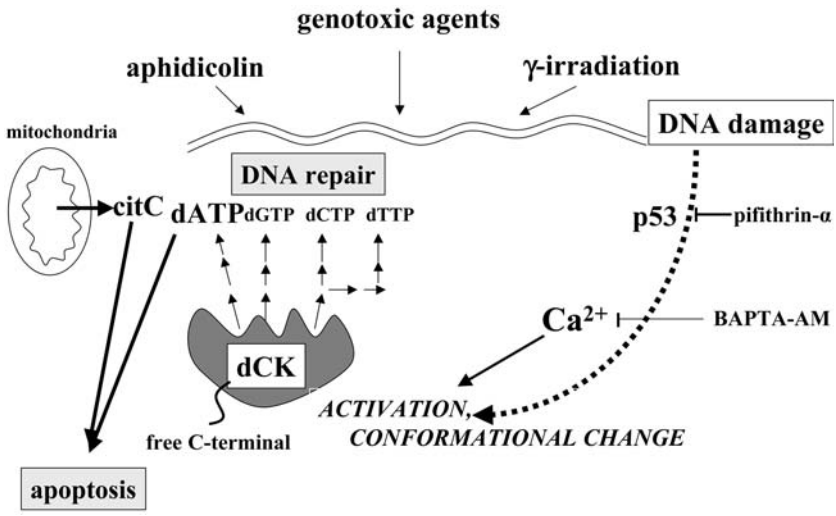


Fig. 5. The mechanism of activation of dCK and induction of apoptosis.

treated with CdA or other genotoxic agents (i.e., DNA polymerase inhibitor aphidicolin) or with the intracellular calcium chelator BAPTA-AM showed that dCK protein levels were not changed during these treatments *(50)*; the enzyme activity was six to eight times higher in cells treated with genotoxic agents than in cells with low Ca^{2+} ion concentration. However, analysis by Western blotting of native gels surprisingly demonstrated a striking correlation between enzyme activity and the intensity of dCK-specific bands. Apparently, the "high-activity" (presumably modified) form of dCK was more efficiently recognized by the antibody than the "low-activity" dCK form existing at low Ca^{2+} levels in BAPTA-AM-treated cells *(50)*. The exact molecular background for this modification process remains to be clarified.

The molecular mechanism of the toxic treatments inducing increase of dCK activity can be seen in Fig. 5. All toxic treatments (nucleoside analogs, UV or γ-irradiation, etc.) caused DNA damage, which needs DNA repair. The dNTP pools were supplied by the increased dCK activity; the activation of dCK needs Ca^{2+} ions and an intact p53 protein. However, the increased dATP pool is ready to induce the apoptotic machinery if DNA cannot be totally repaired (Fig. 5).

5. DEOXYNUCLEOSIDE ANALOGS ACTIVATED BY dCK

In Chapter 3, Munch-Petersen and Piškur list the pharmacologically most important deoxynucleosides and the anabolizing enzymes responsible

for their activation in humans (*see* Chapter 3, Table 2). Here, we shortly summarize the role of dCK in this process; the background is that, as mentioned, dCK is often the rate-limiting enzyme in the activation of these analogs. L-2′3′-Dideoxy-3′-thiacytidine (lamivudine, 3TC); ara-C; CdA; and 2′,2′-difluorodeoxycytidine (gemcitabine, dFdC) (*1–4*, *24*, *57*, and *58* and references therein), the first a human immunodeficiency virus drug and the last three valuable anticancer agents, are all substrates for dCK, and they are between 5% and 50% as efficient as dCyt as substrates for the enzyme. Gemcitabine belongs to a new category of anticancer nucleoside analogs that shows activity against solid tumors; other chapters in this book are devoted to describing the use of this drug as well as the key role of dCK as an anabolizing enzyme.

In addition to the analogs mentioned, the antileukemia drug 2-fluoro-arabinofuranosyladenine (fludarabine, F-ara-A) and the antiviral agents 2′,3′-dideoxycytidine (zalcitabine, ddC) and arabinosyladenine (vidarabine, ara-A) are also phosphorylated by dCK *(1–4,57)*. ddC is about 10–20% as efficient as a substrate as dCyt; the two other arabinosyl analogs are less active.

dCK in not selective in the case of the enantiomeric forms of its substrates and in some cases shows preferential phosphorylation of L-nucleosides *(58–63)*. Several L-nucleosides that are substrates for dCK have been developed as antiviral and anticancer drug. Two of these new L-nucleosides; L-FMAU (2′-fluoro-5-methyl-β-L-arabinofuranosyluracil) and L-OddC (β-L-(–)-dioxolane-cytidine), show relatively high activity with dCK. The former analog has broad antiviral activity, and the second shows good promise as an antitumor agent *(62,64)*.

The fact that α-ddC is a more efficient substrate for dCK than β-ddC *(61)* indicates that dCK prefers nucleoside sugars in the S-conformation (C2′-endo-C3′-exo) because α-ddC adopts that conformation preferentially. This has been verified by direct determination of the structure of the nucleoside bound to dCK using nuclear magnetic resonance methods *(65)*. The results are in agreement with molecular modeling studies with herpes simplex virus type 1 TK and conformationally restricted nucleoside analogs *(66)* and should be considered in the future design of nucleoside analogs.

In addition to the analogs briefly described, there are many other nucleosides known to be phosphorylated by dCK. The overall pattern is that dCK accepts both purine and pyrimidines with many modifications in the base, some of which, like 2-Cl or 2-Br adenosine analogs, are better substrates than the natural compounds. Furthermore, many sugar modifications are accepted, and here fluoro-substituted sugars are often excellent substrates, as are arabionsyl analogs. Both L- and D-analogs are accepted, but acyclic nucleoside analogs are very poor substrates for dCK; this is part of the reason for the antiviral properties of the acyclic analogs.

6. STRUCTURE–ACTIVITY RELATIONSHIPS OF dCK

dCK is composed by two identical polypeptides of 261 amino acids *(67)*, and it shows some significant sequence similarity with the herpes simplex type 1 virus TK, as well as about 40% sequence identity to TK2 and dGK. The structure of a dGK-ATP complex was determined in 2000 *(68)*, and in 2003 the structure of dCK in complex with dCyt and ADP-Mg^{2+} was solved *(69)*. Both dGK and dCK show large similarity in structure, and they have six β-sheets and seven or eight α-helices; the N-terminal phosphate-binding loop is in the center of the active site. In dCK, helices 5 and 6 are short; these are one long helix (helix 5) in the case of dGK *(4,68,69)*, but otherwise the folds of the proteins are almost identical, and differences can only be seen in the active site . Both have the 5′-OH group of the nucleoside held in place by hydrogen bonds to a conserved Glu, the amino group of an Arg, and most likely of a water molecule.

The 3′-OH of the deoxyribose interacts with a Glu and a Tyr, and the latter is "overlapping" the 2′-position so that a hydroxy group from a ribonucleotide is prevented from binding with high affinity. The base is bound by hydrogen bonds to a conserved Gln and a Phe. However, the difference between dGK and dCK apparently stems from an altered hydrogen bond network in the active sites, where an Asp residue interacts with an Arg but only in the case of dGK is there a Ser also participating and stabilizing this network. In contrast, in dCK the Ser is substituted by an Ala, and this means that the Arg residue is in a less-extended conformation and allows another position of the Asp, which thus can form a hydrogen bond with the amino group of dCyt. The "same" Arg residue (R104 in dCK and R118 in dGK) is in both enzymes responsible for interacting with the purine base, and it is assumed that in dCK the flexibility in the active site is such that the Arg can also adopt an extended conformation like that found for dGK.

This very elegant explanation for the mechanism behind the structure–activity relationships of dCK and dGK has been supported by the construction of several different mutants in these key residues, both for dCK *(69)* and the homologs *Drosophila* dNK (*4*,*70*, and Figs. 6 and 7). Overall the understanding of the broad specificity of dCK has made a fundamental advance by the structure determinations of dCK not only in complex with dCyt and ADP-Mg^{2+}, but also in complex with Ara-C-ADP-Mg^{2+} and dFdC-ADP-Mg^{2+} *(69)*. However, still there is no structure of dCK interacting with a phosphate donor or a purine deoxynucleoside, and the structure of the open conformation of dCK is not yet known.

The phosphate donors of dCK most likely bind in the opposite direction compared to the nucleosides, and the β- and γ-phosphates interact with the Lys and Ser residues of the phosphate loop in the N-terminal and two

arginines in the LID region (residues 186–1999) covering the active site *(4,68,69)*. Results regarding the effects of mutations in this region for the specificity of dCK are described next; however, direct structure determinations are needed to clarify the role of the LID region in enzyme catalysis.

A large number of enzyme kinetic studies have been performed with dCK and the reaction kinetics when studied with a wide range of substrate concentrations follows a pattern of negative cooperativity for both the phosphate donor and acceptors, giving Hill coefficients >1 *(1–4,71,72)*. The reaction follows a random bi-bi pathway with ATP; with UTP at low nucleoside concentration, it appears to be ordered with the donor binding first *(70–74)*. This conclusion based on kinetics is difficult to explain in light of the structure of the active site, where the deoxynucleoside is bound in the interior and the donor supposedly binds externally *(4,68,69)*.

Fluorescence quenching, UV difference spectroscopy and near-UV CD spectroscopy demonstrate that substrate binding induces several different conformational changes *(72,74,75)*, with apparently one binding site (or state) for the donors but two binding sites (or states) for the acceptors. These results indicate that the enzyme may exist in different conformational states with different affinities for the substrates, and maybe the open state of the enzyme can bind either substrate alone nonproductively. Only when both bind in the correct subsites simultaneously is a conformational change induced, forming the closed catalytically competent state of dCK.

Feedback inhibitors (e.g., dCTP) act as bisubstrate analogs, binding to both sites in the active cleft, blocking the enzyme *(4,76)*; in case of the *Drosophila* dNK enzyme, the structure of such an enzyme feedback inhibitor complex has been solved *(77)*. It was shown that inorganic tripolyphosphate was a good phosphate donor for dCK when dCyt was the acceptor but not when dAdo was the substrate *(78)*, again indicating that the nature of the donor and acceptor mutually affects its other capacity to participate in the catalytic cycle.

Mouse dCK has a lower capacity to phosphorylate certain deoxynucleosides (e.g., dAdo and ddC) compared with human dCK, which is one reason for that these analogs are less toxic to mice than to humans. The amino acid sequences are 93% identical between mouse and human dCK, but the enzymes show different nucleoside substrate specificity with ATP or UTP used as phosphate donors. The amino acids in the LID region (i.e., in the phosphate donor site) differ in several important positions (particularly Q179R, T184K, H187N) between the mouse and human dCK *(79)*. If these amino acids are changed by mutagenesis, then the specificity of the mutated mouse dCK is indistinguishable from the normal human dCK. Introduction of such a triple mutant (i.e., a humanized form of mouse dCK) into mice may create a better animal model for future nucleoside drug testing.

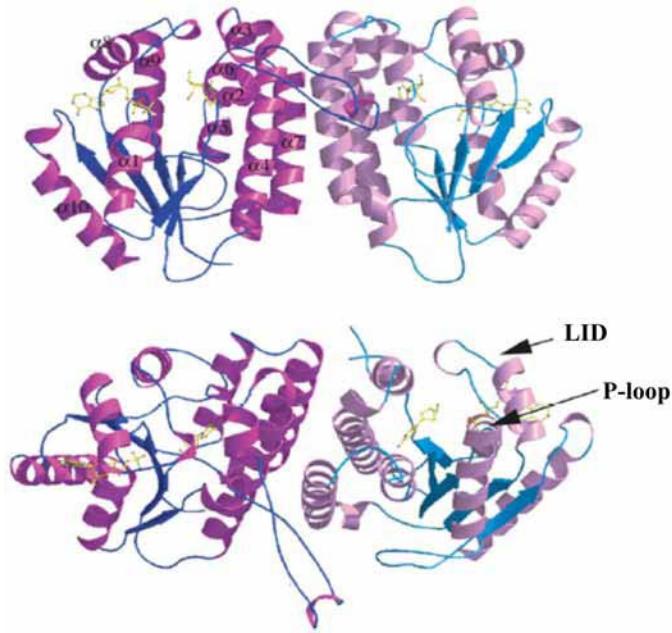


Fig. 6. Comparison of the amino acid sequences of human dCK, dGK, and TK2 and that of the *Drosophila*. Amino acids: identical read, similar: yellow. Structural motives aa:1–10 and β: 1–5 are characteristic for dCK. (From ref. *16*, with permis-

7. dCK IN CELLS RESISTANT TO TOXIC NUCLEOSIDES

Cell lines lacking dCK are resistant to nucleoside analogs, and dCK deficiency was shown to be a reason for ara-C, dFdC, and CdA resistance (and crossresistance) in cultured cells *(23,80–85)*. However, when leukemic cells were isolated from patients with acute AML who were resistant to ara-C, only in 1 of 16 cases showed dCK deficiency *(86)*. In a separate study of ara-C-resistant AML cells, several shorter dCK mRNA variants were found, and these truncated forms of dCK mRNAs lacked one or several exons *(87)*. However, introduction of these alternatively spliced forms of dCK into leukemic cells did not confer the resistance phenotype to the transfected cells; thus, the role of alternate spliced or truncated forms of dCK mRNA in AML cells *(87)* and in dFdC-resistant ovary cancers cells *(80)* is still unclear.

Three-point mutations leading to inactive dCK have been defined by reconstruction and characterization of the recombinant dCK mutants (i.e., G28E in the P loop, Q156R in α-helix 7, and Y99C in α-helix 4) *(68,81,86)*. In these cases, only the Q156R mutation is in an apparently nonconserved residue, but α-helix 7 is responsible for the subunit interactions. Therefore,

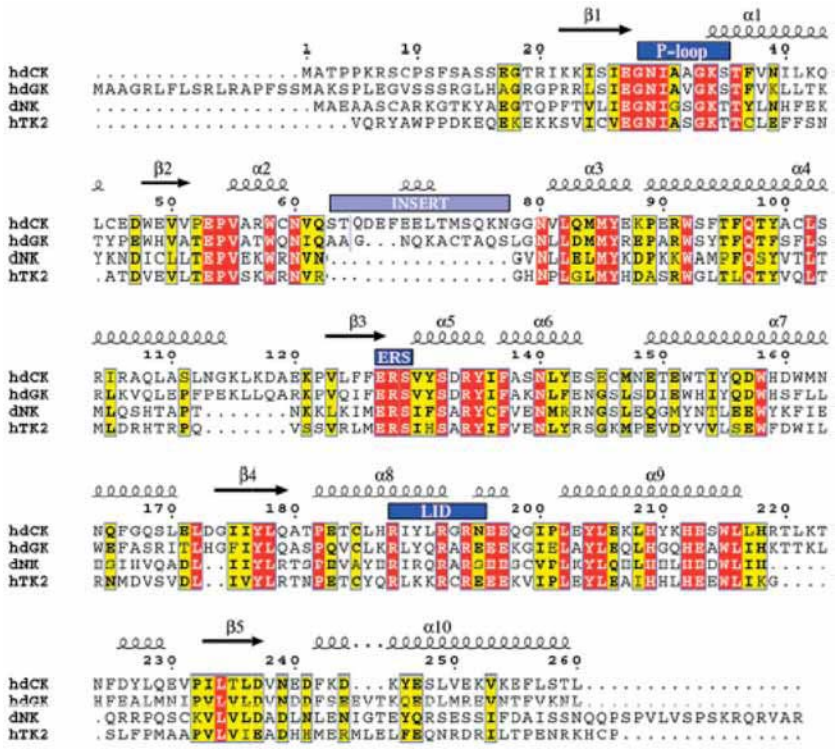


Fig. 7. The homodimer structure of the recombinant dCK. (From ref. *16*, with permission.)

introduction of a charged amino acid may lead to inactivating alterations in the subunit structure of dCK. However, these defined dCK point mutations are only found in a minor fraction of cells resistant to toxic dCK-activated analogs. Apparently, other, more indirect and multiple alteration such as altered splicing and translational and posttranslational mechanisms are involved in the generation in vivo of the resistance phenotype.

In several studies, a correlation between the sensitivity of cells to nucleoside analogs and the level of dCK was observed (e.g., patients responding to CdA had a somewhat higher level of dCK than nonresponders) *(34,88)*. However, the correlation was relatively weak, and in some studies no correlation was observed *(89,90)*. It has been shown that the pretreatment dCK activity or mRNA levels predicted the in vivo sensitivity of transplantable human tumors to treatment with dFdC *(22)*. A significant predictive value of determining the dCK and cNII 5′-nucleotidase levels in AML blast cells at the time of diagnosis has been established in relation to the sensitivity of

treatment of the patients with ara-C *(91)*. Thus, it now seems clear that determinations of dCK levels with highly sensitive and selective methods such as quantitative real-time polymerase chain reaction in the clinical situation is of diagnostic or prognostic value when combined with other parameters, such as the level of catabolic enzymes and other general parameters such as age.

Transfection experiments with dCK gene constructs have shown that higher cellular levels of dCK give higher sensitivity of the cells to cytotoxic nucleosides that serve as substrates for the enzyme *(25,26,92)*. Therefore, dCK gene transfer to tumor cells could be a method to increase the sensitivity for a nucleoside analog. Transfection of glioma cells with vectors containing the dCK gene gave higher sensitivity of the transfected cells to ara-C, and when tested in animal models, it led to tumor regression in response to ara-C chemotherapy *(26)*.

8. CONCLUSIONS

The role of dCK in the supply of DNA precursors is gradually being elucidated, and new knowledge both from enzyme structure and function studies and from studies of deoxynucleoside-resistant cells supports a key function for this enzyme in the deoxynucleoside salvage pathway. The involvement of dCK in liponucleotide metabolism is also obvious even though much remains to be learned about the biological consequences of this pathway. The posttranslational activation of dCK observed after treatment of cells with DNA synthesis inhibitors appears to be a compensatory mechanism induced by metabolic stress signals.

Cells need deoxynucleotides to repair damaged DNA; the increased dCK activity can help supply all four dNTPs, and this may be a unique source of DNA precursors in quiescent cells. The dCK activation process is dependent on the intracellular Ca^{2+} levels and normal function of the p53 protein. The activated form of dCK seems to be an altered stable conformation of the enzyme in which the C-terminal is differently exposed to immunoreagents.

Further studies are needed to define the molecular mechanisms responsible for the activation process, but it is clear that increased understanding in this field may lead to the development of new drug combinations in antitumor or antiviral chemotherapy. The structure of dCK was recently determined, and thus it is now possible to define some of the structure–function relationships of this enzyme and the related expanding dNK enzyme family.

ACKNOWLEDGMENT

Work performed in the authors' laboratories has been supported by funds to M. S. from Hungarian national grants OTKA and from European Communities Grants ETT and to S. E. from the Swedish Research Council,

the Swedish Strategic Research Foundation, the Swedish University for Agricultural Sciences.

REFERENCES

1. Arnér ES, Eriksson S. Mammalian deoxyribonucleoside kinases. Pharmacol Ther 1995;67:155–186.
2. Eriksson S, Arnér ES, Spasokoukotskaja T, et al. Prospective and levels of deoxynucleoside kinases in normal and tumor cells: implications for chemotherapy. Adv Enzyme Regul 1994;34:13–45.
3. Eriksson S, Wang L. The role of the cellular deoxynucleoside kinases in activation of nucleoside analogs used in chemotherapy. In: Chu CK, ed. Recent Advances in Nucleosides: Chemistry and Chemotherapy. Elsevier, 2002:455–475.
4. Eriksson S, Munch-Petersen B, Johansson K, Ekhlund H. Structure and function of cellular deoxyribonucleoside kinases. Cell Mol Life Sci 2002;59:1327–1346.
5. Mathews CK. Deoxycytidylate deaminase, human. In: Wiley Encyclopedia of Molecular Medicine. Vol. 5. Wiley, 2002:1327–1346.
6. Gribaudo G, Riera L, Caposio P, Maley F, Landolfo S. Human cytomegalovirus requires cellular deoxycytidylate deaminase for replication in quiescent cells. J Gen Virol 2003;84:1437–1441.
7. Staub M, Spasokukotskaja T, Benczur M, Antoni F. DNA synthesis and nucleoside metabolism in human tonsillar lymphocyte subpopulations. Acta Otolaryngol 1987;454:118–124.
8. Spasokoukotskaja T, Sasvari-Szekely M, Taljanidisz J, Staub M. Compartmentation of dCTP pools disappears after hydroxyurea or araC treatment in lymphocytes. FEBS Lett 1992;297:151–154.
9. Xu YZ, Huang P, Plunkett W. Functional compartmentation of dCTP pools. J Biol Chem 1995;270:631–637.
10. Anglana M, Apiou F, Bensimon A, Debatisse M. Dynamics of DNA replication in mammalian somatic cells: nucleotide pool modulates origin choice and interorigin spacing. Cell 2003;114:385–394.
11. Spasokoukotskaja T, Spyrou G, Staub M. Deoxycytidine is salvaged not only into DNA but also into phospholipid precursors. Biochem Biophys Research Commun 1988;155:923–929.
12. Spasokukotskaja T, Taljanidisz J, Sasvári-Székely M, Staub M. Deoxycytidine is salvaged not only into DNA but also into phospholipid precursors III. dCDP-Diacylglycerol formation in tonsillar lymphocytes. Biochem Biophys Res Commun 1991;174:680–687.
13. Sasvári-Székely M, Spasokoukotskaja T, Staub M. Deoxyribocytidine is salvaged not only into DNA but also into phospholipid precursors IV. Exogenoue deoxycytidine can be used with the same efficacy as (ribo)cytidine for phospholipid activation. Biochem Biophys Res Commun 1993;194:966–723.
14. Hrabák A, Spasokukotskaja T, Temesi A, Staub M. The salvage of deoxycytidine into dCDP-diacylglycerol by macrophages and lymphocytes. Biochem Biophys Res Commun 1993;193:212–219.
15. Martin DP, Wallace TL, Johnson EM Jr. Cytosine arabinoside kills postmitotic neurons in fashion resembling trophic factor deprivation: evidence that deoxycytidine dependent process may be required for nerve growth factor signal transduction. J Neurosci 1990;10:184–193.

16. Nagy N, Magyar T, Spasokoukotskaja T, Virga S, Oláh I, Staub M. The neurotoxicity of 2-Cl-deoxyadanosine can be released by deoxycytidine. Fund Clin Pharmacol 1999;13:46.
17. Taljanidisz J, Spasokukotskaja T, Sasvari-Szekely M, Antoni F, Staub M. Preferential utilisation of deoxycytidine by undifferentiated (peanut positive) tonsillar lymphocytes. Immunol Lett 1987;15:109–115.
18. Horvath L, Sasvari-Szekely M, Spasokoukotskaja T, Antoni F, Staub M. Different utilisation of deoxycytidine and thymidine by tonsillar lymphocyte subpopulations. Immunol Lett 1989;22:161–166.
19. Taub JW, Huang XM, Matherly LH, et al. Expression of chromosome 21 localised genes in acute myeloid leukemia: differences between Down syndrome and non-Down syndrome blast cells and relationship to in vitro sensitivity to cytosine arabinoside and daunorubicin. Blood 1999;94:1393–1400.
20. Taub JW, Huang X, Ge Y, et al. Cystathione-b-synthase cDNA transfection alters the sensitivity and metabolism of 1-β-D-arabinosylcytosine in CCRF-CEM leukemia cells in vitro and in vivo: a model of leukemia in Down syndrome. Cancer Res 2000;60:6421–6426.
21. van der Wilt CL, Kroep JR, Loves WJ, et al. Expression of deoxycytidine kinase in leukaemic cells compared with solid tumour cell lines, liver metastases and normal liver. Eur J Cancer 2003;39:691–697.
22. Kroep JR, Loves WJ, van der Wilt CL, et al. Pretreatment deoxycytidine kinase levels predict in vivo gemcitabine sensitivity. Mol Cancer Ther 2002;1:371–376.
23. Galmarini CM, Mackey IR, Dumontet C. Nucleoside analogues: mechanism of drug resistance and reversal strategies. Leukemia 2001;15:875–890.
24. Beausejour CM, Tremblay G, Momparler RL. Potential of ribozymes against deoxycytidine kinase to confer drug resistance to cytosine nucleoside analogs. Biochem Biophys Res Commun 2000;278:569–575.
25. Stegmann AP, Honders WH, Willemze R, Ruiz van Haperen VW, Landegent JE. Transfection of wild-type deoxycytidine kinase (dCK) cDNA into an AraC- and DAC-resistant rat leukemic cell line of clonal origin fully restores drug sensitivity. Blood 1995;85:1188–1194.
26. Manome Y, Wen PY, Dong Y, et al. Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effect of cytosine arabinoside in vitro and in vivo. Nat Med 1996;2:567–573.
27. Johansson M, Brismar S, Karlsson A. Human deoxycytidine kinase is located in the cell nucleus. Proc Natl Acad Sci USA 1997;94:11,941–11,945.
28. Hatzis P, Al-Madhoon AS, Jullig M, Petrakis TG, Eriksson S, Talianidis I. The intracellular localization of deoxycytidine kinase. J Biol Chem 1998;273:30,239–30,243.
29. Mohammad RM, Beck FW, Katato K, Hamdy N, Wall N, Al-Katib A. Potentiation of 2-chlorodeoxyadenosine activity by bryostatin 1 in the resistant chronic lymphocytic leukemia cell line (WSU-CLL): association with increased ratios of dCK/5′-NT and Bax/Bcl-2. Biol Chem 1998;379:1253–1261.
30. Sasvari-Szekely M, Spasokoukotskaja T, Szoke M, et al. Activation of deoxycytidine kinase during inhibition of DNA synthesis by 2-chloro-2′-deoxyadenosine (cladribine) in human lymphocytes. Biochem Pharmacol 1998;56: 1175–1179.
31. Csapo Z, Sasvari-Szekely M, Spasokoukotskaja T, Talianidis I, Eriksson S, Staub M. Activation of deoxycytidine kinase by inhibition of DNA synthesis in human lymphocytes. Biochem Pharmacol 2001;61:191–197.

32. Wang LM, Kucera GL. Deoxycytidine kinase is phosphorylated in vitro by protein kinase C alpha. Biochim Biophys Acta 1994;1224:161–167.
33. Spasokoukotskaja T, Csapo Z, Sasvari-Szekely M, et al. Effect of phosphorylation on deoxycytidine kinase activity. Adv Exp Med Biol 2000;486:281–285.
34. Arnér ES, Spasokoukotskaja T, Juliusson G, Eriksson S. Phosphorylation of 2-chlorodeoxyadenosine (CdA) in extracts of peripheral blood mononuclear cells of leukemic patients. Br J Haematol 1994;87:715–718.
35. Sasvari-Szekely M, Piroth Z, Kazimierczuk Z, Staub M. A novel effect of the new antileukemic drug, 2-chloro-2′-deoxyadenosine, in human lymphocytes. Biochem Biophys Res Commun 1994;203:1378–1384.
36. Spasokoukotskaja T, Sasvari-Szekely M, Keszler G, Albertioni F, Eriksson S, Staub M. Treatment of normal and malignant cells with nucleoside analogues and etoposide enhances deoxycytidine kinase activity. Eur J Cancer 1999;35:1862–1867.
37. Ooi K, Ohkube T, Higashigawa M, Kawasaki H, Sakurai M. Increased deoxycytidine kinase activity by etoposide in L1210 murine leukemic cells. Biol Pharm Bull 1996;19:1382–1383.
38. Staub M, Csapó Zs, Spasokoukotskaja T, Sasvári-Székely M. Deoxycytidine kinase can be also potentiated by the G-protein activator NaF in cells. Adv Exp Med Biol 1998;431:425–428.
39. Csapo Z, Sasvari-Szekely M, Spasokoukotskaja T, Staub M. Modulation of human deoxycytidine kinase activity as a response to cellular stress induced by NaF. Acta Biochim Pol 2001;48:251–256.
40. Csapo Zs, Keszler G, Safrany G, et al. Activation of deoxycytidine kinase by gamma-irradiation and inactivation by hyperosmotic shock in human lymphocytes. Biochem Pharmacol 2003;65:2031–2039.
41. Van den Neste E, Smal C, Delacauw A, et al. Activation of deoxycytidine kinase by UV-C irradiation in chronic lymphatic leukemia B-lymphocytes. Biochem Pharmacol 2003;65:573–580.
42. Kreder CN, van Bree C, Peters GJ, Loves PJ, Haveman J. Enhanced levels of deoxycytidine kinase and thymidine kinase 1 and 2 after pulsed low dose rate irradiation as an adaptive response to radiation. Oncol Rep 2002;9:141–144.
43. Ohkubo T, Higashigava M, Kavasaki H, et al. Synergistic interaction between etoposide and 1-β-arabinofuranosylcytosine. Adv Exp Med Biol 1989;253B:355–362.
44. Keszler G, Szikla K, Kazimierczuk Z, Spasokoukotskaja T, Sasvari-Szekely M, Staub M. Selective activation of deoxycytidine kinase by thymidine-5′-thiosulphate and release by deoxycytidine in human lymphocytes. Biochem Pharmacol 2003;65:563–571.
45. Hershfield MS, Mitchell BS. Immunodeficiency diseases caused by adenosine deaminase deficiency and purine nucleotide phosphorylase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The Metabolic and Molecular Bases of Inherited Disease. 7th ed. McGraw-Hill Health Professions, New York, NY, vol. 2. 1995:1725–1769.
46. Keszler G, Spasokoukotskaja T, Csapo Zs, Virga S, Staub M, Sasvari-Szekely M. Selective increase of dATP pools upon activation of deoxycytidine kinase in lymphocytes: implications in apoptosis. Nucleosides Nucleotides Nucleic Acids 2004; 23:1335–1342.
47. Genini D, Budihardjo L, Plunkett W, et al. Nucleotide requirements for the in vitro activation of the apoptosis protein-activating factor-1-mediated caspase pathway. J Biol Chem 2000;275:29–34.

48. Oren M. Decision making by p53: life, death and cancer. Cell Death Differ 2003;10:431–442.
49. Komarov PG, Komarova EA, Kondratov RV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999;285:1733–1737.
50. Keszler G, Spasokoukotskaja T, Csapo Zs, et al. Activation of deoxycytidine kinase in lymphocytes is calcium dependent and involves a conformational change detectable by native immunostaining. Biochem Pharmacol 2004;67;947–955.
51. Tamura RN, Cox GS. Effect of pyrimidine deoxynucleosides and sodium butirate on expression of the glycoprotein hormone a-subunit and placental alkaline phosphatase in HeLa cells. Biochem Biophys Acta 1988;968:151–159.
52. Achanta G, Pelicano H, Feng L, Plunkett W, Huang P. Interaction of p53 and DNA-PK in response to nucleoside analogues: potential roles as a sensor complex for DNA damage. Cancer Res 2001;61:8723–8729.
53. Yamaguchi T, Matsuda K, Sagiya Y, et al. p53R2-dependent pathway for DNA synthesis in p53 regulated cell cycle checkpoint. Cancer Res 2001;61:8256–8261.
54. Smal C, Vertommen D, Bertrand L, et al. Identification of in vivo phosphorylation sites on human deoxycytidine kinase. Biol Chem 2006;281:4887–4893.
55. Tombal B, Denmeade SR, Gillis JM, Isaacs JT. A supramicromolar elevation of intracellular free calcium is consistently required to induce the execution phase of apoptosis. Cell Death Differ 2002;9:561–573.
56. Dieter P, Fitzke E, Duyster J. BAPTA induces a decrease of intracellular free calcium and a translocation and inactivation of protein kinase C in macrophages. Biol Chem Hoppe Seyler 1993;374:171–174.
57. Plunkett W, Gandhi V. Purine and pyrimidine nucleoside analogs. Cancer Chemother Biol Response Modif 2001;19:21–45.
58. Gumina G, Chong Y, Choo H, Song GY, Chu CK. L-Nucleosides: antiviral activity and molecular mechanism. Curr Top Med Chem 2002;2:1065–1086.
59. Chang CN, Skalski V, Zhou JH, Cheng YC. Biochemical pharmacology of (+)- and (–)-2′,3′-dideoxy-3′-thiacytidine as anti-hepatitis B virus agents. J Biol Chem 1992;267:22,414–22,420.
60. Maury G. The enantioselectivity of enzymes involved in current antiviral therapy using nucleoside analogues: a new strategy? Antivir Chem Chemother 2000;11: 165–189.
61. Wang J, Choudhury D, Chattopadhyaya J, Eriksson S. Stereoisomeric selectivity of human deoxyribonucleoside kinases. Biochemistry 1999;38:16,993–16,999.
62. Grove KL, Cheng YC. Uptake and metabolism of the new anticancer compound β-L-(–)-dioxolane-cytidine in human prostate carcinoma DU-145 cells. Cancer Res 1996;56:4187–4191.
63. Verri A, Focher F, Priori G, et al. Lack of enantiospecificity of human 2′-deoxycytidine kinase: relevance for the activation of β-L-deoxycytidine analogs as antineoplastic and antiviral agents. Mol Pharmacol 1997;51:132–138.
64. Liu SH, Grove KL, Cheng YC. Unique metabolism of a novel antiviral L-nucleoside analog, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil: a substrate for both thymidine kinase and deoxycytidine kinase. Antimicrob Agents Chemother 1998;42: 833–839.
65. Maltseva T, Usova E, Eriksson S, Milecki J, Foldesi A, Chattopadhayaya J. An NMR conformational study of the complex of $^{13}C/^{2}H$ double labeled 2′-deoxynucleoside and deoxycytidine kinase. J C S Perkin Trans 2000;2:199–2207.

66. Prota A, Vogt J, Pilger B, et al. Kinetics and crystal structure of the wild-type and the engineered Y101F mutant of Herpes simplex virus type 1 thymidine kinase interacting with (North)-methanocarba-thymidine. Biochemistry 2000;39:9597–9603.
67. Chottiner EG, Shewach DS, Datta NS, et al. Cloning and expression of human deoxycytidine kinase cDNA. Proc Natl Acad Sci USA 1991;88:1531–1535.
68. Johansson K, Ramaswamy S, Ljungcrantz C, et al. Structural basis for substrate specificities of cellular deoxyribonucleoside kinases [erratum in: Nat Struct Biol 2001;8:818–819]. Nat Struct Biol 2001;8:616–620.
69. Sabini E, Ort S, Monnerjahn C, Konrad M, Lavie A. Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. Nat Struct Biol 2003;10:513–519.
70. Knecht W, Sandrini MP, Johansson K, Eklund H, Munch-Petersen B, Piskur J. A few amino acid substitutions can convert deoxyribonucleoside kinase specificity from pyrimidines to purines. EMBO J 2002;21:1873–1880.
71. Ives DH, Durham JP. Deoxycytidine kinase. 3. Kinetics and allosteric regulation of the calf thymus enzyme. J Biol Chem 1970;245:2285–2294.
72. Kierdaszuk B, Rigler R, Eriksson S. Binding of substrates to human deoxycytidine kinase studied with ligand-dependent quenching of enzyme intrinsic fluorescence. Biochemistry 1993;32:699–707.
73. Hughes TL, Hahn TM, Reynolds KK, Shewach DS. Kinetic analysis of human deoxycytidine kinase with the true phosphate donor uridine triphosphate. Biochemistry 1997;36:7540–7547.
74. Turk B, Awad R, Usova EV, Björk I, Eriksson S. A pre-steady state kinetic analysis of substrate binding to human recombinant deoxycytidine kinase: a model for nucleoside kinases. Biochemistry 1999;38:8555–8561.
75. Mani RS, Usova EV, Eriksson S, Cass CE. Hydrodynamic and spectroscopic studies of substrate binding to human recombinant deoxycytidine kinase. Nucleosides Nucleotides Nucleic Acids 2003;22:175–192.
76. Ikeda S, Chakravarty R, Ives DH. Multisubstrate analogs for deoxynucleoside kinases. Triphosphate end products and synthetic bisubstrate analogs exhibit identical modes of binding and are useful probes for distinguishing kinetic mechanisms. J Biol Chem 1986;261:15,836–15,843.
77. Mikkelsen NE, Johansson K, Karlsson A, et al. Structural basis for feedback inhibition of the deoxyribonucleoside salvage pathway: studies of the *Drosophila* deoxyribonucleoside kinase. Biochemistry 2003;42:5706–5712.
78. Krawiec K, Kierdaszuk B, Shugar D. Inorganic tripolyphosphate (PPP(i)) as a phosphate donor for human deoxyribonucleoside kinases. Biochem Biophys Res Commun 2003;301:192–197.
79. Usova EV, Eriksson S. Identification of residues involved in the substrate specificity of human and murine dCK. Biochem Pharmacol 2002;64:1559–1567.
80. Ruiz van Haperen VW, Veerman G, Eriksson S, et al. Development and molecular characterization of a 2′,2′-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. Cancer Res 1994;54:4138–4143.
81. Owens JK, Shewach DS, Ullman B, Mitchell BS. Resistance to 1-β-D-arabinofuranosylcytosine in human T-lymphoblasts mediated by mutations within the deoxycytidine kinase gene. Cancer Res 1992;52:2389–2393.
82. Dumontet C, Fabianowska-Majewska K, Mantincic D, et al. Related common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol 1999;106:78–85.

83. Lotfi K, Månsson E, Spasokoukotskaja T, et al. Biochemical pharmacology and resistance to 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine, a novel analogue of cladrabine in human leukemic cells. Clin Cancer Res 1999;5:2438–2444.
84. Mansson E, Spasokoukotskaja T, Sallstrom J, Eriksson S, Albertioni F. Molecular and biochemical mechanisms of fludarabine and cladribine resistance in a human promyelocytic cell line. Cancer Res 1999;59:5956–5963.
85. Mansson E, Flordal E, Liliemark J, et al. Down-regulation of deoxycytidine kinase in human leukemic cell lines resistant to cladribine and clofarabine and increased ribonucleotide reductase activity contributes to fludarabine resistance. Biochem Pharmacol 2003;65:237–247.
86. Flasshove M, Strumberg D, Ayscue L, et al. Structural analysis of the deoxycytidine kinase gene in patients with acute myeloid leukemia and resistance to cytosine arabinoside. Leukemia 1994;8:780–785.
87. Veuger M, Honders M, Landegent J, Willemze R, Barge R. High incidence of alternatively spliced forms of deoxycytidine kinase. Blood 2000;96:1517–1524.
88. Kawasaki H, Caricia CJ, Carson DA. Quantitative immunoassay of human deoxycytidine kinase in malignant cell. Anal Biochem 1992;207:193–196.
89. Leiby JM, Snider KM, Kraut EH, Metz EN, Malspeis L, Grever MR. Phase II trial of 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate in non-Hodgkin's lymphoma: prospective comparison of response with deoxycytidine kinase activity. Cancer Res 1987;47:2719–2722.
90. Abertioni F, Lindemalm S, Reichlova V, et al. Pharmacokinetics of cladribine in plasma and its 5′-mono and triphosphate in leucemic cells in patients with chronic lymphocytic leukemia. Clin Cancer Res 1998;4:653–658.
91. Galmarini CM, Thomas X, Graham K, et al. Deoxycytidine kinase and cN-II nucleotidase expression in blast cells predict survival in acute myeloid leukaemia patients treated with cytarabine. Br J Haematol 2003;122:53–60.
92. Hapke DM, Stegmann APA, Mitchell BS. Retroviral transfer of deoxycytidine kinase into tumor cells lines enhances nucleoside toxicity. Cancer Res 1996;56:2343–2347.
93. Keszler G, Virga Sz, Spasokuokotskaja T, Bauer PI, Sasvari-Székely M, Staub M. Activation of deoxycytidine kinase by deoxyadenosine: implication in deoxyadenosine mediated cytotoxicity. Arch Biochem Biophys 2005;436:69–77.

3 Deoxynucleoside Kinases and Their Potential Role in Deoxynucleoside Cytotoxicity

Birgitte Munch-Petersen, DSc, and Jure Piškur, PhD

CONTENTS

SUMMARY

Deoxynucleoside kinases are pivotal for the cytotoxic effect of anticancer and antiviral nucleoside analogs. The transfer of a phosphate group from a nucleotide phosphate donor, mostly adenosine triphosphate, to the 5′-OH group of the nucleoside is the key step in the activation of the analog. In human cells, there are four deoxynucleoside kinases with overlapping specificities that together are responsible for the activation of a broad spectrum of nucleoside analogs. Deoxycytidine kinase has the most relaxed specificity and today is the kinase toward which most of the currently used analogs, such as ara-C (cytarabine, Cytosar); dFdC (Gemzar, gemcitabine); CdA (2-chlorodeoxyadenosine, cladribine); and CAFDA (clofarabine,

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

Clofarex) are directed. However, the cytosolic and S-phase-specific thymidine kinase 1 also plays an important role; it is responsible for the activation of the antiviral drug zidovudine (AZT) and, to a lesser degree, d4T (stavudine). Until recently, the two mitochondrial deoxynucleoside kinases, thymidine kinase 2 (TK2) and dGK (deoxyguanosine kinase), have not been in focus as drug-targeting enzymes, but the accumulating knowledge of the role these kinases play for the integrity of mitochondrial deoxyribonucleic acid and their role in apoptosis have increased interest. The efficient cytotoxic effect of drugs like CdA (cladribine) and ara-G (nelarabine) are likely to be caused by the induction of the apoptotic caspase cascade by the corresponding nucleotides. The deoxynucleoside kinases are thought to be evolutionarily closely related to and originate through duplication of a single gene. Then, only a few changes of residues in the active site were necessary for changing the specificity from pyrimidine to purine nucleosides. A thorough and detailed knowledge of the molecular basis for substrate specificity will be a valuable tool for design and development of new and more efficient nucleoside analogs for cancer chemotherapy and viral diseases.

Key Words: Deoxynucleosides; deoxyribonucleosides; kinases; nucleoside analogs; deoxynucleoside kinases; salvage; enzyme evolution; chemotherapy.

1. INTRODUCTION

The overall dominating route for activation of nucleoside analogs is the deoxynucleotide salvage pathway. The key enzymes of this pathway are the deoxynucleoside kinases (dNKs). These enzymes catalyze the transfer of the γ-phosphate group from a nucleotide phosphate donor, usually a nucleoside triphosphate like adenosine triphosphate (ATP), to the 5′-hydroxy group of the nucleoside 2′-deoxyribose. The deoxynucleosides may originate from external sources such as food or degraded dead cells or from internal sources caused by degradation of damaged deoxyribonucleic acid (DNA) during the DNA repair process. The resulting negatively charged deoxynucleoside monophosphates are trapped within the cell and are thereafter phosphorylated by nucleoside monophosphate and diphosphate kinases to the corresponding deoxynucleoside triphosphates (dNTPs), the direct DNA precursors. This rather straightforward route for dNTP biosynthesis must be distinguished from the *de novo* pathway by which the dNTPs are synthesized from small inorganic molecules through a comprehensive number of complex, and far more energy demanding, biosynthetic reactions.

The natural substrates for the dNKs are the four deoxynucleosides: thymidine (dThd), deoxycytidine (dCyd), deoxyadenosine (dAdo), and deoxyguanosine (dGuo). In mammalian cells, they are phosphorylated by four dNKs with overlapping specificity: the cytosolic kinases thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK) and the mitochondrial

kinases thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) *(1,2)*. Most of the nucleoside analogs used in the treatment of cancer and viral diseases are activated by these dNKs. In this chapter, the four human dNKs are discussed according to their origin, substrate, and analog specificity; intracellular localization; regulatory properties; and role in cytotoxicity of nucleoside analogs.

2. THE EVOLUTIONARY ORIGIN OF MULTIPLE dNKs WITH DIFFERENT SUBSTRATE SPECIFICITIES

Gene duplication plays one of the major roles in biological evolution *(3)*. Two genes that are derived from a gene duplication are said to be *paralogous*. In most organisms, genes are continuously duplicated, generating paralogs; often, afterward one of the copies is removed from the genome. However, when a set of parallel genes is created, gene copies can be functionally specialized through accumulation of mutations and afterward permanently preserved in the genome. Preservation can be achieved either by origin of a new function by one of the duplicated genes or by partitioning of the ancestral functions between the two duplicated genes (for additional references, *see* ref. *3*). Mammalian deoxynucleoside kinases represent an excellent model of gene duplication and preservation of the duplicated genes through partitioning of the substrate specificity. On duplication of the progenitor kinase, each kinase copy had to limit the original broad substrate specificity to become specialized for a limited number of native substrates.

Deoxyribonucleoside kinases exhibit much diversity among the presently analyzed organisms. Characterization and comparison of the modern enzymes can help reconstruct the ancient kinases and how they have evolved into the modern forms. The four mammalian deoxyribonucleoside kinases have the following overlapping specificities (*see* Table 1): TK1 phosphorylates only dThd. TK2 phosphorylates both deoxypyrimidines, dThd and dCyd. dCK phosphorylates dCyd, dAdo, and dGuo, and dGK phosphorylates only the purine deoxynucleosides, dAdo and dGuo.

In insects, such as the fruitfly, silk moth, and mosquito, there is only one deoxynucleoside kinase capable of phosphorylating all four natural substrates, as discovered in our laboratories *(4–7)*. The insect dNK was initially assumed to represent a direct descendent of the original enzyme, a dCK/dGK/TK2-like kinase with a broad substrate specificity *(5)*. A few random mutants of *Drosophila melanogaster* dNK (Dm-dNK) were analyzed, and the sequences and the three-dimensional (3D) structures of different kinases were compared, suggesting the key amino acid residues determining the substrate specificity *(8,9)*. These data could be used to reconstruct the evolutionary origin of Dm-dNK.

Table 1
Properties of the Human Deoxynucleoside Kinases

Enzyme name	*EC number*	*Chromosomal localization*	*Intracellular localization*	*Specificity constants*[a] k_{cat}/K_m ($M^{-1}s^{-1}$)			
				dT	*dC*	*dA*	*dG*
dCK	2.7.1.74	4q13.3–21.1	Cytoplasma (nucleus[b])	2×10^2 (0.1)	2×10^5 (100)	8×10^4 (40)	6×10^4 (30)
TK1	2.7.1.21	17q25.2–25.3	Cytoplasma	8×10^6 (100)	—[c]	—	—
TK2	2.7.1.21	16q22	Mitochondrial matrix	9×10^5 (100)	3×10^4 (3.3)	1×10^2 (0.01)	—
dGK	2.7.1.113	2q13	Mitochondrial matrix	—	1×10^3 (1.7)	4×10^3 (6.7)	6×10^4 (100)

The k_{cat} values were calculated from V_{max} values using $V_{max} = k_{cat} \times [E]$, presuming one active site per monomer. Values in parentheses are the relative k_{cat}/K_m with the principal substrate set to 100%.

[a]Values are from ref. *2*.

[b]When overexpressed *(23,24)*.

[c] Values $<10^2$ $M^{-1}s^{-1}$.

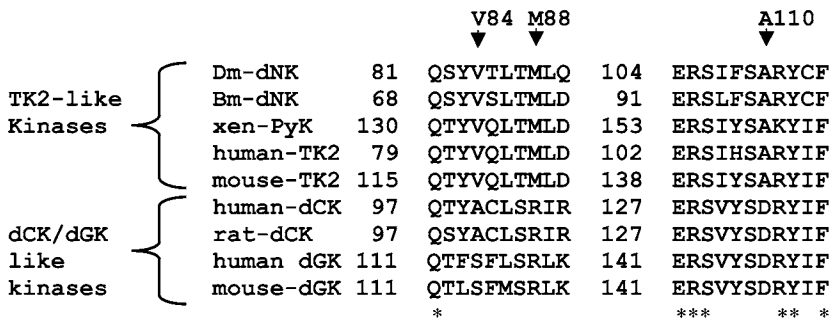


Fig. 1. Multiple alignments of the amino acid sequences from two short domains from various eukaryotic deoxynucleoside kinases. Bm-dNK, *Bombyx mori* multisubstrate dNK; Xen-PyK, *Xenopus laevis* pyrimidine dNK. The three positions in Dm-dNK that were mutated are marked with arrows. The conserved residues are marked with asterisks.

Dm-dNK mutants generated by hypermutagenesis were isolated based on their ability to increase the sensitivity of transformed thymidine kinase-deficient *Escherichia coli* KY895 to various analogs. One mutant, MuC, carrying a Val84Ala mutation, increased the sensitivity of *E. coli* only toward dCyd analogs, like ddC (zalcitabine) and ara-C (cytarabine, Cytosar) *(9,10)*. These results overlapped with the observation that valine at site 84 is present in all TK2-like kinases, whereas all eukaryotic dCKs have alanine at this site (*see* Fig. 1). Furthermore, in Dm-dNK, Val84 is one of the substrate-binding pocket residues (Fig. 2A) *(9,11)*. Thus, this residue may be at least partly responsible for the substrate specificity. When the 3D structures of Dm-dNK and dGK were compared, it was apparent that the predominantly pyrimidine-specific Dm-dNK has a large substrate cleft; purine-specific dGK has a tighter pocket (Fig. 2B,C). Dm-dNK has an extended cleft at the 5-position of the substrate pyrimidine ring, surrounded by Val84, Met88, and Ala110 *(9)*. These residues are conserved in all TK2-like enzymes but not in dCK- and dGK-like enzymes (Figs. 1 and 2). The corresponding hydrophobic cavity of the TK2-like kinases may therefore be necessary to accommodate the methyl group of thymidine.

We attempted to modify a multisubstrate enzyme, Dm-dNK, into different mutant forms with limited substrate specificity. The above-mentioned three amino acid residues, 84/88/110, were mutated into the corresponding residues found among dCK- and dGK-like enzymes (Figs. 1 and 2) *(12)*. As expected, the site-directed mutagenesis of these sites provided mutant enzymes with changed substrate specificity. For example, the triple Dm-dNK mutant, Val84Ser/Met88Arg/Ala110Asp, mimicking the substrate binding site of dGK, also showed dGK substrate specificity, phosphorylating

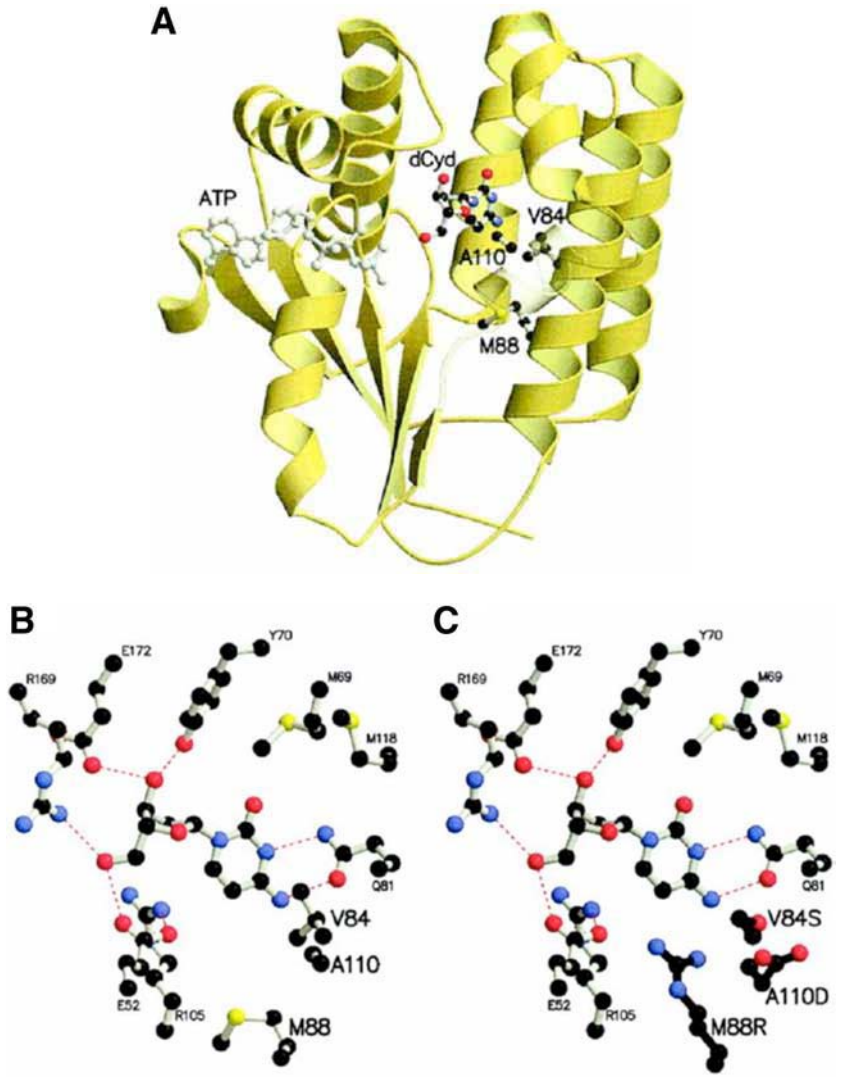


Fig. 2. Crystal structure of the Dm-dNK subunit with dCyd at the active site (pdb code 1J90) *(9)*. (**A**) Mutated residues are shown in ball-and-stick representation. ATP (white) is modeled from the herpes simplex virus TK to show location of the phosphate donor *(127)* (pdb code 1 vtk). (**B**) Close-up view of dCyd bound at the substrate site showing the positions of residues Val84, Met88, and Ala110. The dotted lines represent hydrogen bonds. (**C**) Model of the mutations V84S, M88R, and A100D based on the crystal structures of dNK and dGK. The conformation of these side chains are as in dGK *(9)*. (From ref. *12* with permission.)

exclusively purine substrates. Our results clearly demonstrated that only one to three changes of residues were necessary to convert the substrate specificities from predominantly dThd into dAdo, dCyd, or dGuo as the most-preferred substrate.

Another group solved the 3D crystal structure of dCK. In their work, they mutated two of the three residues corresponding to the above-mentioned Val84, Met88, Aal110 in Dm-dNK back to those of the Dm-dNK, creating the double-dCK mutant Arg104Met, Asp133Ala and thus reversing the mutations done by our group *(13)*. Interestingly, this mutant gained the ability to phosphorylate dThd. Further, the dCK triple-mutant Ala100Val/Arg104Met,Asp133Ala, which was created to imitate the Dm-dNK hydrophobic cavity, was found to have a 30-fold increased k_{cat} with dCyd and 9.5-fold with dFdC (gemcitabine, Gemzar) when compared with the k_{cat} values of the wild-type dCK *(13)*. Thus, the hydrophobicity of the reaction cavity may play a role in release of product from the enzyme, which is reflected in the k_{cat} value. A similar scenario, based on a limited number of single amino acid changes, could have operated during the evolutionary history of eukaryote deoxynucleoside kinases, changing their specificity and reactivity.

We assume that the first deoxynucleoside kinase had a broad substrate specificity, and that the gene was duplicated before the separation of bacteria and eukaryotes. In our model, we propose that the first eukaryote was likely to have a TK1-like kinase and a dCK/dGK/TK2-like kinase, the latter being the progenitor of the modern mammalian dCK, dGK, and TK2 kinases. On further rounds of duplication of the progenitor dCK/dGK/TK2-like kinase, the resulting copies each became specialized for a limited number of substrates. As illustrated from the mutation experiments, only a limited number of point mutations had to accumulate to achieve the final specialization.

Our studies also suggested that Dm-dNK does not represent the original, preduplication stage enzyme, but an enzyme that has undergone retrograde evolution from a specialized TK2-like enzyme toward an enzyme with a broad substrate specificity. The reason could be that insects at some point lost all but the TK2-like kinase. This enzyme was subsequently under evolutionary pressure to broaden the substrate specificity. Again, only a limited number of point mutations were necessary to remodel the specialized enzyme into a modern multisubstrate deoxynucleoside kinase. Our phylogenetic analysis supported this model because the insect dNKs group together with TK2-like pyrimidine-preferring kinases (Fig. 3) and not at the root of the modern dCK, dGK, and TK2 kinases, as would be expected if Dm-dNK represented a living fossil version of the original, nonspecialized, broadly specific kinase *(14)*.

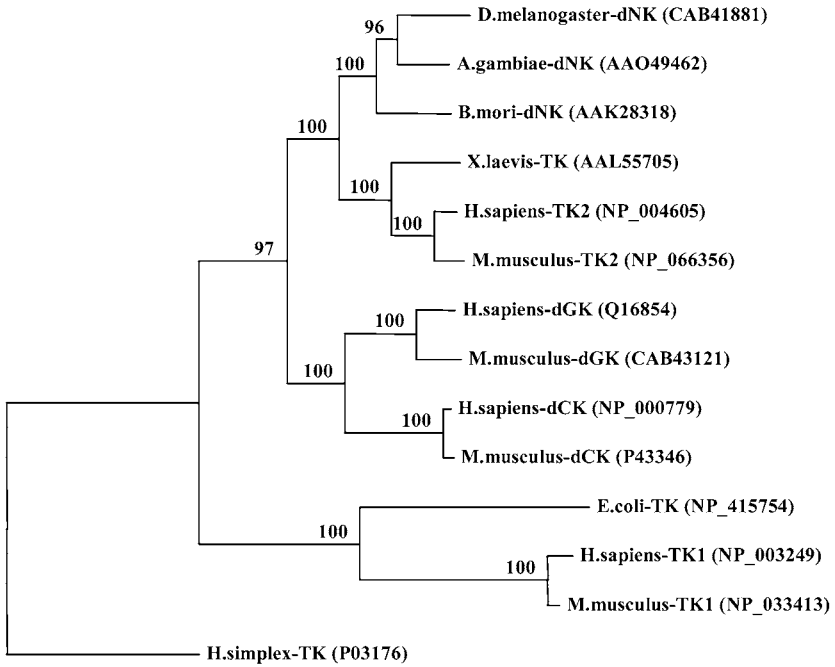


Fig. 3. Phylogenetic relationship among animal deoxynucleoside kinases (amino acid sequence accession numbers in brackets). Insect broadly specific dNKs group together with other animal TK2 kinases that only phosphorylate pyrimidine deoxynucleosides. Apparently, dNK, TK2, dCK, and dGK kinases also share a common progenitor. The phylogenetic analysis was done using *ClustalX 1.81* with standard settings and *TREECON 1.3b* by the neighbor joining method with Poisson correction for distance calculation (*see* also ref. *14*).

3. PROPERTIES OF HUMAN DEOXYNUCLEOSIDE KINASES

3.1. Deoxycytidine Kinase

dCK (NTP:deoxycytidine 5′-phosphotransferase, EC2.7.1.74) has the broadest specificity among the four mammalian dNKs. dCK phosphorylates both purine and pyrimidine deoxynucleosides, and ATP and several other nucleoside triphosphates are efficient phosphate donors *(15)*. Several investigations have indicated that UTP and not ATP is the physiological phosphate donor *(15,16)*, and kinetic investigations indeed showed a more efficient substrate phosphorylation with uridine 5′-triphosphate (UTP) as the phosphate donor compared to ATP *(15,16)*. dCyd is the principal substrate for dCK, but according to the specificity constants (i.e., the k_{cat}/K_m

values), dAdo and dGuo are also efficiently phosphorylated by dCK (*see* Table 1). Even dThd is phosphorylated by dCK, although to a very low degree, having a specificity constant of about 0.1% of that with dCyd.

Active human dCK is composed of two identical polypeptides, each consisting of 260 amino acids and with a calculated molecular weight of 30.5 kDa *(13,17)*; it is located on chromosome 4 (4q13.3–q21.1) *(18)*. dCK does not appear to be cell cycle regulated *(19)*, although the expression and levels of activity vary between different tissue types and are especially high in lymphoid tissue; it appears to be upregulated in some solid tumors *(17,20,21)*. dCK has been found to be activated by posttranslational mechanisms, probably phosphorylation *(22)*. The regulatory and substrate properties of dCK are extensively discussed in Chapter 2.

dCK is generally believed to be a cytoplasmic enzyme, but overexpression of dCK fused to the green fluorescent protein gave a nuclear localization that agreed with the presence of a nuclear import signal in the N-terminal part of the enzyme *(23)*. Another study, however, using a highly specific dCK antibody, showed that the native, endogenous dCK is located in the cytoplasm *(24)*.

3.2. Cytosolic Thymidine Kinase

TK1 (ATP:thymidine 5′-phosphotransferase, EC2.7.1.21) has the most narrow substrate specificity of the four mammalian dNKs. TK1 only phosphorylates thymidine and deoxyuridine *(25)*, but it has the highest specificity constant (k_{cat}/K_m) with its primary substrate, dThd (*see* Table 1), about 40 times higher than that of dCK with dCyd. ATP and deoxyadenosine 5′-triphosphate (dATP) are the preferred phosphate donors, but (deoxy)guanosine 5′-triphosphate (d)GTP, (deoxy)cytidine 5′-triphosphate (d)CTP, and (deoxy)uridine 5′-triphosphate (d)UTP can also serve as phosphate donors, although only with 15–30% of the efficiency with ATP *(26,27)*. Thymidine 5′-triphosphate (dTTP) is not a phosphate donor of TK1 but is an efficient and cooperative feedback inhibitor *(27,28)*.

In the active form, TK1 occurs as a homodimer or homotetramer, with each polypeptide chain consisting of 234 residues with a calculated size of 25.5 kDa *(29,30)*. The chromosomal position of the TK1 gene is 17q25.2–25.3, within a region of chromosomal loss in primary breast tumors and other diseases *(31,32)*. At present, there are no reports on the structure of TK1.

TK1 is located in the cytoplasma and is cell cycle regulated. TK1 activity and enzyme protein are absent in nondividing cells; increase during late G1, reaching maximal activity coinciding with DNA synthesis and DNA polymerase activity; and decline in G2/M *(33–35)*. The regulation of TK1 is very complex and is accomplished at transcriptional, posttranslational, as well as enzymatic levels. A comprehensive number of studies have

shown that the TK1 promoter is under control of S-phase-specific nuclear and cell cycle regulatory factors *(36,37)*.

Furthermore, the regulation of TK1 messenger ribonucleic acid (mRNA) expression appears to be disturbed or defect in some cancer cells. For instance, in lymphocytes from patients with chronic lymphatic leukemia a high level of TK1 mRNA was found, whereas TK1 activity was nearly absent *(38)*, and in HeLa cells, the human TK1 promoter appeared to be differently regulated than in normal human fibroblasts *(39)*.

Also at the posttranscriptional level, TK1 seems to be extensively regulated by proteolytic degradation as well as phosphorylation. The decline of TK1 protein in G2/M was found to be caused by a specific degradation of the protein relying on a signal within the C-terminal 40 residues (but not the last 10 residues). Deletion of the C-terminal part of TK1 prevented the degradation and stabilized TK1 all through the cell cycle *(40,41)*. It is noticeable that, when aligning human TK1 with several viral TK1-like sequences (e.g., from vaccinia virus and poxvirus), there is high identity, about 69%, but the C-terminal residues are missing. It is tempting to suggest that the viral TKs lacking the C-terminal cell-cycle-specific degradation signal escape the S-phase regulating machinery for TK1.

Serine 13 in human TK1 has been shown to be involved in mitotic phosphorylation of TK1 by cyclin-dependent kinases *(42)*, and a report indicated that this phosphorylation may contribute to the degradation signal for human TK1 by the SCF-mediated proteolytic pathway involving ubiquitylation *(43,44)*. The SCF complex catalyzes ubiquitylation of cell cycle regulatory proteins such as cyclines. The ubiquitylated proteins are then degraded in a proteasome. Ke et al. *(43)* shows that cell cycle-specific degradation of TK1 is impaired in *Saccharomyces cerevisiae* having a temperature-sensitive mutation in the SCF complex (Skp1-Cullin-1/CDC53-F-box). But mutation of serine 13 in human Tk1 to alanine results in a TK1 that is more resistant to degradation in wild-type *S. cerevisiae*. However, other signals than phosphorylation at serine 13 seem to be involved in the degradation of TK1, as the Ser13Ala mutant also was degraded in *S. cerevisiae*, although at a slower rate *(43)*.

Even though the transcriptional and posttranscriptional regulation is extensive and complex, TK1 is also regulated at the enzymatic level. Human TK1 can exist either as a dimer with low dThd affinity (K_m about 15 μ*M*) or a tetramer with high-affinity dThd (K_m about 0.5 μ*M*). The two forms have the same k_{cat}, and the transition between them is reversible and depends on ATP and enzyme concentration *(45)*. It is likely that the low-affinity dimer is dominating at the low TK1 concentrations in G1/early S phase, and that the high-affinity form dominates in late S phase, thus constituting a fine-tuning mechanism for regulation of TK1 activity during the cell cycle.

Numerous studies showed that TK1 activity is upregulated in cancerous tissues, which led to the use of TK1 as a prognostic and therapeutic marker as well as an indicator of chemotherapeutic efficiency *(46,47)*.

In the light of the intensive control of TK1 expression and activity, indicating the vital importance of a well-functioning enzyme, it is surprising that genetically modified mice deficient in TK1 were viable, although they had shortened life spans, were very poor breeders, and developed sclerosis of the kidney glomeruli and died prematurely *(48)*.

3.3. Mitochondrial Thymidine Kinase

TK2 (ATP:thymidine 5′-phosphotransferase, EC2.7.1.21 (same EC number as TK1), differs markedly from TK1 by having a broader specificity, with the ability to phosphorylate all three deoxypyrimidine nucleosides (dThd, dUrd, dCyd) with approx the same turnover *(25)* and to be constitutively expressed *(1,27,34,49)*. Even dAdo is phosphorylated, although to a very limited extent (*see* Table 1). The specificity constants for TK2 with dThd and dCyd are about nine- and seven-fold lower than those for TK1 with dThd and for dCK with dCyd, respectively. ATP and dATP are the most efficient phosphate donors, but UTP, (d)CTP, and (d)GTP also can donate the phosphate group, although less efficiently (20–35%). dUTP and dTTP are not phosphate donors; furthermore, dTTP inhibits the reaction in a noncooperative mode, whereas dUTP is without inhibitory effect *(25,27)*.

The TK2 gene is located at chromosome 16q22, but the initial reports on the cloning disagreed about the length of the polypeptide and the N-terminal sequence *(50,51)*. Although early studies indicated TK2 was a mitochondrial enzyme *(52,53)*, the cloned TK2 sequences did not contain a mitochondrial leader sequence. However, the complementary DNA (cDNA) 5′-end of the human TK2 constituting exon 1 of the 45-kb TK2 gene was cloned *(54)* and shown to have high homology to the mouse TK2 N-terminal sequence that directed the import of the mouse TK2 into mitochondria *(55)*. The entire human TK2 cDNA encoded a protein of 265 residues, of which the leader sequence constituted 33 residues. Based on the sequence data and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) experiments of the pure native and recombinant TK2s, the size of the peptide is about 30 kDa *(25,51)*.

There has been some uncertainty whether TK2 occurs as a monomer or dimer. The discrepancies seem to be caused by the choice of chromatographic material in the gel filtration experiments. Native TK2 partly purified from human lymphocytes eluted from Sephadex G-200 had a size of 60 kDa indicating a dimer *(27)*, but in other experiments, both native and cloned human TK2 eluted from Superose 12 and Superdex 200 had an apparent size of 30 kDa, which points to a monomer form *(25,54)*. When recombinant TK2 from mouse was analyzed by gel filtration on both

Superose and Sephadex, it behaved as a monomer on Superose 12 but as a dimer on Superdex 200 *(55)*. A similar discrepancy has been observed with the closely related *D. melanogaster* dNK, which appeared as a monomer or dimer, depending on the type of size determination technique applied *(4,6)*. However, the 3D crystal structure of Dm-dNK shows that it is a dimer *(9)*, and gel filtration experiments in our laboratory using Superdex 200 also showed a dimer structure (unpublished results). The complex behavior of these enzymes may be caused by the hydrophobic character of the enzymes.

There are no reports on the regulation of the transcription of TK2, but enzymatic activity is regulated through the negative cooperativity observed with dThd *(25,27,34,51)*. With dCyd, the kinetics is noncooperative. As seen in Table 1, the specificity constant with dCyd is only 3% of that with dThd, although the maximal velocities with the two substrates are in the same range, about 0.6–0.9 µmol/min/mg. But, because of the negative cooperative behavior with dThd, the velocity at low substrate concentrations is higher with dThd than with dCyd *(25,60)*.

The importance of TK2 has been demonstrated by the finding of two homozygous point mutations in TK2 in four children with the mitochondrial DNA (mtDNA) depletion syndrome *(58)*. The level of TK2 in the affected children was diminished, and they died between 19 months and 4 yr of age from devastating myopathy. In an analysis of the effect of the mutations on the activity of TK2, one of the mutations, His90Asn, was shown to change the negative cooperativity of TK2 to a hyperbolic reaction mechanism *(54,61)*. This is the first observation that clearly underlines the importance and significance of the enzyme kinetic regulation of DNA precursor enzymes for maintenance of genome integrity. In two new screening studies of the TK2 gene of patients with the mitochondrial deletion syndrome, the same mutations were identified, as were novel mutations *(62,63)*.

3.4. Mitochondrial Deoxyguanosine Kinase

dGK (NTP:deoxyguanosine 5′-phosphotransferase, EC2.7.1.113) phosphorylates the purine deoxynucleosides dAdo, dGuo, and deoxyinosine (dIno), with dGuo the preferred substrate. As seen in Table 1, although very limited, it also has activity with dCyd, as reflected by the specificity constant, which is about 2% of that with dGuo. The phosphate donor specificity is rather relaxed, with ATP and UTP the most efficient, and dATP and dGTP are feedback inhibitors.

dGK does not appear to be cell cycle regulated, and Northern blot analysis revealed a major 1.35-dGK mRNA transcript in most tissues, proliferating as well as nonproliferating *(64,65)*. The human dGK gene is located to chromosome 2p13 *(66)*, a region reported to be involved in translocations in some patients with lymphoproliferative diseases such as acute and chronic leukemia *(67)*. The human dGK cDNA encodes a polypeptide of 260 residues

with a calculated molecular weight of 30 kDa, plus an N-terminal part of 17 residues with a mitochondrial leader sequence *(65,68)*. This N-terminal sequence was shown to direct a dGK fused to the green fluorescent protein into the mitochondria *(23)*. The localization of dGK to the mitochondrial matrix was verified by biochemical studies of subcellular fractions and by *in situ* immunohistochemical analysis, and these studies showed no significant cytosolic signals *(69)*. It is, however, surprising and unexpected that dGK was observed to leak out of the mitochondrial matrix simultaneously with cytochrome c after induction of apoptosis in human epithelial and lymphoblastic cells *(70)*. These observations indicate an as-yet-unidentified role of dGK in apoptosis, but there may be an association to its role in synthesis of dATP necessary for formation of the apoptotic complex required to initiate the "caspase cascade" *(71)*.

Together with TK2, dGK is responsible for the supply of nucleotide precursors for mtDNA synthesis. Because no mitochondrial ribonucleotide reductase activity has been clearly demonstrated, mitochondria do not seem to have *de novo* deoxynucleotide synthesis. Therefore, the two mitochondrial dNKs, TK2 and dGK, must be regarded as very important for mtDNA synthesis and repair. As described Section 3.3, TK2 was found to be mutated in several individuals with mitochondrial deletion syndrome with fatal effect. In three other kindreds with the early onset hepatocerebral variant of the disease, a homozygous mutation in the dGK gene was found *(68)*. The mutation was a frameshift resulting from deletion of a single nucleotide. No dGK protein or activity was detectable in the patients, who died in early age by liver failure, encephalopathy, and mtDNA deletion in their liver mitochondria, but not in the muscle mitochondria. In another study, a novel homozygous nonsense mutation in exon 3 of the dGK gene was revealed *(72)*.

4. NUCLEOSIDE ANALOG SPECIFICITY

The antiviral or anticancer effects of the nucleoside analogs are generally exerted by their phosphorylated derivatives, through incorporation into DNA, thereby acting as elongation terminators; by distorting the helix structure; or by causing misincorporation of nucleotides on the opposite strand (*73,74*; *see* also Chapters 8 and 15). Other analogs act by interfering with the viral or cellular DNA polymerases or the dNTP *de novo* synthesis through inhibition of ribonucleoside reductase (RNR) *(75)*.

Zidovudine (AZT)-triphosphate has been shown to inhibit repair of ultraviolet-induced DNA strand breaks and mitogen-stimulated growth of human lymphocytes *(76)*. Some analogs, FIAU (fialuridine), AZT, d4T (stavudine), ddI (ddA), and 3TC (lamivudine) have been shown to interfere with mtDNA polymerase-γ or to be incorporated in mtDNA, thereby causing depletion of mtDNA, which is responsible for the myopathy and bone marrow suppression observed after long-term treatment with these analogs *(77–80)*. Lately, deoxyadenosine analogs such as CdA (cladribine),

Table 2
Properties of Nucleoside Analogs

Analog			Action mechanism	Target disease	Principal phosphorylating dNK(s)	k_{cat}/K_m (analog) ($M^{-1}s^{-1}$)[a]	Relative analog specificity[b]
Abbreviation	*Chemical name*	*Clinical name*					
Ara-C	Cytosine-β-D-arabinofuranoside	Cytosar	Inhibition of DNA synthesis	Hematological malignancies	dCK TK2	5×10^4 3×10^2	0.13 0.0003
Ara-G	Guanin-9-β-D-arabinofuranoside	Nelarabine	DNA chain termination	T-cell malignancies	dCK dGK	1×10^4 3×10^4	0.03 0.32
AZT	3′-Azido-2′,3′-dideoxythymidine	Zidovudine	DNA chain termination	HIV	TK1 TK2	3×10^6 8×10^4	0.27 0.079
CdA	2-chloro-deoxyadenosine	Cladribine	RNR inhibition, apoptosis induction	Hematological diseases	dCK dGK	6×10^4 5×10^4	0.15 0.43
ddC	2′,3′-Dideoxycytidine	Zalzitabine	DNA chain termination	HIV	dCK	1×10^4	0.03
dFdC	2′,2′-Difluoro-deoxycytidine	Gemzar	RNR inhibition, DNA pausing, DNA structure	Hematological malignancies	dCK TK2	4×10^4 2×10^4	0.11 0.02
F-Ara-A	2-Fluoroadenine-β-D-arabino-furanoside	Fludarabine	Inhibits RNR, DNA synthesis, apoptosis induction	Hematological malignancies	dCK dGK	1×10^3 4×10^3	0.003 0.06
FdU	5-Fluoro-2′-deoxyuridine		Inhibition of TMP synthase	Cancer	TK1 TK2	2×10^6 3×10^5	0.2 0.24
FLT	3′-Fluoro-3′-deoxythymidine	Alovudine	DNA chain termination	HIV	TK1	6×10^5	0.07
3TC (L-SddC)	β-L-2′,3′-Dideoxy-3′-thia-cytidine	Lamivudine	DNA chain termination	HIV and HBV	dCK TK2	1×10^5 1×10^2	0.23 0.0002

5-Aza dC	5-Aza-2′-deoxycytidine	Decitabine	Inhibits DNA chain elongation and DNA methylation	Cancer	dCK	—	—
CAFDA	2-Chloro-2-deoxy-fluoro-β-D-arabino-furanosyl)adenine	Clofarabine, Clofarex	Inhibition of DNA synthesis and RNR	Hematological malignancies	dCK dGK —	— —	—
ddI	2′,3′-Dideoxy-inosine	Didanosine	DNA chain termination (ddATP)	HIV	dCK	—	—
d4T	2′,3′-Dideoxythymidine	Stavudine	DNA chain termination	HIV	(TK1)	—	—
FIAU	1-(2′-Deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil	Fialuridine	Incorporated into mtDNA	HBV	TK2	—	—
L-FMAU	2′-Fluoro-5-methyl-β-L-arabino-furanosyl-uracil	Clevudine	HBV DNA pol inhibition	HBV	TK1 TK2 dCK	— —	— —
FMdC	(E)-2′-Deoxy-2′-(fluoromethyl)-cytidine	Tezacitabine	DNA chain termination, RNR inhibition	Cancer	dCK	—	—
L-OddC	L-(—)-Deoxy-3′-oxacytidine	Troxacitabine	DNA chain termination	HIV, HBV	dCK	—	—

—, data not available.

[a]The (k_{cat}/K_m) values for the analogs are from ref. 2, except for FLT, which is from ref. 25.

[b]The relative analog specificity for the individual enzyme is calculated using the following expression:

$$\frac{[k_{cat}/K_m \text{ (nucleoside analog)}]}{[k_{cat}/K_m \text{ (dAdo)} + k_{cat}/K_m \text{ (dGuo)} + k_{cat}/K_m \text{ (dCyd)} + k_{cat}/K_m \text{ (dThd)} + k_{cat}/K_m \text{ (nucleoside analog)}]}$$

CAFDA (clofarabine, Clofarex), and F-Ara-A (fludarabine) have been shown to induce apoptosis through activation of the apoptotic caspase cascade, leading to programmed cell death *(81–83)*. Table 2 summarizes these properties for a number of the most frequently used analogs.

4.1. Comparison of Substrate Specificities

The best tool for comparison of velocity and affinity toward various substrates is the k_{cat}/K_m value, that is, the specificity constant. This figure gives the velocity at substrate concentrations below K_m for the substrate and therefore the efficiency of phosphorylation at low and often physiological substrate concentrations. Moreover, when comparing the efficiency of an enzyme toward different analogs or between different enzymes toward the same analog, the simultaneous presence of all four deoxynucleosides in the cellular compartment should be considered. This can be done using the expression given in the footnote to Table 2 and as proposed by Kokoris et al. *(84)*. This equation reflects the relative velocity with the enzyme/analog in question at low and often physiological substrate concentrations and was used for the calculations in Table 2. The equation and its interpretation indirectly assume that the concentration of the competing native substrates in the cell is similar, but this is not necessarily true. Therefore, this equation may sometimes give a wrong impression of the in vivo situation.

4.1.1. Cytosolic Thymidine Kinase 1

TK1 has a very restricted substrate specificity and tolerates only a few substitutions. The analog now known to be most efficiently phosphorylated by TK1 is AZT, widely used in human immunodeficiency virus (HIV) therapy together with other nucleoside analogs and protease inhibitors *(85,86)*. Only the high-affinity tetramer form of TK1 seems to phosphorylate AZT, indicating that efficient phosphorylation of AZT takes place in the S phase *(28)*. FdU (5-fluoro-2′-deoxyuridine) is nearly as efficient as AZT (*see* Table 2), and quite a few 5-substituted dUrd analogs are also good substrates (5-propenyl, 5-chloro, 5-iodo, 5-bromo, and 5-ethyl dUrd); larger substitutions are not tolerated [5-propenyl, 5-(2-chloroethyl), 5-(2-bromovinyl)] *(87)*.

In another study of the binding of TK1 to thymidine model compounds linked to affinity matrices, it was found that TK1 bound better to thymidine fixed to the matrix through the 5 and 5′ positions than when fixed through the 3 and 3′ positions *(88)*. However, there seems to be some tolerance for changes at the N3 position because a number of large substitutions at the N3 position, such as *o*-carboranyl-alkyl dT, were found to be good substrates for TK1 *(89)*.

The anti-HIV drug d4T is a very poor substrate for TK1 and expectedly showed considerably less antiviral effect than AZT *(90)*. The fluorinated

pyrimidine analog FIAU and its in vivo metabolite FMAU (2′-fluoro-5-methyl-β-D-arabino-furanosyl-uracil) have been efficient substrates for both TK1 and TK2 *(91)* and initially proved to be effective against hepatitis B virus (HBV) *(92)*. However, clinical trials were halted because of severe toxicity, and the clinical signs indicated mitochondrial dysfunctions *(93)*. Later research showed that FIAU was phosphorylated in vivo by mitochondrial enzymes and interfered with mtDNA synthesis by inhibiting mtDNA polymerase and by incorporation into DNA, leading to mtDNA depletion *(94–96)*. Another novel fluoropyrimidine, L-FMAU, was also active against HBV as well as Epstein-Barr virus, but without the toxic effects of its D-analog, and did not deplete cells of mtDNA *(97,98)*. Interestingly, L-FMAU was shown to be phosphorylated by TK1, TK2, and dCK *(99)*.

4.1.2. Deoxycytidine Kinase

dCK has a very broad substrate specificity, and a large number of nucleoside analogs are phosphorylated efficiently (*see* Table 2). The antileukemic drug ara-C is probably the most used dCK-targeted drug *(100)*, but other drugs that are less susceptible for degradation by cellular deaminases have been developed, such as dFdC and CdA *(74,101)*. The action of dFdC seems to involve interference with ribonucleotide reductase *(102)* and affects the ribonucleotide pools *(103)*. A newly developed purine analog with promising potential is the hybrid analog CAFDA, which is CdA with a fluorine at the arabinosyl configuration at the 2′-position of the ribose (*see* Table 2) *(104)*. In contrast to CdA, this drug is resistant to degradation by acids and bacterial phosphorylases in the digestive channels and can therefore be given orally *(105)*. It is activated by dCK, and it seems to have higher cytotoxic effects than CdA *(106,107)*. It is at present in clinical trials against acute leukemias and solid tumors and has shown promising results *(108,109)*. It was found that dCK accepts different enantiomeric forms of the nucleosides and even in some cases prefers L-nucleosides *(110)*. dCK appears to prefer nucleosides with the sugar in the S-conformation (C2′-endo-C3′-exo) because α-ddC, which mostly is in the S-conformation, is a better substrate for dCK than β-ddC *(111)*.

4.1.3. Mitochondrial Thymidine Kinase 2

TK2, like dCK, covers a broad spectrum of substrate specificity. FdU is phosphorylated as efficiently, and the relative specificity for FdU is slightly higher for TK2 (0.24) than for TK1 (0.2) (Table 2). Arabinofuranosyl compounds with thymine or uracil as the base (AraT, AraU) are efficiently phosphorylated *(25,112)*, and even ara-C is accepted, although to a low degree *(113)*. The relative analog specificity of TK2 for ara-C is more than 400-fold lower than that of dCK (Table 2). TK2, in contrast to TK1, accepts relatively large hydrophobic substitutions at the 5-position of the base but has a low capacity to phosphorylate dideoxynucleosides like ddC, ddT, and AZT *(114)*.

It has been discussed whether TK2 plays a role in the severe side effects of AZT in spite of the low phosphorylation capacity. AZT has been found to inhibit HIV replication efficiently in macrophages even more than in dividing H9 cells *(115)*. This is in spite of the fact that TK2 is the only kinase in the nondividing macrophages *(116)*. The surprisingly good anti-HIV effect of AZT in the macrophages may be explained by the absence of catabolization of AZT combined with a low TTP pool and therefore a diminished feedback inhibition *(117)*.

Only a few inhibitors of dNKs have been reported, but a series of efficient and selective nonnucleoside inhibitors were developed. One of these, KIN-52, ((E)-5-(2-bromovinyl)-1-[(Z)-4-(triphenylmethoxy-2-butenyl]uracil), selectively and efficiently inhibited TK2 (IC_{50} = 1.3 μ*M*) and not Herpes simplex virus TK and *D. melanogaster* dNK *(118)*. Like dCK, TK2 has relaxed stereospecificty and phosphorylates the L forms of dThd and dCyd as well as L-FMAU and L-BVDU (bromovinyl-deoxyuridine) *(119,120)*.

4.1.4. Deoxyguanosine Kinase

dGK can phosphorylate many of the analogs phosphorylated by dCK (*see* Table 2), but mostly with lower efficiency. Therefore, dGK has not been the focus of interest as an analog-activating enzyme. Ara-G, however, a nucleoside analog with high efficacy toward T-cell acute lymphoblastic leukemia, is phosphorylated by dGK with a higher catalytic efficiency than dCK *(65,121)*. The relative specificity for ara-G is about 20-fold higher for dGK than for dCK (Table 2). This is in agreement with the observation that in vivo dGK seems to be the responsible activating kinase at low concentrations, and dCK seems responsible at high ara-G concentrations *(122)*. Another study showed that ara-G was indeed predominantly incorporated into mtDNA of human lymphoblastic cell lines *(123)*.

In a study of the role of purine nucleoside phosphorylase deficiency for immunedeficiency syndromes, mitochondria have been suggested to play a role in the T-cell-specific toxicity of deoxyguanosine and its analogs, which are resistant to degradation by purine nucleoside phosphorylase *(124)*. Another analog with higher specificity for dGK than dCK is CdA (Table 2), and it has been shown that the sensitivity to CdA as well as ara-G was increased by overexpression of dGK in a human pancreatic cell line *(125)*. Also, CAFDA seems to be a good substrate for dGK with a specificity constant that is about 60% of that with dGuo *(126)*.

5. SUMMARY AND CONCLUSION

Deoxyribonucleoside kinases are pivotal for the cytotoxic effect of anticancer and antiviral nucleoside analogs. The transfer of a phosphate group from a nucleotide phosphate donor, mostly ATP to the 5′-OH group of the

nucleoside, is the key step in the activation of the analog. In human cells, there are four dNKs with overlapping specificities that together are responsible for the activation of a broad spectrum of nucleoside analogs. dCK has the most relaxed specificity, and today it is the kinase toward which most of the currently used analogs, such as ara-C (Cytosar), dFdC (Gemzar), CdA (cladribine), and CAFDA (clofarabine, Clofarex) are directed. However, the cytosolic and S-phase-specific thymidine kinase TK1 also plays an important role because it is responsible for the activation of the antiviral drug AZT and, to a low degree d4T (stavudine).

Until recently, the two mitochondrial dNKs, TK2 and dGK, have not been a focus as drug-targeting enzymes, but the accumulating knowledge of the role these kinases play for the integrity of mtDNA and their role in apoptosis have increased interest. The efficient cytotoxic effect of drugs like CdA (cladribine) and ara-G (nelarabine) are likely to be caused by induction of the apoptotic caspase cascade by the corresponding nucleotides.

The non-TK1 dNKs are thought to be evolutionary closely related and to originate through duplication of a single gene. Then, only a few changes of residues in the active site were necessary for changing the specificity from pyrimidine to purine nucleosides. Thorough and detailed knowledge of the molecular basis for substrate specificity will be a valuable tool for design and development of new and more efficient nucleoside analogs for cancer chemotherapy and viral diseases.

REFERENCES

1. Arnér ESJ, Eriksson S. Mammalian deoxyribonucleoside kinases. Pharmacol Ther 1995;67:155–186.
2. Eriksson S, Munch-Petersen B, Johansson K, Eklund H. Structure and function of cellular deoxyribonucleoside kinases. Cell Mol Life Sci 2002;59:1327–1346.
3. Langkjaer RB, Cliften PF, Johnston M, Piskur J. Yeast genome duplication was followed by asynchronous differentiation of duplicated genes. Nature 2003;421: 848–852.
4. Munch-Petersen B, Piskur J, Sondergaard L. Four deoxynucleoside kinase activities from *Drosophila melanogaster* are contained within a single monomeric enzyme, a new multifunctional deoxynucleoside kinase. J Biol Chem 1998;273:3926–3931.
5. Munch-Petersen B, Knecht W, Lenz C, Sondergaard L, Piskur J. Functional expression of a multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster* and its C-terminal deletion mutants. J Biol Chem 2000;275:6673– 6679.
6. Knecht W, Petersen GE, Munch-Petersen B, Piskur J. Deoxyribonucleoside kinases belonging to the thymidine kinase 2 (TK2)-like group vary significantly in substrate specificity, kinetics and feed-back regulation. J Mol Biol 2002;315:529–540.
7. Knecht W, Petersen GE, Sandrini MP, Sondergaard L, Munch-Petersen B, Piskur J. Mosquito has a single multisubstrate deoxyribonucleoside kinase characterized by unique substrate specificity. Nucleic Acids Res 2003;31:1665–1672.

8. Knecht W, Munch-Petersen B, Piskur J. Identification of residues involved in the specificity and regulation of the highly efficient multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster.* J Mol Biol 2000;301:827–837.
9. Johansson K, Ramaswamy S, Ljungcrantz C, et al. Structural basis for substrate specificities of cellular deoxyribonucleoside kinases. Nat Struct Biol 2001;8: 616–620.
10. Solaroli N, Bjerke M, Johansson M, Karlsson A. Investigation of the substrate recognition of *Drosophila melanogaster* nucleoside kinase by site directed mutagenesis. Nucleosides Nucleotides Nucleic Acids 2004;23(8–9):1527–1529.
11. Mikkelsen NE, Johansson K, Karlsson A, et al. Structural basis for feedback inhibition of the deoxyribonucleoside salvage pathway: studies of the *Drosophila* deoxyribonucleoside kinase. Biochemistry 2003;42:5706–5712.
12. Knecht W, Sandrini MP, Johansson K, Eklund H, Munch-Petersen B, Piskur J. A few amino acid substitutions can convert deoxyribonucleoside kinase specificity from pyrimidines to purines. EMBO J 2002;21:1873–1880.
13. Sabini E, Ort S, Monnerjahn C, Konrad M, Lavie A. Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. Nat Struct Biol 2003;10:513–519.
14. Piskur J, Sandrini MP, Knecht W, Munch-Petersen B. Animal deoxyribonucleoside kinases: "forward" and "retrograde" evolution of their substrate specificity. FEBS Lett 2004;560:3–6.
15. Krawiec K, Kierdaszuk B, Eriksson S, Munch-Petersen B, Shugar D. Nucleoside triphosphate donors for nucleoside kinases: donor properties of UTP with human deoxycytidine kinase. Biochem Biophys Res Commun 1995;216:42–48.
16. Hughes TL, Hahn TM, Reynolds KK, Shewach DS. Kinetic analysis of human deoxycytidine kinase with the true phosphate donor uridine triphosphate. Biochemistry 1997;36:7540–7547.
17. Chottiner EG, Shewach DS, Datta NS, et al. Cloning and expression of human deoxycytidine kinase cDNA. Proc Natl Acad Sci USA 1991;88:1531–1535.
18. Song JJ, Walker S, Chen E, et al. Genomic structure and chromosomal localization of the human deoxycytidine kinase gene. Proc Natl Acad Sci USA 1993;90:431–434.
19. Hengstschlager M, Denk C, Wawra E. Cell cycle regulation of deoxycytidine kinase. Evidence for post-transcriptional control. FEBS Lett 1993;321:237–240.
20. Eriksson S, Arnér ESJ, Spasokoukotskaja T, et al. Properties and levels of deoxynucleoside kinases in normal and tumor cells; implications for chemotherapy. Adv Enzyme Regul 1994;34:13–25.
21. Spasokoukotskaja T, Arnér ESJ, Brosjo O, et al. Expression of deoxycytidine kinase and phosphorylation of 2- chlorodeoxyadenosine in human normal and tumour cells and tissues. Eur J Cancer 1995;31A:202–208.
22. Csapo Z, Sasvari-Szekely M, Spasokoukotskaja T, Talianidis I, Eriksson S, Staub M. Activation of deoxycytidine kinase by inhibition of DNA synthesis in human lymphocytes. Biochem Pharmacol 2001;61:191–197.
23. Johansson M, Brismar S, Karlsson A. Human deoxycytidine kinase is located in the cell nucleus. Proc Natl Acad Sci USA 1997;94:11,941–11,945.
24. Hatzis P, Al Madhoon AS, Jullig M, Petrakis TG, Eriksson S, Talianidis I. The intracellular localization of deoxycytidine kinase. J Biol Chem 1998;273: 30,239–30,243.

25. Munch-Petersen B, Cloos L, Tyrsted G, Eriksson S. Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. J Biol Chem 1991;266:9032–9038.
26. Lee L-S, Cheng Y-C. Human deoxythymidine kinase II: substrate specificity and kinetic behavior of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia. Biochemistry 1976;15: 3686–3690.
27. Munch-Petersen B. Differences in the kinetic properties of thymidine kinase isoenzymes in unstimulated and phytohemagglutinin-stimulated human lymphocytes. Mol Cell Biochem 1984;64:173–185.
28. Munch-Petersen B, Tyrsted G, Cloos L, Beck RA, Eger K. Different affinity of the two forms of human cytosolic thymidine kinase towards pyrimidine analogs. Biochim Biophys Acta 1995;1250:158–162.
29. Bradshaw HD Jr, Deininger PL. Human thymidine kinase gene: molecular cloning and nucleotide sequence of a cDNA expressible in mammalian cells. Mol Cell Biol 1984;4:2316–2320.
30. Flemington E, Bradshaw HD Jr, Traina-Dorge V, Slagel V, Deininger PL. Sequence, structure and promoter characterization of the human thymidine kinase gene. Gene 1987;52:267–277.
31. Petty EM, Miller DE, Grant AL, Collins EE, Glover TW, Law DJ. FISH localization of the soluble thymidine kinase gene (TK1) to human 17q25, a region of chromosomal loss in sporadic breast tumors. Cytogenet Cell Genet 1996;72:319–321.
32. Kalikin LM, George RA, Keller MP, et al. An integrated physical and gene map of human distal chromosome 17q24-proximal 17q25 encompassing multiple disease loci. Genomics 1999;57:36–42.
33. Tyrsted G, Munch-Petersen B. Early effects of phytohemagglutinin on induction of DNA polymerase, thymidine kinase, deoxyribonucleoside triphosphate pools and DNA synthesis in human lymphocytes. Nucleic Acids Res 1977;4:2713–2723.
34. Munch-Petersen B, Tyrsted G. Induction of thymidine kinases in phytohemagglutinin-stimulated human lymphocytes. Biochim Biophys Acta 1977;478:364–375.
35. Sherley JL, Kelly TJ. Regulation of human thymidine kinase during the cell cycle. J Biol Chem 1988;263:8350–8358.
36. Kim YK, Kim KS, Park KS. A 50-base-pair G1/S-regulated region in the promoter of the human thymidine kinase gene and its binding to factors. Mol Cells 1995;5:126–133.
37. Knight GB, Gudas JM, Pardee AB. Cell-cycle-specific interaction of nuclear DNA-binding proteins with a CCAAT sequence from the human thymidine kinase gene. Proc Natl Acad Sci USA 1987;84:8350–8354.
38. Kristensen T, Jensen HK, Munch-Petersen B. Overexpression of human thymidine kinase mRNA without corresponding enzymatic activity in patients with chronic lymphatic leukemia. Leuk Res 1994;18:861–866.
39. Chang ZF, Huang DY, Lai TC. Different regulation of the human thymidine kinase promoter in normal human diploid IMR-90 fibroblasts and HeLa cells. J Biol Chem 1995;270:27,374–27,379.
40. Kauffman MG, Kelly TJ. Cell cycle regulation of thymidine kinase: residues near the carboxyl terminus are essential for the specific degradation of the enzyme at mitosis. Mol Cell Biol 1991;11:2538–2546.

41. Kauffman MG, Rose PA, Kelly TJ. Mutations in the thymidine kinase gene that allow expression of the enzyme in quiescent (G0) Cells. Oncogene 1991;6:1427– 1435.
42. Chang ZF, Huang DY, Chi LM. Serine 13 is the site of mitotic phosphorylation of human thymidine kinase. J Biol Chem 1998;273:12,095–12,100.
43. Ke PY, Yang CC, Tsai IC, Chang ZF. Degradation of human thymidine kinase is dependent on serine-13 phosphorylation: involvement of the SCF-mediated pathway. Biochem J 2003;370(pt 1):265–273.
44. Ke PY, Kuo YY, Hu CM, Chang ZF. Control of dTTP pool size by anaphase promoting complex/cyclosome is essential for the maintenance of genetic stabilty. Genes Dev 2005;19:1920–1933.
45. Munch-Petersen B, Tyrsted G, Cloos L. Reversible ATP-dependent transition between two forms of human cytosolic thymidine kinase with different enzymatic properties. J Biol Chem 1993;268:15,621–15,625.
46. Mizutani Y, Wada H, Yoshida O, Fukushima M, Nakao M, Miki T. Significance of thymidine kinase activity in renal cell carcinoma. J Urol 2003;169:706–709.
47. O'Neill KL, Buckwalter MR, Murray BK. Thymidine kinase: diagnostic and prognostic potential. Expert Rev Mol Diagn 2001;1:428–433.
48. Dobrovolsky VN, Bucci T, Heflich RH, Desjardins J, Richardson FC. Mice deficient for cytosolic thymidine kinase gene develop fatal kidney disease. Mol Genet Metab 2003;78:1–10.
49. Kit S. Thymidine kinase. Microbiol Sci 1985;2:369–375.
50. Johansson M, Karlsson A. Cloning of the cDNA and chromosome localization of the gene for human thymidine kinase 2. J Biol Chem 1997;272:8454–8458.
51. Wang L, Munch-Petersen B, Herrstrom SA, et al. Human thymidine kinase 2: molecular cloning and characterisation of the enzyme activity with antiviral and cytostatic nucleoside substrates. FEBS Lett 1999;443:170–174.
52. Kit S, Leung W-C. Submitochondrial localization and characteristics of thymidine kinase molecular forms in parental and kinase-deficient HeLa cells. Biochem Genet 1974;11:231–247.
53. Lee L-S, Cheng Y-C. Human deoxythymidine kinase. I. Purification and general properties of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia. J Biol Chem 1976;251:2600–2604.
54. Wang L, Saada A, Eriksson S. Kinetic properties of mutant human thymidine kinase 2 suggest a mechanism for mitochondrial DNA depletion myopathy. J Biol Chem 2003;278:6963–6968.
55. Wang L, Eriksson S. Cloning and characterization of full-length mouse thymidine kinase 2: the N-terminal sequence directs import of the precursor protein into mitochondria. Biochem J 2000;351(pt 2):469–476.
56. Petrakis TG, Ktistaki E, Wang L, Eriksson S, Talianidis I. Cloning and characterization of mouse deoxyguanosine kinase. Evidence for a cytoplasmic isoform. J Biol Chem 1999;274:24,726–24,730.
57. Jansson O, Bohman C, Munch-Petersen B, Eriksson S. Mammalian thymidine kinase 2. Direct photoaffinity labeling [^{32}P]dTTP of the enzyme from spleen, liver, heart, and brain. Eur J Biochem 1992;206:485–490.
58. Saada A, Shaag A, Mandel H, Nevo Y, Eriksson S, Elpeleg O. Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. Nat Genet 2001;29:342–344.

59. Herrstrom Sjoberg A, Wang L, Eriksson S. Antiviral guanosine analogs as substrates for deoxyguanosine kinase: implications for chemotherapy. Antimicrob Agents Chemother 2001;45:729–742.
60. Wehelie R, Eriksson S, Wang L. Effect of fluoropyrimidines on the growth of *Ureaplasma urealyticum*. Nucleosides Nucleotides Nucleic Acids 2004;23: 1499–1502.
61. Wang L, Limongelli A, Vila MR, Carrara F, Zeviani M, Eriksson S. Molecular insight into mitochondrial DNA depletion in two patients with novel mutations in the deoxyguanosine kinase and thymidine kinase 2 genes. Mol Genet Metab 2005;84:75–82.
62. Mancuso M, Salviati L, Sacconi S, et al. Mitochondrial DNA depletion: mutations in thymidine kinase gene with myopathy and SMA. Neurology 2002;59:1197–1202.
63. Carrozzo R, Bornstein B, Lucioli S, et al. Mutation analysis in 16 patients with mtDNA depletion. Hum Mutat 2003;21:453, 454.
64. Johansson M, Karlsson A. Cloning and expression of human deoxyguanosine kinase cDNA. Proc Natl Acad Sci USA 1996;93:7258–7262.
65. Wang L, Hellman U, Eriksson S. Cloning and expression of human mitochondrial deoxyguanosine kinase cDNA. FEBS Lett 1996;390:39–43.
66. Johansson M, Bajalica-Lagercrantz S, Lagercrantz J, Karlsson A. Localization of the human deoxyguanosine kinase gene to chromosome 2p13. Genomics 1996;38:450, 451.
67. Berkowicz M, Toren A, Rosner E, et al. Translocation (2; 14)(p13; q32) in CD10+; CD13+ acute lymphatic leukemia. Cancer Genet Cytogenet 1995;83:140–143.
68. Mandel H, Szargel R, Labay V, et al. The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. Nat Genet 2001;29:337–341.
69. Jullig M, Eriksson S. Mitochondrial and submitochondrial localization of human deoxyguanosine kinase. Eur J Biochem 2000;267:5466–5472.
70. Jullig M, Eriksson S. Apoptosis induces efflux of the mitochondrial matrix enzyme deoxyguanosine kinase. J Biol Chem 2001;276:24,000–24,004.
71. Purring-Koch C, McLendon G. Cytochrome c binding to Apaf-1: the effects of dATP and ionic strength. Proc Natl Acad Sci USA 2000;97:11,928–11,931.
72. Taanman JW, Kateeb I, Muntau AC, Jaksch M, Cohen N, Mandel H. A novel mutation in the deoxyguanosine kinase gene causing depletion of mitochondrial DNA. Ann Neurol 2002;52:237–239.
73. Macchi B, Mastino A. Pharmacological and biological aspects of basic research on nucleoside-based reverse transcriptase inhibitors. Pharmacol Res 2002;46:473–482.
74. Plunkett W, Huang P, Searcy CE, Gandhi V. Gemcitabine: preclinical pharmacology and mechanisms of action. Semin Oncol 1996;23:3–15.
75. Ostruszka LJ, Shewach DS. The role of DNA synthesis inhibition in the cytotoxicity of 2′,2′-difluoro-2′-deoxycytidine. Cancer Chemother Pharmacol 2003.
76. Munch-Petersen B. Azidothymidine inhibits mitogen stimulated growth and DNA-repair in human peripheral lymphocytes. Biochem Biophys Res Commun 1988;157:1369–1375.
77. Richman DD, Fischl MA, Griego MH, et al. The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. N Engl J Med 1987;317:192–197.

78. Arnaudo E, Dalakas M, Shanske S, Moraes CT, DiMauro S, Schon EA. Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy. Lancet 1991;337:508–510.
79. Johnson AA, Ray AS, Hanes J, et al. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. J Biol Chem 2001;276:40,847–40,857.
80. Kleiner DE, Gaffey MJ, Sallie R, et al. Histopathologic changes associated with fialuridine hepatotoxicity. Mod Pathol 1997;10:192–199.
81. Carson DA, Wasson DB, Esparza LM, Carrera CJ, Kipps TJ, Cottam HB. Oral antilymphocyte activity and induction of apoptosis by 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine. Proc Natl Acad Sci USA 1992;89:2970–2974.
82. Genini D, Adachi S, Chao Q, et al. Deoxyadenosine analogs induce programmed cell death in chronic lymphocytic leukemia cells by damaging the DNA and by directly affecting the mitochondria. Blood 2000;96:3537–3543.
83. Carson DA, Leoni LM. Hairy-cell leukaemia as a model for drug development. Best Pract Res Clin Haematol 2003;16:83–89.
84. Kokoris MS, Sabo P, Adman ET, Black ME. Enhancement of tumor ablation by a selected HSV-1 thymidine kinase mutant. Gene Therapy 1999;6:1415–1426.
85. Furman PA, Fyfe JA, St Clair MH, et al. Phosphorylation of 3′-azido-3′deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc Natl Acad Sci USA 1986;83:8333–8337.
86. Fischl MA, Richman DD, Griego MH, et al. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. N Engl J Med 1987;317:185–191.
87. Eriksson S, Kierdaszuk B, Munch-Petersen B, Oberg B, Johansson NG. Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. Biochem Biophys Res Commun 1991;176:586–592.
88. Beck RA, Munch-Petersen B, Dolker M, Cloos L, Tyrsted G, Eger K. Ligands for the affinity chromatography of mammalian thymidine kinase. 1: Strategy, synthesis and evaluation. Pharm Acta Helv 1996;71:279–291.
89. Lunato AJ, Wang J, Woollard JE, Anisuzzaman AK, Ji W, Rong FG, et al. Synthesis of 5-(carboranylalkylmercapto)-2′-deoxyuridines and 3-(carboranylalkyl)thymidines and their evaluation as substrates for human thymidine kinases 1 and 2. J Med Chem 1999;42:3378–3389.
90. Balzarini J, Herdewijn P, De Clercq E. Differential patterns of intracellular metabolism of 2′,3′- didehydro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. J Biol Chem 1989;264:6127–6133.
91. Wang J, Eriksson S. Phosphorylation of the anti-hepatitis B nucleoside analog 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil (FIAU) by human cytosolic and mitochondrial thymidine kinase and implications for cytotoxicity. Antimicrob Agents Chemother 1996;40:1555–1557.
92. Lewis W, Levine ES, Griniuviene B, et al. Fialuridine and its metabolites inhibit DNA polymerase gamma at sites of multiple adjacent analog incorporation, decrease mtDNA abundance, and cause mitochondrial structural defects in cultured hepatoblasts. Proc Natl Acad Sci USA 1996;93:3592–3597.
93. McKenzie R, Fried MW, Sallie R, et al. Hepatic failure and lactic acidosis to fialuridine (FIAU), an investigational nucleoside analogue for chronic hepatitis B. N Engl J Med 1995;333:1099–1105.

94. Lewis W, Levine ES, Griniuviene B, et al. Fialuridine and its metabolites inhibit DNA polymerase gamma at sites of multiple adjacent analog incorporation, decrease mtDNA abundance, and cause mitochondrial structural defects in cultured hepatoblasts. Proc Natl Acad Sci USA 1996;93:3592–3597.
95. Horn DM, Neeb LA, Colacino JM, Richardson FC. Fialuridine is phosphorylated and inhibits DNA synthesis in isolated rat hepatic mitochondria. Antiviral Res 1997;34:71–74.
96. Lewis W, Copeland WC, Day BJ. Mitochondrial DNA depletion, oxidative stress, and mutation: mechanisms of dysfunction from nucleoside reverse transcriptase inhibitors. Lab Invest 2001;81:777–790.
97. Ma T, Pai SB, Zhu YL, et al. Structure–activity relationships of 1-(2-deoxy-2-fluoro-β-L-arabinofuranosyl)pyrimidine nucleosides as anti-hepatitis B virus agents. J Med Chem 1996;39:2835–2843.
98. Aguesse-Germon S, Liu SH, Chevallier M, et al. Inhibitory effect of 2′-fluoro-5-methyl-beta-L-arabinofuranosyl-uracil on duck hepatitis B virus replication. Antimicrob Agents Chemother. 1998;42:369–376.
99. Liu SH, Grove KL, Cheng YC. Unique metabolism of a novel antiviral L-nucleoside analog, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil: a substrate for both thymidine kinase and deoxycytidine kinase. Antimicrob Agents Chemother 1998;42:833–839.
100. Freund A, Boos J, Harkin S, et al. Augmentation of 1-β-D-arabinofuranosylcytosine (ara-C) cytotoxicity in leukaemia cells by co-administration with antisignalling drugs. Eur J Cancer 1998;34:895–901.
101. Gandhi V, Plunkett W. Modulatory activity of 2′,2′-difluorodeoxycytidine on the phosphorylation and cytotoxicity of arabinosyl nucleosides. Cancer Res 1990;50: 3675–3680.
102. Ostruszka LJ, Shewach DS. The role of DNA synthesis inhibition in the cytotoxicity of 2′,2′-difluoro-2′-deoxycytidine. Cancer Chemother Pharmacol 2003;52: 325–332.
103. van Haperen VW, Veerman G, Vermorken JB, Pinedo HM, Peters G. Regulation of phosphorylation of deoxycytidine and 2′,2′-difluorodeoxycytidine (gemcitabine); effects of cytidine 5′-triphosphate and uridine 5′-triphosphate in relation to chemosensitivity for 2′,2′-difluorodeoxycytidine. Biochem Pharmacol 1996;51:911–918.
104. Parker WB, Shaddix SC, Chang CH, et al. Effects of 2-chloro-9-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)adenine on K562 cellular metabolism and the inhibition of human ribonucleotide reductase and DNA polymerases by its 5′-triphosphate. Cancer Res 1991;51:2386–2394.
105. Cottam HB, Wasson DB, Shih HC, et al. New adenosine kinase inhibitors with oral antiinflammatory activity: synthesis and biological evaluation. J Med Chem 1993;36:3424–3430.
106. Mansson E, Flordal E, Liliemark J, et al. Down-regulation of deoxycytidine kinase in human leukemic cell lines resistant to cladribine and clofarabine and increased ribonucleotide reductase activity contributes to fludarabine resistance. Biochem Pharmacol 2003;65:237–247.
107. Lindemalm S, Liliemark J, Gruber A, et al. Comparison of cytotoxicity of 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (clofarabine) with cladribine in mononuclear cells from patients with acute myeloid and chronic lymphocytic leukemia. Haematologica 2003;88:324–332.

108. Kantarjian H, Gandhi V, Cortes J, et al. Phase 2 clinical and pharmacologic study of clofarabine in patients with refractory or relapsed acute leukemia. Blood 2003;102:2379–2386.
109. Kantarjian HM, Gandhi V, Kozuch P, et al. Phase I clinical and pharmacology study of clofarabine in patients with solid and hematologic cancers. J Clin Oncol 2003;21:1167–1173.
110. Wang J, Choudhury D, Chattopadhyaya J, Eriksson S. Stereoisomeric selectivity of human deoxyribonucleoside kinases. Biochemistry 1999;38:16,993–16,999.
111. Van Roey P, Taylor EW, Chu CK, Schinazi RF. Correlation of molecular conformation and activity of reverse transcriptase inhibitors. Ann N Y Acad Sci 1990; 616:29–40.
112. Eriksson S, Kierdaszuk B, Munch-Petersen B, Oberg B, Johansson NG. Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. Biochem Biophys Res Commun 1991;176:586–592.
113. Wang L, Munch-Petersen B, Herrstrom SA, et al. Human thymidine kinase 2: molecular cloning and characterisation of the enzyme activity with antiviral and cytostatic nucleoside substrates. FEBS Lett 1999;443:170–174.
114. Eriksson S, Kierdaszuk B, Munch-Petersen B, Oberg B, Johansson NG. Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. Biochem Biophys Res Commun 1991;176:586–592.
115. Perno CF, Yarchoan R, Cooney DA, et al. Inhibition of human immunodeficiency virus (HIV-1/HTLV-III$_{Ba-L}$) replication in fresh and cultured human peripheral blood monocytes/macrophages by azidothymidine and related 2′,3′- dideoxynucleosides. J Exp Med 1988;168:1111–1125.
116. Arner ES, Valentin A, Eriksson S. Thymidine and 3′-azido-3′-deoxythymidine metabolism in human peripheral blood lymphocytes and monocyte-derived macrophages. A study of both anabolic and catabolic pathways. J Biol Chem 1992;267:10,968–10,975.
117. Munch-Petersen B. Azidothymidine inhibits mitogen stimulated growth and DNA-repair in human peripheral lymphocytes. Biochem Biophys Res Commun 1998;157:1369–1375.
118. Balzarini J, Hernandez AI, Roche P, et al. Non-nucleoside inhibitors of mitochondrial thymidine kinase (TK-2) differentially inhibit the closely related herpes simplex virus type 1 TK and *Drosophila melanogaster* multifunctional deoxynucleoside kinase. Mol Pharmacol 2003;63:263–270.
119. Zhu YL, Dutschman DE, Liu SH, Bridges EG, Cheng YC. Anti-hepatitis B virus activity and metabolism of 2′,3′-dideoxy-2′,3′-didehydro-beta-L(-)-5-fluorocytidine. Antimicrob Agents Chemother 1998;42:1805–1810.
120. Verri A, Focher F, Priori G, et al. Lack of enantiospecificity of human 2′-deoxycytidine kinase: relevance for the activation of β-L-deoxycytidine analogs as antineoplastic and antiviral agents. Mol Pharmacol 1997;51:132–138.
121. Lotfi K, Mansson E, Peterson C, Eriksson S, Albertioni F. Low level of mitochondrial deoxyguanosine kinase is the dominant factor in required resistance to 9-beta-D-arabinofuranosylguanine cytotoxicity. Biochem Biophys Res Commun 2002;293:1489–1496.
122. Rodriguez CO Jr, Mitchell BS, Ayres M, Eriksson S, Gandhi V. Arabinosylguanine is phosphorylated by both cytoplasmic deoxycytidine kinase and mitochondrial deoxyguanosine kinase. Cancer Res 2002;62:3100–3105.

123. Curbo S, Zhivotovsky B, Johansson M, Karlsson A. Effects of 9-β-D-arabinofuranosylguanine on mitochondria in CEM T-lymphoblast leukemia cells. Biochem Biophys Res Commun 2003;307:942–947.
124. Arpaia E, Benveniste P, Di Cristofano A, et al. Mitochondrial basis for immune deficiency. Evidence from purine nucleoside phosphorylase-deficient mice. J Exp Med 2000;191:2197–2208.
125. Zhu C, Johansson M, Permert J, Karlsson A. Enhanced cytotoxicity of nucleoside analogs by overexpression of mitochondrial deoxyguanosine kinase in cancer cell lines. J Biol Chem 1998;273:14,707–14,711.
126. Sjoberg AH, Wang L, Eriksson S. Substrate specificity of human recombinant mitochondrial deoxyguanosine kinase with cytostatic and antiviral purine and pyrimidine analogs. Mol Pharmacol 1998;53:270–273.
127. Wild K, Bohner T, Folkers G, Schulz GE. The structures of thymidine kinase from herpes simplex virus type 1 in complex with substrates and a substrate analogue. Protein Sci 1997;6:2097–2106.

4 Nucleotidases and Nucleoside Analog Cytotoxicity

Sally Anne Hunsucker, PhD,
Beverly S. Mitchell, MD, PhD
and Jozef Spychala, PhD

CONTENTS

SUMMARY

Nucleoside analogs are an important part of therapeutic strategies in a broad range of diseases, especially cancer and viral infections. Most nucleoside analogs need to be phosphorylated to attain full clinical potency; thus, knowledge of the metabolism of this class of drugs is required to improve their clinical use. The 5′-nucleotidases are a family of enzymes that catalyze the final dephosphorylation step of nucleotides in the cell and, by opposing the activation step catalyzed by nucleoside kinases, initiate subsequent purine and pyrimidine catabolism. They also catalyze a critical step in nucleotide analog degradation; therefore, their expression and regulation in various tissues will likely have an impact on a nucleoside drug's half-life in the human body. Numerous studies in vitro and in vivo indicate that increased expression of 5′-nucleotidase may decrease nucleoside analog activation and thereby contribute to drug resistance. Because cloned 5′-nucleotidases have

From: *Cancer Drug Discovery and Development:*
Deoxynucleoside Analogs in Cancer Therapy
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

been described in human tissues, it is not always possible to assess which particular 5′-nucleotidase is important in nucleoside drug catabolism. In this chapter, we review the properties of all cloned 5′-nucleotidases and the important role of these enzymes in nucleoside analog metabolism and clinical resistance.

Key Words: Drug metabolism; drug resistance; nucleoside analogs; 5′-nucleotidase.

1. INTRODUCTION

The deoxyribonucleoside triphosphates (dNTPs) required for DNA synthesis are either produced *de novo* from ribonucleoside diphosphates by ribonucleotide reductase, or salvaged from the extracellular or intracellular pools of deoxynucleosides by phosphorylation to the triphosphate form in a stepwise cascade of kinase reactions. Because the maintenance of balanced dNTP pools is critical for accurate DNA replication and repair (reviewed in ref. *1*), regulatory mechanisms operate at multiple levels in both the *de novo* and salvage pathways. Among these, the least understood are the mechanisms that regulate deoxynucleotide catabolism. The most critical step in the catabolic pathway is the dephosphorylation of deoxynucleoside monophosphates catalyzed by 5′-nucleotidases, which ultimately leads to the release of purine and pyrimidine bases from cells. The important role of these enzymes in nucleotide pool regulation is emphasized by the severe perturbations in pyrimidine nucleotide pools that result from deficiency of 5′-nucleotidase in erythrocytes, leading to hereditary hemolytic anemia *(2)*.

In combination with nucleoside kinases (described in detail in other chapters of this book), 5′-nucleotidases form substrate cycles that seem to be critical for the accurate maintenance of deoxynucleotide pools (Fig. 1). Nucleoside analogs are designed to mimic the natural nucleosides and take advantage of the kinases of the deoxyribonucleoside salvage pathway for phosphorylation to the triphosphate form. Analog monophosphates are also substrates of the 5′-nucleotidases, and the relative level of activating kinase to catabolic nucleotidase will determine in part the therapeutic efficiency of these compounds.

Seven human 5′-nucleotidases have been cloned; one encodes an extracellularly oriented nucleotidase, one encodes a mitochondrial nucleotidase, and the remaining five encode cytosolic enzymes (Table 1). These nucleotidases have different, but overlapping, substrate specificities, providing a way for nucleotide metabolism to be regulated to meet the metabolic needs of different cell types. This chapter discusses the role of 5′-nucleotidases in the maintenance of nucleotide pools, their properties in relation to deoxynucleotide metabolism, and the evidence that these enzymes may be involved in clinical resistance to nucleoside analogs.

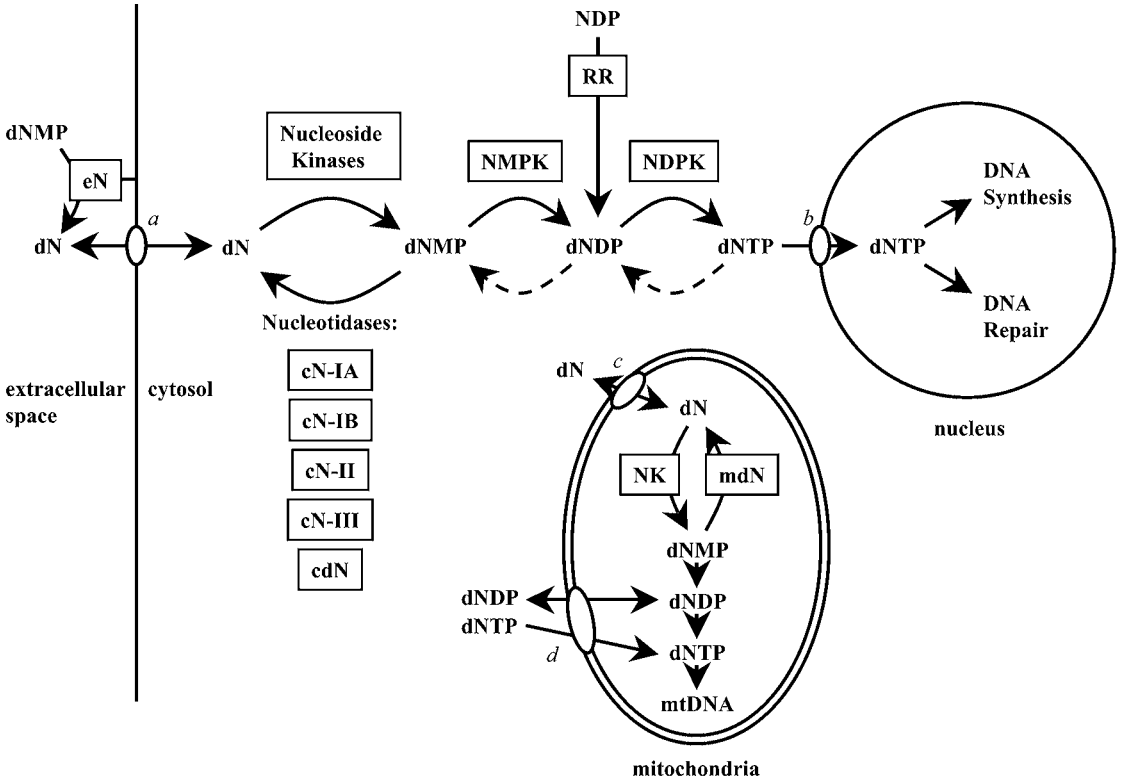


Fig. 1. Scheme depicting the anabolic and catabolic pathways leading to deoxyribonucleoside triphosphates in different cellular compartments. dN, deoxynucleoside; dNMP; deoxynucleoside monophosphate; dNDP; deoxynucleoside diphosphate; dNTP, deoxynucleoside triphosphate; NK, nucleoside kinase; NMPK, nucleoside monophosphate kinase; NDPK, nucleoside diphosphate kinase; RR, ribonucleotide reductase; *a*, nucleoside transporter; *b*, nuclear pore; *c*, mitochondrial nucleoside transporter; *d*, mitochondrial deoxynucleotide carrier.

Table 1
5′-Nucleotidases

5′-Nucleotidase (gene name)	*Description*	*Protein structure*	*Human cytogenetic localization*	*GenBank accession numbers*	*Aliases*	*References*
eN (NT5E)	5′-Specific nucleotidase that hydrolyzes extracellular purine and pyrimidine deoxy- and ribo-nucleoside monophosphates; variable expression in most tissues and cells.	Glycosylphos -phatidylinositol-linked membrane glycoprotein (dimer) subunits: 63 kDa; has a PLC-cleaved soluble variant	6q14–q21	Human, NM_002526; murine, NM_011851	Ecto-5′-NT, NT5, eNT, low K_m 5′-NT, CD73	*52*, *125*, *126*
cN-IA (NT5C1A)	5′-Specific nucleotidase that hydrolyzes purine and pyrimidine deoxyribonucleotides and AMP; highly expressed in skeletal and heart muscle and brain	Cytosolic (tetramer) subunits: 41 kDa	1p34.3-p33	Human, AF331801 and NM_032526; pigeon, AJ131243	AMP-specific 5′-NT, cN-I	*66*, *67*
cN-IB (NT5C1B)	5′-Specific nucleotidase that hydrolyzes AMP; highly expressed in testis	Cytosolic	2p24.3	Human, AF417165, NM_033253, and NM_001002006; murine, NM_027588	Homolog of cN-I, cN-I, AIRP	*39*

cN-II (NT5C2)	5′-Specific nucleotidase that hydrolyzes purine (inosine and guanosine) monophosphates; possesses phosphotransferase activity; ubiquitous expression	Cytosolic (tetramer) subunits: 65 kDa	10q24.33	Human, D38524 and NM_012229; bovine, U73690	High K_m 5′-NT, purine 5′-NT, GMP-IMP-specific 5′-NT	*42, 75, 77, 79, 127*
cN-III (NT5C3)	5′-Specific nucleotidase that hydrolyzes preferentially CMP and UMP; possesses phosphotransferase activity; most likely ubiquitous	Cytosolic (monomer) 36 kDa	7p14.3	Human, AF312735 and NM_016489	PN-I, P5′N-1, UMPH-1	*87, 94, 128*
cdN (NT5C)	Preferentially hydrolyzes 5′-phosphates of deoxynucleosides and 2′(3′)-phosphates of ribonucleosides; some preparations have phospho-transferase activity; ubiquitous; highly expressed in pancreas, heart, and skeletal muscle	Cytosolic (dimer) subunits: 23 kDa	17q25. 321–23	Human, AF154829 and NM_014595; murine, AF078840 and NM_015807	dNT-1, PN-II, P5′N-II, UMPH-2	*97, 99*

(Continued)

Table 1 *(Continued)*

5′-Nucleotidase (gene name)	*Description*	*Protein structure*	*Human cytogenetic localization*	*GenBank accession numbers*	*Aliases*	*References*
mdN (NT5M)	Mitochondrial homolog to cdN with pronounced specificity for uracil and thymine nucleoside monophosphates; highly expressed in heart, skeletal muscle, and brain	Mitochondrial (dimer) subunits: 26 kDa with mitochondrial import sequence	17p11.2	Human, AF210652 and NM_020201	dNT-2	*102*

GenBank accession numbers relate to the earliest deposition and include a unified NM numeration (reference sequences), where available. Molecular size of the subunit derived from human cDNA (does not include posttranslational modifications such as glycosylation, signal peptide cleavage, etc.). Chromosomal localization derived from the Human Genome Project and approved gene symbols from HUGO Gene Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature).

2. MAINTENANCE OF DEOXYNUCLEOTIDE POOLS

Deoxynucleotide pools provide the necessary substrates for nuclear and mitochondrial DNA (mtDNA) synthesis and repair *(3)*, and deoxycytidine triphosphate (dCTP) may also contribute to the synthesis of phospholipids *(4,5)*. Thus, the maintenance of deoxynucleotide pools has an important bearing on broad aspects of cell integrity and survival. Perturbations in the supply of DNA precursors may cause spontaneous and induced genetic instability, misincorporation of nucleotides, reduced fidelity of DNA synthesis, decreased effectiveness of DNA repair, and a decreased frequency of immunoglobulin gene rearrangement in B cells *(6,7)*. The imbalance in deoxynucleotides may also cause enhanced sensitivity to mutagens, chromosome breakage, and other genetic aberrations (reviewed in ref. *8*).

2.1. Biosynthetic vs Catabolic Pathways and Substrate Cycles

The pathways leading to dNTP synthesis have been fairly well defined. The *de novo* pathway uses ribonucleoside diphosphates from the general ribonucleotide pools and transforms them into deoxynucleoside diphosphates in a single step catalyzed by ribonucleotide reductase *(9)*, followed by phosphorylation by nucleoside diphosphate kinases (NDPKs). The salvage pathway uses preformed deoxynucleosides and a cascade of deoxynucleoside kinases, deoxynucleoside monophosphate kinases, and NDPKs. Interestingly, the two biosynthetic pathways are not equivalent in their ability to provide specific precursors for DNA synthesis and repair. For example, inhibition of ribonucleotide reductase (and thus the *de novo* synthesis pathway) with hydroxyurea leads primarily to depletion of purine deoxynucleotides and a twofold increase in the deoxythymidine 5′-triphosphate (dTTP) pool *(10–13)*.

The increased levels of deoxyadenosine and deoxyguanosine found in adenosine deaminase (ADA) deficiency and purine nucleoside phosphorylase (PNP) deficiency, respectively, result in decreased purine catabolism and high deoxyadenosine 5′-triphosphate (dATP) and deoxyguanosine 5′-triphosphate (dGTP) levels, severely perturbing DNA synthesis as well as impairing lymphocyte development *(14–17)*. On the other hand, dCTP pools expand more readily in the presence of excess cytidine than in the presence of exogenous deoxycytidine *(18)* and seem to be functionally compartmentalized: dCTP for DNA synthesis originates mainly from the *de novo* pathway, and dCTP for DNA repair arises from the salvage pathway *(5,19,20)*. Unlike other nucleoside kinases, expression of cytosolic thymidine kinase 1 (TK1) is limited to S phase; accordingly, thymidine salvage outside S phase and in resting cells can only occur through mitochondrial thymidine kinase 2 (TK2) *(21,22)*.

Another example of differential regulation of deoxynucleotide pools has been demonstrated in myeloid cells, where withdrawal of the cytokine

interleukin 3 led to a rapid decrease in dATP, dGTP, and dTTP pools without affecting dCTP levels *(23)*, reminiscent of the unique accumulation of dCTP in V79 hamster fibroblasts lacking deoxycytidylate (dCMP) deaminase *(24)*. These examples illustrate the complexities of deoxynucleotide metabolism and show that much needs to be done to understand fully the regulatory mechanisms of *de novo* and salvage pathways.

Much less information is available on the catabolic pathways of deoxynucleotides. Although the extracellular ecto-adenosine triphosphatase and ecto-adenosine phosphatase that are involved in the breakdown of free nucleotides outside the cell have been studied in detail *(25)*, little is known about intracellular deoxyribonucleoside diphosphate and triphosphate hydrolases that may also (if present) regulate deoxynucleotide pools. A nuclear deoxyribonucleotide triphosphatase has been described with a preference for dATP and dGTP, but the role that this enzyme plays in deoxynucleotide metabolism is still unclear *(26,27)*.

Ribonucleotide and deoxyribonucleotide pools have different metabolic fates in cells. DNA turns over several orders of magnitude more slowly than ribonucleic acid (RNA), and with the exception of DNA repair, degradation occurs only after cell death. Consequently, catabolic pathways are likely to be less pronounced in deoxyribonucleotide than ribonucleotide degradation, and this may have a significant impact in cases of overexpansion of dNTP pools in pathological situations. Increased catabolism of deoxyribonucleotides could lead to the accumulation of deoxynucleoside monophosphates and consequently to the generation of nucleosides through intracellular 5′-nucleotidases.

The involvement of deoxyribonucleotide monophosphate dephosphorylation in countering the imbalance of dNTP pools was elegantly demonstrated in V79 cells that lack dCMP deaminase *(24)*. In these cells, in contrast to the wild-type strain, there is a large expansion of the dCTP pool and a massive excretion of deoxycytidine, implying a role for 5′-deoxynucleotidase. The accumulation of deoxyribonucleoside monophosphates will also depend on the energy status of cells: Under conditions of insufficient adenosine triphosphate (ATP) supply, deoxynucleotide mono- and diphosphates will be ineffectively phosphorylated by ribonucleoside monophosphate kinases and NDPKs, respectively, and increased deoxyribonucleotide dephosphorylation will ensue *(28)*.

There are several deoxyribonucleoside monophosphate-specific 5′-nucleo-tidases that appear to be important in the regulation of the first step in deoxynucleotide synthesis by the salvage pathway (Table 1). In combination with deoxyribonucleoside kinases, they form substrate cycles that, depending on relative supply of either deoxynucleosides or deoxyribonucleoside mono-phosphates, may have both anabolic and catabolic functions (Fig. 1) *(29)*. Consequently, the alterations in the activity of either kinase

or nucleotidase may bring about net excretion or uptake of deoxyribonucleosides *(30)*. The existence of specific cytosolic and mitochondrial 5′-nucleotidases and deoxynucleoside kinases provides a basis for the independent operation of substrate cycles in these cellular compartments.

2.2. Separation of Cytosolic, Mitochondrial, and Extracellular Deoxynucleotide Pools

Several studies have shown indirect evidence for functional compartmentation of dNTP pools in the nucleus, suggesting that some enzymes of the *de novo* pathway may be localized close to the DNA replication site *(1)*. However, in spite of great experimental effort, there is no convincing biochemical evidence to date for the nuclear localization of key enzymes participating in the salvage and *de novo* pathways. Furthermore, no dNTP concentration gradient has been found across the nuclear membrane *(31)*. However, the association of ribonucleotide reductase with the nuclear membrane of rat liver cells may provide argument for the possibility of channeling dNTPs to the transcription machinery from outside the nucleus *(32)*.

In contrast to the more porous nuclear membrane, the mitochondrial inner membrane is not permeable to nucleotides and other charged small molecules, instead requiring the presence of specific nucleotide transporters. Although there is evidence that the cytosolic and mitochondrial dNTP pools are separate (e.g., depletion of cytosolic dNTPs does not affect mitochondrial dNTP levels *(33)*), there is more recent evidence that deoxynucleoside di- and triphosphates as well as the phosphorylated analog 2′,3′-dideoxycytidine triphosphate (ddCTP) can enter the mitochondria through transporters such as the mitochondrial deoxynucleotide carrier, which exchanges cytosolic deoxyribonucleoside diphosphates (dNDPs) and dNTPs for adenosine 5′-diphosphate (ADP), ATP, and dNDPs *(34–36)*. While studies so far have indicated that nucleoside analogs phosphorylated in the mitochondria are mainly incorporated into mtDNA and are not exported to the cytosol *(37)*, the same is not true for the natural nucleosides. Studies of cytosolic and mitochondrial dTTP pools using cells deficient in cytosolic TK1 demonstrated that salvaged thymidine phosphorylated in the mitochondria by TK2 can be exported to the cytosol for use in nuclear DNA synthesis *(21)*. Conversely, import of *de novo* synthesized nucleotides from the cytosol appears to be a major source of mitochondrial dTTP in cycling cells *(21)*. It is likely that an equilibrium also exists between the cytosolic and mitochondrial pools for other nucleotides as well *(21)*.

3. MAMMALIAN 5′-NUCLEOTIDASES

Seven 5′-nucleotidases have been cloned and characterized (Table 1). Although there is high homology between cytosolic 5′(3′)-deoxyribonucleotidase (cdN) and mitochondrial 5′(3′)-deoxyribonucleotidase (mdN)

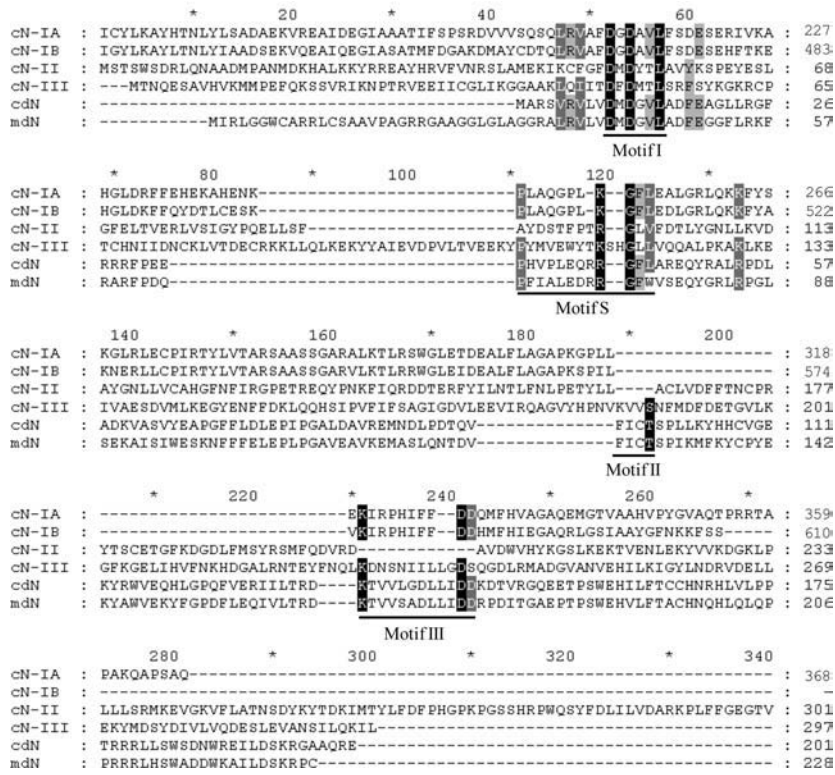


Fig. 2. Alignment of the conserved catalytic motifs of the intracellular nucleotidases identified from the crystal structure of mdN (based on the alignment by Rinaldo-Matthis et al. in ref. *40*). Motif I, DXDX[T/V][L/V/I]; motif S, $P(X)_{7-8}$[R/K]XGF[W/L]; motif II, three hydrophobic residues followed by [T/S]; and motif III, $K(X)_5D(X)_3DD$. Motif II has not been identified in cN-IA, cN-IB, or cN-II, and motif III has not been identified in cN-II. Conserved residues in and around the motifs are shaded. cN-III, cdN, and mdN are shown full length; cN-II has been truncated at 301 amino acids, and the N-terminal portion of cN-IA and cN-IB has been removed.

(38) and between cytosolic 5′-nucleotidase IA (cN-IA) and cytosolic 5′-nucleotidase IB (cN-IB) *(39)*, there is little overall homology among the other nucleotidases. Despite the lack of overall sequence similarity, alignment of the intracellular nucleotidases revealed three conserved motifs containing the catalytic residues (motifs I, II, and III) and one conserved motif containing amino acids involved in substrate recognition (motif S) *(40)* (Fig. 2).

Because of the conservation of catalytic residues among the intracellular nucleotidases, it seems likely that they may share a common catalytic

mechanism involving the formation of a phosphoenzyme intermediate, with the phosphate group covalently bound to aspartate *(40,41)*. In the nucleotidase reaction, the phosphoenzyme intermediate is hydrolyzed by water, releasing orthophosphate. Cytosolic 5′-nucleotidase II (cN-II), cytosolic 5′-nucleotidase III (cN-III), and cdN can also catalyze a phosphotransferase reaction by which phosphate is transferred to an acceptor nucleoside, forming a nucleoside monophosphate, instead of being released as free phosphate *(42–44)*.

Although all cloned 5′-nucleotidases display different substrate specificities, there is some degree of overlap, indicating limited functional redundancy. Because of overlapping substrate specificities, it is difficult to assess the expression of individual nucleotidases by activity assays. Recently, specific assays have been proposed to distinguish between many of the nucleotidases *(45)*, and quantitative reverse transcriptase polymerase chain reaction has been useful in determining nucleotidase messenger RNA (mRNA) expression levels from patient samples *(46–48)*.

3.1. Ecto-5′-Nucleotidase

Ecto-5′-nucleotidase (eN) is ubiquitously expressed; however, its level of expression may vary significantly between different cell types in the same tissue. For example, eN is expressed only on endothelial cells in liver, kidney, and spleen *(49)*; expression of eN on B cells correlates with cell maturity *(50)*. eN expression is highly variable in cancers and may correlate with a more aggressive phenotype *(51)*. eN exists as a dimer linked to the plasma membrane by a glycosylphosphatidylinositol anchor and co-localizes with detergent-resistant and glycolipid-rich membrane subdomains called lipid rafts *(52,53)*.

eN dephosphorylates both purine and pyrimidine ribo- and deoxyribonucleoside monophosphates with high affinity. Of the nucleotidases characterized to date, it has the highest affinity for adenosine 5′-monophosphate (AMP), with a K_m of 3–15 μ*M* *(54–56)*. eN is inhibited by both ADP and ATP as well as other nucleoside di- and triphosphates and the ADP analog α,β-methyleneadenosine diphosphate *(54)*. eN is responsible for the generation of extracellular adenosine to activate local adenosine receptors that mediate responses as diverse as vasodilation, neurotransmission, inhibition of immune responses, and angiogenesis *(57–60)*. Adenosine produced both extracellularly by eN and intracellularly by cytosolic 5′-nucleotidase IA is cardioprotective under conditions of metabolic stress in the heart through activation of G protein-coupled adenosine receptors *(61,62)*.

The ectocellular orientation of eN, high substrate affinity and broad substrate specificity make this enzyme unique in deoxynucleotide metabolism in that it may ensure complete and rapid local dephosphorylation and salvage of released deoxynucleotides from dead and apoptotic cells. Aside from

its nucleotidase activity, eN is also involved in the adhesion of lymphocytes to vascular endothelial cells during migration to sites of inflammation *(63)*.

3.2. Cytosolic 5′-Nucleotidase IA

AMP-selective 5′-nucleotidase (cN-IA) was first purified from pigeon and rabbit hearts and is proposed to have a role in adenosine generation under ischemic conditions because of its dependence on ADP as an activator *(64,65)*. Both pigeon and human cN-IA have been cloned *(66,67)*. In pigeon tissues, cN-IA mRNA is expressed at high levels in heart muscle, skeletal muscle, and brain and at a lower level in liver and kidney *(66)*. mRNA expression in human tissues is similar, with a high level in skeletal muscle; a lower level in heart, brain, and pancreas; and a much lower level in kidney, testis, and uterus *(67)*. cN-IA is active as a tetramer *(68)* and appears to be associated with contractile elements of cardiac muscle *(66,68)*.

cN-IA enzymes purified from pigeon, rat, and rabbit hearts have a K_m that ranges from 1.2 to 8.3 m*M* for AMP and demonstrate the highest V_{max} with this substrate *(64,69–72)*. Relative selectivity toward AMP at millimolar concentrations suggested that this is the major physiological substrate, and overexpression of cN-IA in the COS-7 and H9c2 cell lines significantly increased the production of adenosine *(73)*. More detailed kinetic studies with the purified rabbit enzyme revealed that cN-IA possesses high affinity toward deoxypyrimidine monophosphates (K_m of 20 μ*M* for dTMP [thymidylate] and 61 μ*M* for dCMP) as well as deoxypurine monophosphates (K_m of 130 μ*M* for dAMP (deoxyadenylate) and 120 μ*M* for dGMP [deoxyguanylate]) *(69)*. Thus, it seems that cN-IA is a dual-specificity 5′-nucleotidase and may participate in both the regulation of deoxynucleotide pools and, under ischemic conditions with elevated AMP and ADP concentrations, in the generation of signaling adenosine *(64,67,73)*.

3.3. Cytosolic 5′-Nucleotidase IB

cN-IB was identified as a nucleotidase by its high sequence homology to cN-IA. Murine cN-IB has been cloned and, like cN-IA, is activated by ADP and catalyzes the production of adenosine from AMP, although the range of substrates accepted by this enzyme has not yet been defined *(39)*. There are multiple possible translation start sites in both the mouse and human cN-IB mRNAs, and it is currently unclear which may be used in vivo, although three of the initiation sites in murine cN-IB were shown to produce active enzymes when expressed in cultured cells *(39)*. Using reverse transcriptase polymerase chain reaction, cN-IB mRNA was shown to be present in all tissues tested, with the highest level of expression in testis; lower levels in placenta, pancreas, liver, heart, lung, and kidney; and barely detectable amounts in skeletal muscle and brain *(39)*.

3.4. Cytosolic 5′-Nucleotidase II

The 6-hydroxypurine-specific 5′-nucleotidase cN-II was first described in chicken liver, where it is expressed at a high level *(74)*. The cloned human cN-II *(75)* shows extremely high amino acid conservation throughout evolution, with homology of 98% to cN-II from other mammalian species, 66% to the enzyme from *Drosophila*, and 51% to the protein from *Caenorhabditis elegans*. The unusually high sequence conservation and ubiquitous expression suggest an important and conserved function of cN-II in purine catabolism. Data from gel filtration chromatography suggest that both purified and recombinant cN-II are tetramers *(76,77)*, but cN-II can oligomerize into an octamer in the presence of ATP, which may account for its increased activity with this activator *(76)*.

cN-II is allosterically regulated by ATP, inorganic phosphate, and substrate in a complex manner *(77–79)*. cN-II may also be regulated by diadenosine tetraphosphate or glycerate 2,3-bisphosphate in a tissue-specific manner *(80,81)*. In the presence of ATP as an activator, cN-II has an apparent K_m of 200 μ*M* for IMP and 1.8 m*M* for AMP *(72)*. IMP (inosinate), GMP (guanylate), dIMP (deoxyinosinate), dGMP, and XMP (xanthylate) serve as the best substrates; AMP, dAMP, and pyrimidine monophosphates are poor substrates *(77)*. Because cN-II preferentially hydrolyzes IMP, a precursor of both AMP and GMP on the *de novo* synthesis pathway, this nucleotidase is critically positioned at the crossroad of biosynthetic and catabolic pathways leading to adenylate and guanylate pools *(30,73,77–79)*.

Interestingly, cN-II was found to catalyze, under physiological conditions, the reverse reaction involving the transfer of the phosphoryl group from one purine nucleoside onto another *(42)*. Although the physiological significance of this reaction is unclear, it is responsible for phosphorylation, and thus activation, of several antiviral and anticancer nucleoside analogs *(82–84)*. The reaction intermediate formed during catalysis is phosphoaspartate at position 52 *(41)*. The phosphate may either be released from the enzyme, leading to hydrolysis, or be transferred to a suitable nucleoside acceptor, thus leading to transphosphorylation. The rate-limiting step of nucleotidase activity is hydrolysis of the enzyme-phosphate bond. Addition of a phosphate acceptor, inosine, increases the speed of the nucleotidase reaction, suggesting that transfer of the phosphate to inosine occurs more rapidly than hydrolysis of the enzyme-phosphate bond *(42,43)*. It has been suggested that, under physiological conditions, cN-II acts mainly as a phosphotransferase *(85)*.

3.5. Cytosolic 5′-Nucleotidase III

The kinetic properties of cN-III are uniquely suited to function in the erythrocyte. During the maturation of erythrocytes, the nucleus is expelled and

RNA is degraded, producing large quantities of nucleotides. cN-III is upregulated during erythrocyte maturation and plays the critical role of breaking down uridine and cytidine ribonucleotides *(2,86)*. Deficiency of this enzyme leads to the accumulation of cytidine and uridine phosphates, which in turn can interfere with glycolysis, leading to hemolytic anemia *(2,87)*.

Although cN-III expression was originally thought to be restricted to erythrocytes, enzyme activity has also been found in multiple mouse and rat tissues *(88,89)*, and the Expressed sequence tags (EST) database shows expression in a wide variety of human tissues *(90,91)*. Three alternatively spliced mRNA transcripts are produced from the cN-III gene, predicting proteins of 285, 286, and 297 amino acids that are identical throughout the majority of the protein and differ only in the number of amino acids at the N-terminus *(91,92)*. The cloned 286-amino acid form of cN-III has the same characteristics as cN-III purified from erythrocytes; the other two forms have not been cloned *(93)*. Unlike other nucleotidases, cN-III is a monomer *(44)*.

Catalytic activity of cN-III is restricted to pyrimidines: uridylate (UMP; K_m 330 μ*M*), deoxyuridylate (dUMP; K_m 400 μ*M*), cytidylate (CMP; K_m 10–150 μ*M*), dCMP (K_m 580 μ*M*), and dTMP (K_m 1.0 m*M*) *(87,94)*. cN-III activity is dependent on Mg^{2+}, is not affected by ADP and ATP, and is inhibited by nucleosides and phosphate *(94)*. cN-III also displays phosphotransferase activity, preferring uridine, cytidine, and deoxycytidine as phosphate acceptors, although the role that this reaction plays in vivo is unclear *(94)*.

3.6. Cytosolic 5′(3′)-Deoxyribonucleotidase

The cdN has been purified from rat liver *(95,96)*, human placenta *(97)*, and erythrocytes *(98)*. cdN is ubiquitously expressed, with a high level of mRNA in pancreas, heart, and skeletal muscle; a lower level in kidney and liver; and low expression in brain and lung *(99)*. cdN is unique among the cytosolic nucleotidases in its preference for uridine and thymidine monophosphates. The best overall substrates for the human enzyme, as defined by the V_{max}/K_m ratio, are 3′-UMP, 3′-dTMP, and 3′-dUMP with K_m values of 250, 300, and 200 μ*M*, respectively *(100)*. 5′-dUMP (K_m 410 μ*M* to 1.5 m*M*) and 5′-dTMP (K_m 1.5 m*M*) also serve as substrates *(100,101)*. Although cdN can use some purine ribonucleotides as substrates, AMP is a very poor substrate *(99)*. cdN functions as a dimer and is dependent on Mg^{2+} for activity *(97)*.

Although cdN purified from erythrocytes was shown to catalyze phosphotransferase reactions, using 3′-UMP and 3′-dUMP as phosphate donors and deoxyuridine and deoxythymidine as acceptors *(44)*, the recombinant mouse cdN did not demonstrate any phosphotransferase activity *(99)*. The overall substrate specificity of cdN suggests an important role in protecting against deoxyuridine triphosphate (dUTP) and dTTP pool expansion and in

elimination of atypical 2′ and 3′ nucleoside monophosphates. Overexpression of mouse cdN in 293 cells caused increased excretion of thymidine, uridine, deoxycytidine, and even cytidine, although there was no activity of the enzyme with CMP in vitro *(30)*.

3.7. Mitochondrial 5′(3′)-Deoxyribonucleotidase

The mdN is encoded by nuclear DNA and has a mitochondrial import signal that is cleaved in the mature protein *(102)*. Not including the mitochondrial leader sequence, mdN has 52% amino acid identity with cdN, and like cdN, mdN is a dimer. The expression pattern for mdN is similar to that of cN-IA, with high expression in heart, brain, and skeletal muscle; barely detectable expression in kidney and pancreas; and no expression in lung, placenta, or liver *(102)*. mdN has a striking preference for dUMP and dTMP and shows high activity with 5′-, 2′-, and 3′-UMP and 3′-dTMP. Only residual activity with dAMP, dGMP, and dCMP has been demonstrated *(102)*.

mdN forms a substrate cycle with TK2 to regulate thymidine salvage in the mitochondria *(103)*. Mitochondrial export of thymidine phosphates salvaged by TK2 to cytosolic pools and import of dTDP synthesized *de novo* in the cytosol appear to reach a dynamic equilibrium *(103)*. Overexpression of mdN, in contrast to overexpression of cdN, did not lead to increased excretion of deoxynucleosides from the cell, demonstrating that mdN activity does not regulate cytosolic deoxynucleotide levels in cycling cells *(104)*. In noncycling cells, where TK1 is absent and *de novo* synthesis of dTTP is turned off, dTTP can only be provided through salvage of thymidine by TK2. In this case, it is possible that the mdN/TK2 ratio may affect both mitochondrial and cytosolic dTTP levels *(21,103)*.

4. 5′-NUCLEOTIDASES AND DRUG RESISTANCE

Although nucleoside analogs have been essential components of anticancer and antiviral therapies for years *(105)*, the cellular mechanisms responsible for clinical resistance to this class of compounds are still not completely understood. To maximize cytotoxic effects, the ideal nucleoside analog is readily phosphorylated, stable as a triphosphate, and poorly catabolized. The mechanisms of nucleoside analog resistance are likely to involve changes in a number of independent pathways, including enzymes of nucleoside metabolism and proteins involved in apoptosis *(106)*.

Because 5′-nucleotidases are critical for dephosphorylation of ribonucleoside and deoxyribonucleoside monophosphates, they also are likely to inactivate nucleoside analogs before they have a chance to be phosphorylated into di- and triphosphates, thereby limiting their therapeutic potency. The substrate cycles described between nucleoside kinases and nucleotidases are

likely to play a major role in nucleoside analog metabolism, with the balance between the kinases and nucleotidases determining the extent to which analogs are phosphorylated to triphosphate form. Reduced nucleoside kinase activity has already been defined as an important mechanism of nucleoside analog resistance *(107)*, and altered activity of 5′-nucleotidases is now emerging as a related resistance mechanism *(105)*. In this respect, the role of cN-II in drug resistance has been studied most extensively; however, the kinetic properties of several other nucleotidases suggest that they may also play a role in nucleoside analog resistance.

4.1. cN-II in Nucleoside Analog Metabolism

Several studies have implicated increased cN-II activity in nucleoside analog resistance both in vivo and in vitro. Increased cN-II activity has been reported in HL60 (acute promyelocytic leukemia) cells selected for cladribine (2-chloro-2′-deoxyadenosine; CdA) resistance *(108)*, CEM (acute lymphocytic leukemia) cells selected for deoxyadenosine resistance *(109)*, K562 (chronic myelogenous leukemia) cells selected for resistance to cytarabine (1-β-D-arabinofuranosylcytosine; Ara-C), CdA, fludarabine phosphate (2-fluoroadenine-9-β-D-arabinofuranosine monophosphate; F-AraAMP), or gemcitabine (dFdC) *(110)*, and even CEM cells selected for etoposide resistance *(111)*. Many of these cells are crossresistant to other nucleoside analogs, including the CEM cells selected for etoposide resistance that are also resistant to CdA, Ara-C, and to a lesser extent dFdC *(111)*. Along with cN-II upregulation, some of these cell lines had reduced deoxycytidine kinase (dCK) expression, suggesting that the dCK/cN-II ratio is important for determining nucleoside analog phosphorylation.

In WSU-CLL (chronic lymphocytic leukemia) cells that have a low dCK/cN-II ratio, treatment with bryostatin 1, a protein kinase C activator that induces differentiation, both increased dCK and decreased cN-II, thus sensitizing the cells to CdA *(112)*. Bryostatin 1 pretreatment of severe combined immunodeficient mice with WSU-CLL xenografts also improved tumor growth inhibition by CdA, suggesting that modulation of the dCK /cN-II ratio in vivo may improve cancer treatment *(112)*. The nucleoside analog monophosphates FdUMP (5-fluorodeoxyuridylate), AZTMP (3′-azido-2′,3′-dideoxythymidylate; zidovudine monophosphate), CdAMP (CdA monophosphate), Ara-GMP (9-β-D-arabinofuranosylguanylate), BVdUMP ([E]-5-[2-bromovinyl]-2-deoxyuridylate), and ddCMP (2′-,3′-dideoxycytidylate; zalcitabine monophosphate) are all substrates of purified recombinant human cN-II *(101)*. Of interest, Ara-CMP (Ara-C monophosphate) was not a substrate of cN-II in this study, suggesting that Ara-C resistance in cell lines with increased cN-II activity may be caused by other factors, such as changes in dNTP pools. In the etoposide-resistant CEM cells with increased cN-II activity that were crossresistant to CdA and

Ara-C, the dCTP pool was increased and likely reduced incorporation of Ara-CTP (Ara-C triphosphate) into DNA *(111)*.

A limited number of reports are available on the expression of cN-II in the clinical setting. Studies of cN-II in patients with leukemia have focused on the prognostic value of cN-II mRNA expression or activity prior to treatment. The dCK/cN-II ratio was higher in patients with refractory chronic lymphocytic leukemia who responded to CdA than patients who did not respond *(113)*. Patients diagnosed with acute myeloid leukemia (AML) who had higher cN-II mRNA levels had shorter disease-free and overall survival when treated with Ara-C; dCK mRNA levels did not correlate with survival *(114)*.

A study identified cN-II as an independent prognostic factor in AML treated with a combination of Ara-C and anthracyclines *(46)*. In this study, elevated cN-II activity was associated with shorter disease-free survival in the entire patient population and shorter overall survival in patients under 57 years. Furthermore, a follow-up study revealed that both dCK and cN-II activities may better predict survival in AML patients treated with Ara-C *(48)*. Further studies are necessary to confirm or extend these observations.

As a phosphotransferase, cN-II can also phosphorylate several clinically relevant drugs, including 2′,3′-dideoxyinosine (ddI) *(83)*, tiazofurin *(82)*, and acyclovir *(115)*, using IMP as a phosphate donor. For the anti-human immunodeficiency virus analog ddI, phosphorylation by cN-II to ddIMP is the main route for activation *(83)*. Two anticancer agents in development, tiazofurin and benzamide riboside, with active dinucleotide forms that inhibit inosine monophosphate dehydrogenase, are phosphorylated to the monophosphate form by cN-II, adenosine kinase, and nicotinamide ribonucleoside kinase *(82,116,117)*. cN-II can also phosphorylate the antiviral guanosine analogs acyclovir and ganciclovir *(115,118)*. These drugs are usually phosphorylated by the herpes simplex virus-encoded thymidine kinase and not by cellular thymidine kinases, allowing them to be specifically activated only in virus-infected cells *(119)*. However, low-level activation has also been seen in uninfected cells and is caused by cN-II phosphotransferase activity, although this activity plays little role in the antiviral activity of these compounds *(118)*. Increasing the cellular concentration of IMP, the preferred phosphoryl donor in the phosphotransferase reaction, may increase the efficiency of drug activation by this route. For example, the inhibition of IMP dehydrogenase limits the use of IMP for the synthesis of guanylates and thereby increases IMP levels; this leads to increased phosphorylation of ddI by cN-II *(120)*.

4.2. cN-IA in Nucleoside Analog Resistance

cN-IA has far more favorable kinetic properties toward pyrimidine and purine deoxyribonucleoside monophosphates than other cytosolic nucleotidases, suggesting that cN-IA could play a significant role in nucleoside

Table 2
Activity of Cytosolic 5′-Nucleotidases With Deoxynucleoside Monophosphates

Nucleotidase	*Substrate*	V_{max} (µmol/mg/min)	K_m (mM)	V_{max}/K_m	V_{max} (µmol/mg/min)	K_m (mM)	V_{max}/K_m
		Rabbit heart enzyme[a]			*Human recombinant enzyme*		
cN-IA	dAMP	14.9	0.130	115	—	—	—
	dGMP	8.5	0.120	71	—	—	—
	dCMP	12.8	0.061	210	49.5	0.012	4125
	dTMP	9.9	0.020	495	61.5	0.020	3075
		Chicken liver enzyme[a]			*Chicken heart enzyme*[a]		
cN-II	dAMP	27	41.0	0.66	3.3	23.0	0.1
	dGMP	29	3.4	8.5	5.7	1.0	5.7
	dCMP	—	—	—	—	—	—
	dTMP	22	54.0	0.41	5.1	18.0	0.3
	dIMP	27	2.0	13.5	6.4	0.4	16.1
		Human erythrocyte enzyme					
cN-III	dCMP	15.0	0.580	25.9			
	dTMP	12.6	1.0	12.6			
	dUMP	4.1	0.4	10.3			
		Mouse recombinant enzyme			*Human placenta enzyme*		
cdN	dAMP	53.6	1.0	52	14	3.0	4.5
	dGMP	195	1.2	171	73	3.3	22
	dCMP	165	3.6	46	—	—	—
	dTMP	347	1.25	277	83	7.0	12
	dUMP	313	0.42	745	107	2.6	41
	dIMP	—	—	—	294	4.4	67

Data compiled from refs. *67*,*69*,*94*,*97*,*99*, and *129*.
[a]Recalculated from refs. *69* and *129*.

analog resistance. The substrate affinities of the cytosolic 5′-nucleotidases for deoxyribonucleotides are shown in Table 2. The affinity of cN-IA toward dCMP and dTMP, approximately 50- to 100-fold lower than for the other intracellular 5′-nucleotidases, would suggest that cN-IA may be more efficient in dephosphorylating both natural deoxyribonucleotide substrates and nucleoside analog monophosphates at low physiological concentrations *(67,69)*. Overexpression of cN-IA in HEK293 and Jurkat cells led to resistance to CdA in both cell lines, to dFdC in HEK293 cells, and to ddC in Jurkat cells, supporting a role for this enzyme in nucleoside analog metabolism *(67)*. In addition, cN-IA can dephosphorylate AZTMP (K_m 8 μ*M*) with high efficiency (S. A. Hunsucker, unpublished data).

Thus, it will be extremely interesting to test whether clinically developed resistance to nucleoside analogs is associated with increased activity of cN-IA. In addition, cN-IA is not ubiquitously expressed by mRNA analysis, and confirmation of protein expression in tissues other than skeletal and heart muscle is needed to fully assess its role in drug resistance. In a preliminary study with mononuclear cells from patients with AML and chronic lymphocytic leukemia, dephosphorylation of CdAMP in extracts correlated best with cN-I activity *(121)*.

4.3. cdN in Nucleoside Analog Resistance

Unexpectedly, it was demonstrated that low expression of cdN correlates with both shorter overall survival and shorter disease-free survival in patients treated with Ara-C for AML *(122)*. While purified recombinant human cdN uses FdUMP, AZTMP, BVdUMP, d4TMP (2′,3-didehydro-3′-deoxythymidylate; stavudine monophosphate), dFdCMP (dFdC monophosphate), 3TCMP (-)2′-deoxy-3′-thiacytidylate; lamivudine monophosphate), ddCMP, and Ara-GMP as substrates, Ara-CMP was not a substrate *(101)*. Although it is possible that Ara-C is a substrate for cdN phosphotransferase activity, this is not likely to be a major route of Ara-C activation, and it is more likely that a reduction of cdN leads to an increased level of dCTP that competes with Ara-CTP for incorporation into DNA *(122)*. dCTP also inhibits dCK, the enzyme responsible for the initial phosphorylation of Ara-C, and activates cytidine deaminase, thus increasing Ara-C catabolism *(122)*. The finding that reduced expression of cdN can cause resistance to an analog that is not a substrate highlights the complexities inherent in studying nucleoside metabolism.

4.4. Other Nucleotidases

Although their activity with nucleoside analogs has not been studied in detail, cN-III and cN-IB are also potential candidates for mediating nucleoside analog resistance. cN-III uses both AZTMP and Ara-CMP as substrates, suggesting a potential role of this enzyme in drug resistance *(100)*. Although cN-IB has not been investigated in this respect, its low-level

presence in main tissues may potentially make this nucleotidase another candidate for involvement in drug metabolism *(39)*.

Several nucleoside analogs used in antiviral therapies show striking mitochondrial toxicities, most likely resulting from the incorporation of the toxic triphosphate form of these drugs into mtDNA *(123)*. The recently solved crystal structure of mdN may aid in the design of new compounds that will be more susceptible to dephosphorylation in the mitochondria and thus limit toxicity to this organelle without affecting activation of the drug in the cytosol. Purified recombinant human mdN uses FdUMP, BVdUMP, d4TMP, AZTMP, and Ara-TMP (1-β-D-arabinosylthymidylate) as substrates; ddCMP, dFdCMP, Ara-CMP, 3TCMP, CdAMP, and Ara-GMP were not hydrolyzed *(101)*.

Because of low solubility of the nucleoside form, fludarabine is administered as a monophosphate F-Ara-AMP that requires dephosphorylation to be taken up by target cells. Although eN is unlikely to be involved in nucleoside analog resistance because of its extracellular orientation, it may catalyze the initial step in fludarabine activation *(124)*. Because some solid tumors have increased expression of eN *(60)*, this may provide a novel therapeutic opportunity for tumor site-specific activation and uptake of anticancer drugs.

5. 5′-NUCLEOTIDASES: NEW POTENTIAL DRUG TARGETS?

Progress in our understanding of the role of 5′-nucleotidases in the regulation of nucleotide and deoxynucleotide pools led to the realization that they may have similar functions in metabolism of nucleoside-based drugs. As stated, ideal drug candidates must be readily metabolized by nucleoside kinases and be stable in the triphosphate form. Thus, it is important that the catabolism of phosphorylated nucleoside analogs is slow, especially at the step catalyzed by 5′-nucleotidases. Although this theoretical framework is well established, much remains to be done to fully understand the role of 5′-nucleotidases in drug metabolism. However, given their prominent role in deoxyribonucleotide metabolism, it is already clear that they constitute potential drug targets.

In this respect, there are two areas for further research. First, novel nucleoside analogs should be tested as potential substrates of 5′-nucleotidases to avoid developing drugs that are metabolized and inactivated by this pathway. Second, development of specific 5′-nucleotidase inhibitors would likely increase the pharmacological efficacy of existing nucleoside-based drugs. In this regard, knowledge of structure–function relationships of human 5′-nucleotidases could facilitate the development of more selective and potent drug candidates.

REFERENCES

1. Kunz, B. A., Kohalmi, S. E., Kunkel, T. A., Mathews, C. K., McIntosh, E. M., and Reidy, J. A. Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. *Mutat. Res.* 1994;318:1–64.
2. Valentine, W. N., Fink, K., Paglia, D. E., Harris, S. R., and Adams, W. S. (1974). Hereditary hemolytic anemia with human erythrocyte pyrimidine 5′-nucleotidase deficiency. *J. Clin. Invest.* 54, 866–879.
3. Reichard, P. (1988). Interactions between deoxyribonucleotide and DNA synthesis. *Annu. Rev. Biochem.* 57, 349–374.
4. Kennedy, E. P., Borkenhagen, L. F., Smith, S. W. (1959). Possible metabolic functions of deoxycytidine diphosphate choline and deoxycytidine diphosphate ethanolamine. *J. Biol. Chem.* 234,1998–2000.
5. Spyrou, G. A., and Reichard, P. (1989). Intracellular compartmentation of deoxycytidine nucleotide pools in S phase mouse 3T3 fibroblasts. *J. Biol. Chem.* 264, 960–964.
6. Haynes, R. H., and Kunz, B. A. (1986). The influence of thymine nucleotide depletion on genetic stability and change in eucaryotic cells. *Current Sci.* 55, 1–11.
7. Gangi-Peterson, L., Sorscher, D. H., Reynolds, J. W., Kepler, T. B., and Mitchell, B. S. (1999). Nucleotide pools imbalance and adenosine deaminase deficiency induce alterations of N-region insertions during V(D)J recombination. *J. Clin. Invest.* 103, 833–841.
8. Kunz, B. A., and Kohalmi, S. E. (1991). Modulation of mutagenesis by deoxyribonucleotide levels. *Annu. Rev. Genet.* 25, 339–359.
9. Jordan, A., and Reichard, P. (1998). Ribonucleotide reductases. *Annu. Rev. Biochem.* 67, 71–98.
10. Plagemann, P. G., and Erbe, J. (1974). Intracellular conversions of deoxyribonucleosides by Novikoff rat hepatoma cells and effects of hydroxyurea. *J. Cell Physiol.* 83, 321–336.
11. Snyder, R. D. (1984). Deoxyribonucleoside triphosphate pools in human diploid fibroblasts and their modulation by hydroxyurea and deoxynucleosides. *Biochem. Pharmacol.* 33, 1515–1518.
12. Bianchi, V., Pontis, E., and Reichard, P. (1986). Interrelations between substrate cycles and de novo synthesis of pyrimidine deoxyribonucleoside triphosphates in 3T6 cells. *Proc. Natl. Acad. Sci. U. S. A.* 83, 986–990.
13. Bianchi, V., Pontis, E., and Reichard, P. (1986). Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. *J. Biol. Chem.* 261, 16,037–16,042.
14. Mitchell, B. S., Mejias, E., Daddona, P. E., Kelley, W. N. (1978). Purinogenic immunodeficiency diseases: selective toxicity of deoxyribonucleosides for T cells. *Proc. Natl. Acad. Sci. U. S. A.* 75, 5011–5014.
15. Mitchell, B. S., Edwards, N. L., and Koller, C. A. (1983). Deoxyribonucleoside triphosphate accumulation by leukemic cells. *Blood* 62, 419–424.
16. Cohen, A., Hirschhorn, R., Horowitz, S. D., et al. (1978). Deoxyadenosine triphosphate as a potentially toxic metabolite in adenosine deaminase deficiency. *Proc. Natl. Acad. Sci. U. S. A.* 75, 472–476.
17. Wortmann, R., L, Mitchell, B., S, Edwards, N., L, and Fox, I., H. (1979). Biochemical basis for differential deoxyadenosine toxicity to T and B lymphoblasts: role for 5′-nucleotidase. *Proc. Natl. Acad. Sci. U. S. A.* 76, 2434–2437.

18. Bianchi, V., Borella, S., Rampazzo, C., et al. (1997). Cell cycle-dependent metabolism of pyrimidine deoxynucleoside triphosphates in CEM cells. *J. Biol. Chem.* 272, 16,118–16,124.
19. Snyder, R. D. (1984). The role of deoxynucleoside triphosphate pools in the inhibition of DNA-excision repair and replication in human cells by hydroxyurea. *Mutat. Res.* 131, 163–172.
20. Xu, Y.-Z., Huang, P., and Plunkett, W. (1995). Functional compartmentation of dCTP pools. Preferential utilization of salvaged deoxycytidine for DNA repair in human lymphoblasts. *J. Biol. Chem.* 270, 631–637.
21. Pontarin, G., Gallinaro, L., Ferraro, P., Reichard, P., and Bianchi, V. (2003). Origins of mitochondrial thymidine triphosphate: dynamic relations to cytosolic pools. *Proc. Natl. Acad. Sci. U. S. A.* 100, 12,159–12,164.
22. Sherley, J. L., and Kelly, T. J. (1988). Regulation of human thymidine kinase during the cell cycle. *J. Biol. Chem.* 263, 8350–8358.
23. Oliver, F. J., Collins, M. K., and Lopez-Rivas, A. (1996). Regulation of salvage pathway of deoxynucleotide synthesis in apoptosis induced by growth factor deprivation. *Biochem. J.* 316, 431–425.
24. Bianchi, V., Pontis, E., and Reichard, P. (1987). Regulation of pyrimidine deoxyribonucleotide metabolism by substrate cycles in dCMP deaminase-deficient V79 hamster cells. *Mol. Cell. Biol.* 7, 4218–4224.
25. Zimmermann, H. (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch. Pharmacol.* 362, 299–309.
26. Dalton, A., Hornby, D. P., Langston, S. P., and Blackburn, G. M. (1992). Characterization and purification of a novel dATP-binding protein in eukaryotes. *Biochem. J.* 287, 871–879.
27. Ford, K. G. (2000). The dNTPase enzyme activity is inhibited by nucleic acids and contains a heat-insensitive component. *Biochem. Biophys. Res. Commun.* 276, 823–829.
28. Barankiewicz, J., and Cohen, A. (1984). Evidence for distinct catabolic pathways of adenine ribonucleotides and deoxyribonucleotides in human T lymphoblastoid cells. *J. Biol. Chem.* 259, 15,178–15,181.
29. Bianchi, V., Ferraro, P., Borella, S., Bonvini, P., and Reichard, P. (1994). Effects of mutational loss of nucleoside kinases on deoxyadenosine 5′-phosphate/deoxyadenosine substrate cycle in cultured CEM and V79 cells. *J. Biol. Chem.* 269, 16,677–16,683.
30. Gazziola, C., Ferraro, P., Moras, M., Reichard, P., and Bianchi, V. (2001). Cytosolic high *K*(m) 5′-nucleotidase and 5′(3′)-deoxyribonucleotidase in substrate cycles involved in nucleotide metabolism. *J. Biol. Chem.* 276, 6185–6190.
31. Leeds, J. M., Slabaugh, M. B., and Mathews, C. K. (1985). DNA precursor pools and ribonucleotide reductase activity: distribution between the nucleus and cytoplasm of mammalian cells. *Mol. Cell Biol.* 5, 3443–3450.
32. Sikorska, M., Brewer, L. M., Youdale, T., et al. (1990). Evidence that mammalian ribonucleotide reductase is a nuclear membrane associated glycoprotein. *Biochem. Cell Biol.* 68, 880–888.
33. Bestwick, R. K., and Mathews, C. K. (1982). Unusual compartmentation of precursors for nuclear and mitochondrial DNA in mouse L cells. *J. Biol. Chem.* 257, 9305–9308.
34. Chen, C. H., and Cheng, Y. C. (1992). The role of cytoplasmic deoxycytidine kinase in the mitochondrial effects of the anti-human immunodeficiency virus compound, 2′,3′-dideoxycytidine. *J. Biol. Chem.* 267, 2856–2859.

35. Bridges, E. G., Jiang, Z., and Cheng, Y. C. (1999). Characterization of a dCTP transport activity reconstituted from human mitochondria. *J. Biol. Chem.* 274, 4620–4625.
36. Dolce, V., Fiermonte, G., Runswick, M. J., Palmieri, F., and Walker, J. E. (2001). The human mitochondrial deoxynucleotide carrier and its role in the toxicity of nucleoside antivirals. *Proc. Natl. Acad. Sci. U. S. A.* 98, 2284–2288.
37. Zhu, C., Johansson, M., and Karlsson, A. (2000). Incorporation of nucleoside analogs into nuclear or mitochondrial DNA is determined by the intracellular phosphorylation site. *J. Biol. Chem.* 275, 26,727–16,731.
38. Rampazzo, C., Kost-Alimova, M., Ruzzenente, B., Dumanski, J. P., and Bianchi, V. (2002). Mouse cytosolic and mitochondrial deoxyribonucleotidases: cDNA cloning of the mitochondrial enzyme, gene structures, chromosomal mapping and comparison with the human orthologs. *Gene* 294, 109–117.
39. Sala-Newby, G. B., and Newby, A. C. (2001). Cloning of a mouse cytosolic 5′-nucleotidase-I identifies a new gene related to human autoimmune infertility-related protein. *Biochim. Biophys. Acta* 1521, 12–18.
40. Rinaldo-Matthis, A., Rampazzo, C., Reichard, P., Bianchi, V., and Nordlund, P. (2002). Crystal structure of a human mitochondrial deoxyribonucleotidase. *Nat. Struct. Biol.* 9, 779–787.
41. Allegrini, S., Scaloni, A., Ferrara, L., et al. (2001). Bovine cytosolic 5′-nucleotidase acts through the formation of an aspartate 52-phosphoenzyme intermediate. *J. Biol. Chem.* 276, 33,526–33,532.
42. Worku, Y., and Newby, A. C. (1982). Nucleoside exchange catalysed by the cytoplasmic 5′-nucleotidase. *Biochem. J.* 205, 503–510.
43. Tozzi, M. G., Camici, M., Pesi, R., Allegrini, S., Sgarrella, F., and Ipata, P. L. (1991). Nucleoside phosphotransferase activity of human colon carcinoma cytosolic 5′-nucleotidase. *Arch. Biochem. Biophys.* 291, 212–217.
44. Amici, A., Emanuelli, M., Magni, G., Raffaelli, N., and Ruggieri, S. (1997). Pyrimidine nucleotidases from human erythrocyte possess phosphotransferase activities specific for pyrimidine nucleotides. *FEBS Lett.* 419, 263–267.
45. Rampazzo, C., Mazzon, C., Reichard, P., and Bianchi, V. (2002). 5′-Nucleotidases: specific assays for five different enzymes in cell extracts. *Biochem. Biophys. Res. Commun.* 293, 258–263.
46. Galmarini, C. M., Graham, K., Thomas, X., et al. (2001). Expression of high K_m 5′-nucleotidase in leukemic blasts is an independent prognostic factor in adults with acute myeloid leukemia. *Blood* 98, 1922–1926.
47. Galmarini, C. M., Thomas, X., Calvo, F., et al. (2002). In vivo mechanisms of resistance to cytarabine in acute myeloid leukaemia. *Br. J. Haematol.* 117, 860–868.
48. Galmarini, C. M., Thomas, X., Graham, K., et al. (2003). Deoxycytidine kinase and cN-II nucleotidase expression in blast cells predict survival in acute myeloid leukaemia patients treated with cytarabine. *Br. J. Haematol.* 122, 53–60.
49. Thompson, L. F. (1991). 5′-Nucleotidase—an overview of the last 3 years. *Adv. Exp. Med. Biol.* 309B, 145–150.
50. Thompson, L. F., and Ruedi, J. M. (1988). Synthesis of immunoglobin G by pokeweed mitogen or Epstein-Barr virus-stimulated human B cells in vitro is restricted to the ecto-5′-nucleotidase positive subset. *J. Clin. Invest.* 82, 902–905.
51. Spychala, J., Lazarowski, E., Ostapkowicz, A., Ayscue, L. H., Jin, A., and Mitchell, B. S. (2004). Role of estrogen receptor in the regulation of ecto-5′-nucleotidase (eN) expression and extracellular adenosine generation in breast cancer. *Clin. Cancer Res.* 10, 708–717.

52. Misumi, Y., Ogata, S., Ohkubo, K., Hirose, S., and Ikehara, Y. (1990). Primary structure of human placental 5′-nucleotidase and identification of the glycolipid anchor in the mature form. *Eur. J. Biochem.* 191, 563–569.
53. Muller, G., Jung, C., Frick, W., Bandlow, W., and Kramer, W. (2002). Interaction of phosphatidylinositolglycan(-peptides) with plasma membrane lipid rafts triggers insulin-mimetic signaling in rat adipocytes. *Arch. Biochem. Biophys.* 408, 7–16.
54. Burger, R., M, and Lowenstein, J., M. (1975). 5′-Nucleotidase from smooth muscle of small intestine and from brain. Inhibition of nucleotides. *Biochemistry* 14, 2362–2366.
55. Spychala, J., Madrid-Marina, V., Nowak, P. J., and Fox, I. H. (1989). AMP and IMP dephosphorylation by soluble high- and low-K_m 5′-nucleotidases. *Am. J. Physiol.* 256, E386–E391.
56. Naito, Y., and Lowenstein, J., M. (1981). 5′-Nucleotidase from rat heart. *Biochemistry* 20, 5188–5194.
57. Resta, R., Yamashita, Y., and Thompson, L. F. (1998). Ecto-enzyme and signaling functions of lymphocyte CD73. *Immunol. Rev.* 161, 95–109.
58. Nemoto, E., Kunii, R., Tada, H., Tsubahara, T., Ishihata, H., and Shimauchi, H. (2004). Expression of CD73/ecto-5′-nucleotidase on human gingival fibroblasts and contribution to the inhibition of interleukin-1α-induced granulocyte-macrophage colony stimulating factor production. *J. Periodontal Res.* 39, 10–19.
59. Duarte-Araujo, M., Nascimento, C., Alexandrina Timoteo, M., Magalhaes-Cardoso, T., and Correia-de-Sa, P. (2004). Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A(2A) and extrajunctional inhibitory A(1) receptors. *Br. J. Pharmacol.* 141, 925–934.
60. Spychala, J. (2000). Tumor-promoting functions of adenosine. *Pharmacol. Ther.* 87, 161–173.
61. Headrick, J. P., Hack, B., and Ashton, K. J. (2003). Acute adenosinergic cardioprotection in ischemic-reperfused hearts. *Am. J. Physiol. Heart Circ. Physiol.* 285, H1797–H1818.
62. Kitakaze, M., Minamino, T., Node, K., et al. (1999). Adenosine and cardioprotection in the diseased heart. *Jpn. Circ. J.* 63, 231–243.
63. Airas, L., Niemela, J., and Jalkanen, S. (2000). CD73 engagement promotes lymphocyte binding to endothelial cells via a lymphocyte function-associated antigen-1-dependent mechanism. *J. Immunol.* 165, 5411–5417.
64. Skladanowski, A. C., and Newby, A. C. (1990). Partial purification and properties of an AMP-specific soluble 5′-nucleotidase from pigeon heart. *Biochem. J.* 268, 117–122.
65. Yamazaki, Y., Truong, V. L., and Lowenstein, J. M. (1991). 5′-Nucleotidase-I from rabbit heart. *Biochemistry* 30, 1503–1509.
66. Sala-Newby, G. B., Skladanowski, A. C., and Newby, A. C. (1999). The mechanism of adenosine formation in cells. Cloning of cytosolic 5′-nucleotidase-I. *J. Biol. Chem.* 274, 17,789–17,793.
67. Hunsucker, S. A., Spychala, J., and Mitchell, B. S. (2001). Human cytosolic 5′-nucleotidase I: characterization and role in nucleoside analog resistance. *J. Biol. Chem.* 276, 10,498–10,504.
68. Darvish, A., and Metting, P. J. (1993). Purification and regulation of an AMP-specific cytosolic 5′-nucleotidase from dog heart. *Am. J. Physiol.* 264, H1528–H1534.

69. Garvey, E. P., Lowen, G. T., and Almond, M. R. (1998). Nucleotide and nucleoside analogues as inhibitors of cytosolic 5′-nucleotidase I from heart. *Biochemistry* 37, 9043–9051.
70. Skladanowski, A. C., Hoffmann, C. S., Krass, J. D., Makarewicz, W., and Jastorff, B. (1995). Different substrate specificity of two isozymes of cytosolic 5′-nucleotidase from rabbit heart. *Adv. Exp. Med. Biol.* 370, 617–621.
71. Skladanowski, A. C., Smolenski, R. T., Tavenier, M., de Jong, J. W., Yacoub, M. H., and Seymour, A. M. (1996). Soluble forms of 5′-nucleotidase in rat and human heart. *Am. J. Physiol.* 270, H1493–H1500.
72. Truong, V. L., Collinson, A. R., and Lowenstein, J. M. (1988). 5′-Nucleotidase in rat heart. Evidence for the occurrence of two soluble enzymes with different substrate specificities. *Biochem. J.* 253, 117–121.
73. Sala-Newby, G. B., Freeman, N. V., Skladanowski, A. C., and Newby, A. C. (2000). Distinct roles for recombinant cytosolic 5′-nucleotidase-I and -II in AMP and IMP catabolism in COS-7 and H9c2 rat myoblast cell lines. *J. Biol. Chem.* 275, 11,666–11,671.
74. Itoh, R., Mitsui, A., and Tsushima, K. (1967). 5′-Nucleotidase of chicken liver. *Biochim. Biophys. Acta* 146, 151–159.
75. Oka, J., Matsumoto, A., Hosokawa, Y., Inoue, S. (1994). Molecular cloning of human cytosolic purine 5′-nucleotidase. *Biochem. Biophys. Res. Commun.* 205, 917–922.
76. Spychala, J., Chen, V., Oka, J., and Mitchell, B. S. (1999). ATP and phosphate reciprocally affect subunit association of human recombinant high K_m 5′-nucleotidase. Role of C-terminal polyglutamic acid tract in subunit association and catalytic activity. *Eur. J. Biochem.* 259, 851–858.
77. Spychala, J., Madrid-Marina, V., and Fox, I. H. (1988). High K_m soluble 5′-nucleotidase from human placenta. Properties and allosteric regulation by IMP and ATP. *J. Biol. Chem.* 263, 18,759–18,765.
78. Van den Berghe, G., Van Pottersberghe, C., and Hers, H.-G. (1977). A kinetic study of the soluble 5′-nucleotidase of rat liver. *Biochem. J.* 162, 611–616.
79. Itoh, R. (1993). IMP-GMP 5′-nucleotidase. *Comp. Biochem. Physiol. [B]* 105, 13–19.
80. Pinto, R., M, Canales, J., Gunther, S., M, A, and Sillero, A. (1986). Diadenosine tetraphosphate activates cytosol 5′-nucleotidase. *Biochem. Biophys. Res. Commun.* 138, 261–267.
81. Bontemps, F., Van den Berghe, G., and Hers, H.-G. (1988). 5′-Nucleotidase activities in human erythrocytes. Identification of purine 5′-nucleotidase stimulated by ATP and glycerate 2,3-bisphosphate. *Biochem. J.* 250, 687–696.
82. Fridland, A., Connelly, M. C., and Robbins, T. J. (1986). Tiazofurin metabolism in human lymphoblastoid cells: evidence for phosphorylation by adenosine kinase and 5′-nucleotidase. *Cancer Res.* 46, 532–537.
83. Johnson, M. A., and Fridland, A. (1989). Phosphorylation of 2′,3′-Dideoxyinosine by cytosolic 5′-nucleotidase of human lymphoid cells. *Mol. Pharmacol.* 36, 291–295.
84. Baiocchi, C., Pesi, R., Camici, M., Itoh, R., and Tozzi, M. G. (1996). Mechanism of reaction catalyzed by cytosolic 5′-nucleotidase/phosphotransferase: formation of a phosphorylated intermediate. *Biochem. J.* 317, 797–801.
85. Pesi, R., Turriani, M., Allegrini, S., et al. (1994). The bifunctional cytosolic 5′-nucleotidase: regulation of the phosphotransferase and nucleotidase activities. *Arch. Biochem. Biophys.* 312, 75–80.

86. Dragon, S., Hille, R., Gotz, R., and Baumann, R. (1998). Adenosine 3′:5′-cyclic monophosphate (cAMP)-inducible pyrimidine 5′-nucleotidase and pyrimidine nucleotide metabolism of chick embryonic erythrocytes. *Blood* 91, 3052–3058.
87. Paglia, E. D., and Valentine, W. N. (1975). Characteristics of pyrimidine-specific 5′-nucleotidase in human erythrocytes. *J. Biol. Chem.* 250, 7973–7979.
88. Swallow, D. M., Turner, V. S., and Hopkinson, D. A. (1983). Isozymes of rodent 5′-nucleotidase: evidence for two independent structural loci Umph-1 and Umph-2. *Ann. Hum. Genet.* 47(pt. 1), 9–17.
89. Beutler, E., and West, C. (1982). Tissue distribution of pyrimidine-5′-nucleotidase. *Biochem. Med.* 27, 334–341.
90. Lu, M. M., Chen, F., Gitler, A., et al. (2000). Cloning and expression analysis of murine lupin, a member of a novel gene family that is conserved through evolution and associated with lupus inclusions. *Dev. Genes Evol.* 210, 512–517.
91. Marinaki, A. M., Escuredo, E., Duley, J. A., et al. (2001). Genetic basis of hemolytic anemia caused by pyrimidine 5′ nucleotidase deficiency. *Blood* 97, 3327–3332.
92. Kanno, H., Takizawa, T., Miwa, S., and Fujii, H. (2004). Molecular basis of Japanese variants of pyrimidine 5′-nucleotidase deficiency. *Br. J. Haematol.* 126, 265–271.
93. Rees, D. C., Duley, J. A., and Marinaki, A. M. (2003). Pyrimidine 5′ nucleotidase deficiency. *Br. J. Haematol.* 120, 375–383.
94. Amici, A., and Magni, G. (2002). Human erythrocyte pyrimidine 5′-nucleotidase, PN-1. *Arch. Biochem. Biophys.* 397, 184–190.
95. Fritzson, P., and, and Smith, I. (1971). A new nucleotidase of rat liver with activity toward 3′- and 5′-nucleotides. *Biochim. Biophys. Acta* 235, 128–141.
96. Magnusson, G. (1971). Deoxyribonucleotide phosphatase from rat liver and cultured mouse fibroblasts. *Eur. J. Biochem.* 20, 225–230.
97. Hoglund, L., and Reichard, P. (1990). Cytoplasmic 5′(3′)-nucleotidase from human placenta. *J. Biol. Chem.* 265, 6589–6595.
98. Amici, A., Emanuelli, M., Ferretti, E., Raffaelli, N., Ruggieri, S., and Magni, G. (1994). Homogeneous pyrimidine nucleotidase from human erythrocytes: enzymic and molecular properties. *Biochem. J.* 304, 987–992.
99. Rampazzo, C., Johansson, M., Gallinaro, L., et al. (2000). Mammalian 5′(3′)-deoxyribonucleotidase, cDNA cloning, and overexpression of the enzyme in *Escherichia coli* and mammalian cells. *J. Biol. Chem.* 275, 5409–5415.
100. Amici, A., Emanuelli, M., Ruggieri, S., Raffaelli, N., and Magni, G. (2002). Kinetic evidence for covalent phosphoryl-enzyme intermediate in phosphotransferase activity of human red cell pyrimidine nucleotidases. *Meth. Enzymol.* 354, 149–159.
101. Mazzon, C., Rampazzo, C., Scaini, M. C., et al. (2003). Cytosolic and mitochondrial deoxyribonucleotidases: activity with substrate analogs, inhibitors and implications for therapy. *Biochem. Pharmacol.* 66, 471–479.
102. Rampazzo, C., Gallinaro, L., Milanesi, E., Frigimelica, E., Reichard, P., and Bianchi, V. (2000). A deoxyribonucleotidase in mitochondria: involvement in regulation of dNTP pools and possible link to genetic disease. *Proc. Natl. Acad. Sci. U. S. A.* 97, 8239–8244.
103. Rampazzo, C., Ferraro, P., Pontarin, G., Fabris, S., Reichard, P., and Bianchi, V. (2004). Mitochondrial deoxyribonucleotides, pool sizes, synthesis, and regulation. *J. Biol. Chem.* 279, 17,019–17,026.

104. Gallinaro, L., Crovatto, K., Rampazzo, C., et al. (2002). Human mitochondrial 5′-deoxyribonucleotidase. Overproduction in cultured cells and functional aspects. *J. Biol. Chem.* 277, 35,080–35,087.
105. Galmarini, C. M., Mackey, J. R., and Dumontet, C. (2002). Nucleoside analogues and nucleobases in cancer treatment. *Lancet Oncol.* 3, 415–424.
106. Bergman, A. M., Pinedo, H. M., and Peters, G. J. (2002). Determinants of resistance to 2′,2′-difluorodeoxycytidine (gemcitabine). *Drug. Resist. Updat.* 5, 19–33.
107. van der Wilt, C. L., Kroep, J. R., Loves, W. J., et al. (2003). Expression of deoxycytidine kinase in leukaemic cells compared with solid tumour cell lines, liver metastases and normal liver. *Eur. J. Cancer* 39, 691–697.
108. Schirmer, M., Stegmann, A. P., Geisen, F., and Konwalinka, G. (1998). Lack of cross-resistance with gemcitabine and cytarabine in cladribine-resistant HL60 cells with elevated 5′-nucleotidase activity. *Exp. Hematol.* 26, 1223–1228.
109. Carson, D. A., Carrera, C. J., Wasson, D. B., and Iizasa, T. (1991). Deoxyadenosine-resistant human T-lymphoblasts with elevated 5′-nucleotidase activity. *Biochim. Biophys. Acta* 1091, 22–28.
110. Dumontet, C., Fabianowska-Majewska, K., Mantincic, D., et al. (1999). Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. *Br. J. Haematol.* 106, 78–85.
111. Lotfi, K., Mansson, E., Chandra, J., et al. (2001). Pharmacological basis for cladribine resistance in a human acute T lymphoblastic leukaemia cell line selected for resistance to etoposide. *Br. J. Haematol.* 113, 339–346.
112. Mohammad, R. M., Beck, F. W., Katato, K., Hamdy, N., Wall, N., and Al-Katib, A. (1998). Potentiation of 2-chlorodeoxyadenosine activity by bryostatin 1 in the resistant chronic lymphocytic leukemia cell line (WSU-CLL): association with increased ratios of dCK/5′-NT and Bax/Bcl-2. *Biol. Chem.* 379, 1253–1261.
113. Kawasaki, H., Carrera, C. J., Piro, L. D., Saven, A., Kipps, T. J., and Carson, D. A. (1993). Relationship of deoxycytidine kinase and cytoplasmic 5′-nucleotidase to the chemotherapeutic efficacy of 2-chlorodeoxyadenosine. *Blood* 81, 597–601.
114. Galmarini, C. M., Thomas, X., Calvo, F., et al. (2002). Potential mechanisms of resistance to cytarabine in AML patients. *Leuk. Res.* 26, 621–629.
115. Keller, P. M., McKee, S.A., and Fyfe, J.A. (1985). Cytoplasmic 5′-nucleotidase catalyzes acyclovir phosphorylation. *J. Biol. Chem.* 260, 8664–8667.
116. Saunders, P. P., Spindler, C. D., Tan, M. T., Alvarez, E., and Robins, R. K. (1990). Tiazofurin is phosphorylated by three enzymes from Chinese hamster ovary cells. *Cancer Res.* 50, 5269–5274.
117. Jager, W., Salamon, A., and Szekeres, T. (2002). Metabolism of the novel IMP dehydrogenase inhibitor benzamide riboside. *Curr. Med. Chem* 9, 781–786.
118. Agbaria, R., Mullen, C. A., Hartman, N. R., et al. (1994). Effects of IMP dehydrogenase inhibitors on the phosphorylation of ganciclovir in MOLT-4 cells before and after herpes simplex virus thymidine kinase gene transduction. *Mol. Pharmacol.* 45, 777–782.
119. Smee, D. F., Campbell, N. L., and Matthews, T. R. (1985). Comparative antiherpesvirus activities of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, acyclovir, and two 2′-fluoropyrimidine nucleosides. *Antiviral Res.* 5, 259–267.
120. Balzarini, J., Lee, C. K., Herdewijn, P., and Declercq, E. (1991). Mechanism of the potentiating effect of ribavirin on the activity of 2′,3′-dideoxyinosine against human immunodeficiency virus. *J. Biol. Chem.* 266, 21,509–21,514.

121. Razmara, M., Eriksson, S., and Albertioni, F. (2003). 5′-Nucleotidases levels measured in peripheral blood cells from patients with chronic and acute leukemia. In *Abstracts, Joint 11th International and 9th European Symposium on Purines and Pyrimidines in Man, Egmond aan Zee,* Peters, G. J., ed., June 9–13. Abstract P82, Drukkerij Peters and VU University Medical Center, Amsterdam, The Netherlands.
122. Galmarini, C. M., Cros, E., Graham, K., Thomas, X., Mackey, J. R., and Dumontet, C. (2004). 5-(3)-Nucleotidase mRNA levels in blast cells are a prognostic factor in acute myeloid leukemia patients treated with cytarabine. *Haematologica* 89, 617–619.
123. Lewis, W. A., and Dalakas, M. C. (1995). Mitochondrial toxicity of antiviral drugs. *Nat. Med.* 1, 417–422.
124. Gandhi, V., and Plunkett, W. (2002). Cellular and clinical pharmacology of fludarabine. *Clin. Pharmacokinet.* 41, 93–103.
125. Zimmermann, H. (1992). 5′-nucleotidase: molecular structure and functional aspects. *Biochem. J.* 285, 345–365.
126. Resta, R., Hooker, S. W., Hansen, K. R., et al. (1993). Murine ecto-5′-nucleotidase (CD73)—cDNA cloning and tissue distribution. *Gene* 133, 171–177.
127. Allegrini, S., Pesi, R., Tozzi, M. G., Fiol, C. J., Johnson, R. B., and Eriksson, S. (1997). Bovine cytosolic IMP/GMP-specific 5′-nucleotidase: cloning and expression of active enzyme in *Escherichia coli*. *Biochem. J.* 328, 483–487.
128. Amici, A., Emanuelli, M., Raffaelli, N., Ruggieri, S., Saccucci, F., and Magni, G. (2000). Human erythrocyte pyrimidine 5-nucleotidase, PN-I, is identical to p36, a protein associated to lupus inclusion formation in response to α-interferon. *Blood* 96, 1596–1598.
129. Itoh, R., and Oka, J. (1985). Evidence for existence of a cytosol 5′-nucleotidase in chicken heart: comparison of some properties of heart and liver enzymes. *Comp. Biochem. Physiol.* 81B, 159–163.

5 Pumping Out Drugs

*The Potential Impact of ABC Transporters on Resistance to Base, Nucleoside, and Nucleotide Analogs**

Piet Borst, MD, PhD
and Peter Wielinga, PhD

CONTENTS

SUMMARY

Several adenosine triphosphate-binding cassette transporters can transport nucleoside analogs out of cells against steep concentration gradients, resulting in resistance to these drugs. At least three members of the family of human multidrug resistance-associated proteins (MRPs) (MRP4, 5, and 8) are able to transport not only the cyclic nucleotides cyclic adenosine 5′-monophosphate and cyclic guanosine 5′-monophosphate but also some

*This is a shortened and adapted version of a review by Borst et al. *(1)*.

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

nucleoside–monophosphate analogs. This can result in resistance to their base, nucleoside, or nucleotide precursors, at least in cell lines with high levels of the transporter. MRP4- and MRP5-transfected cells showed resistance (although at a low level) to the thiopurines 6-mercaptopurine and 6-thioguanine and to the antiviral agent adefovir. MRP8-transfected cells also show resistance to adefovir, dideoxycytidine, and the fluoropyrimidines 5-fluorouracil and 5-fluorodeoxyuridine, possibly because MRP8 can efflux the monophosphate derivative of these analogs. However, the affinity of these transporters for the nucleotide analogs studied thus far is relatively low (millimolar rather than micromolar), and this limits their potential impact on resistance. This review summarizes briefly how adenosine triphosphate-binding cassette transporters in general, and MRPs in particular, could affect the disposition and cellular accumulation of anticancer agents.

Key Words: ABC transporters; BCRP; efflux pumps; MRP4; MRP5; multidrug resistance proteins; nucleoside monophosphates; PMEA; thiopurines.

1. INTRODUCTION

The number of transporters able to transport drugs through membranes against a steep concentration gradient has rapidly risen in recent years. The transporters that use adenosine triphosphate (ATP) hydrolysis to energize transport belong to the superfamily of ATP-binding cassette (ABC) proteins *(1–4)* (Fig. 1):

1. *P-Glycoproteins (P-gps), exemplified by the P-gps encoded by the human* MDR1 *gene and the murine* Mdr1a *and* Mdr1b *genes, in formal new nomenclature ABCB1*. These proteins transport a range of amphipathic (natural product) drugs, including many major anticancer drugs, such as taxanes, vinca alkaloids, and anthracyclines. P-gps prefer neutral and weakly basic drugs as substrate, and they appear to take these drugs from the inner leaflet of the plasma membrane and expel them from the cell. High expression of P-gp genes results in multidrug resistance (MDR) of cancer cells.

   There are three other P-gps known, but these appear to play no role in drug resistance. The MDR3 (also called MDR2) human P-gp and its mouse homolog encoded by the Mdr2 gene (both now ABCB4) are dedicated phosphatidylcholine (PC) transporters, essential for transport of PC into bile. The human homolog still has some of the drug transport capacity of ABCB1 *(5)*, but is not known to be involved in MDR.

   The second MDR1 P-gp homolog was originally called “sister of P-gp” *(6)* but is now known as BSEP *(7)*, the bile salt export protein (ABCB11). It has low transport activity toward paclitaxel *(8)*. The third P-gp homolog (ABCB5) was recently described and still needs to be characterized *(9)*.

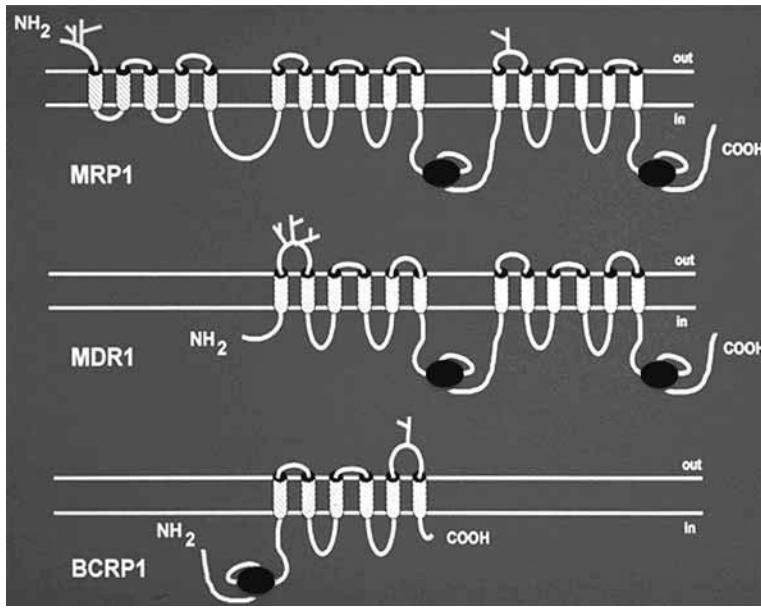


Fig. 1. Putative two-dimensional membrane topology of representative ABC transporters. *See* ref. *3* for background references. MRP1, multidrug resistance (-associated) protein 1 (ABCC1); MDR1, the P-glycoprotein (ABCB1) encoded by the human *MDR1* gene; BCRP1, the breast cancer resistance protein (ABCG2). BCRP1 functions as a homodimer.

2. *The multidrug resistance (-associated) proteins or MRPs (ABCC group), a large transporter family containing nine members in humans (2,10–12).* Some MRPs have five extra transmembrane segments not present in P-gps, as indicated in Fig. 1, but these do not seem to be essential for transport activity, and they are missing in MRP4, MRP5, MRP8, and MRP9. The MRPs studied thus far are organic anion transporters; that is, they transport organic compounds with one or more negative charges, such as drugs conjugated with glutathione, glucuronide, or sulfate. Whereas MRP1 can cause MDR and may play a substantial role in anticancer drug resistance, this is much less clear for the other MRPs. Some of these transporters appear to fulfill essential roles in normal metabolism. An example is MRP2, located in the canalicular membrane of the hepatocyte and the main transporter for the excretion of bilirubin glucuronides into bile. MRP4, MRP5, and MRP8 can transport nucleotide analogs, and these MRPs are discussed in more detail in this review.
3. *The breast cancer resistance protein (BCRP), or ABCG2, but also known under various other names (3,4).* ABCG2 is a half-transporter

(Fig. 1) and functions as a homodimer. Its substrate specificity is wider than initially thought and overlaps with both P-gp and the MRPs. Like the MDR1 P-gp, BCRP is located apically in epithelial cells and plays a role in preventing drugs from entering the body and entering the fetus through the feto-maternal barrier.

2. TRANSPORT OF NUCLEOSIDE/NUCLEOTIDE ANALOGS BY MRP4 AND MRP5

The first indication that MRP5 might be able to transport nucleotide analogs came from Wijnholds et al. *(13)*, who found that cells transfected with an *MRP5* gene construct were resistant to thiopurines. Schuetz et al. *(14)* showed that cells selected for resistance to adefovir (PMEA) overproduced MRP4, and subsequent work has established that MRP4 *(14–20)* and MRP5 *(20–22)* can confer resistance to a range of base, nucleoside, and nucleotide analogs, as summarized in Table 1. For the thiopurines and PMEA, a detailed metabolite analysis has shown that resistance is caused by transport by MRP4/5 of the nucleoside-monophosphate, and not the base, nucleoside, nucleoside-diphosphate, or -triphosphate *(19,20)*. By inference, we assume that the same holds for all other compounds listed in Table 1.

The resistance levels in Table 1 are low. The main reason for this is the low affinity of MRP4/5 for all nucleotide analogs tested thus far. This has been demonstrated in vesicular transport experiments *(20)*, but it also follows from the relatively high intracellular nucleotide analog concentrations required for a difference between resistant and sensitive cells. In fact, the human T-lymphoid cell line CEM-resistant1 (CEM-r1), selected for high resistance to PMEA, has a partial defect in mitochondrial adenylate kinase in addition to high overexpression of MRP4 *(14)*. The adenylate kinase is required for phosphorylation of PMEA. Hence, PMEA accumulates to high levels in these cells.

The Human Embryonic Kidney 293 (HEK293) cells often used to screen for MRP function have a high intrinsic resistance to several nucleoside analogs. This is not because of the absence of nucleoside transporters (unpublished results) but presumably sluggish phosphorylation of the nucleoside analogs in HEK293 cells. This may explain why the HEK cells are not affected by zidovudine, stavudine (d4T), and 3TC (lamivudine) *(20)*, whereas the MRP4-overproducing CEM-r1 cells of Schuetz et al. *(14)* are partially resistant to the anti-human immunodeficiency virus activity of these drugs. Even in the CEM-r1 cells, however, substantial resistance to the cytostatic effect of ganciclovir was only obtained when the CEM-r1 and the control parental cells were both transfected with a herpesvirus thymidine kinase gene construct to allow efficient phosphorylation of ganciclovir *(17)*.

Table 1
Resistance Against Drugs Caused by MRP4 and MRP5 in Transfected Cells

Base/nucleoside/nucleotide	*HEK293* IC_{50} *(μM)*	*Resistance factor* *MRP4*	*MRP5*
Altered purine ring			
6-mercaptopurine	5	3	3
Thioguanine	1	3	2
Altered pyrimidine ring		No resistance found	
Purine nucleoside analog			
adefovir (PMEA)	84	4	3
PMEG	2	2	1
PME diaminopurine (PMEDAP)	64	5	2
Cyclopropyl-PMEDAP (cPr-PMEDAP)	2	10	1
didanosine (ddI)	>250	n.d.	n.d.
ddA	>400	1	1
Abacavir (ABC)	244	2	2
Cladribine	2	2	2
Ganciclovir[a] (GCV)	12	5	n.d.
Pyrimidine nucleoside analog			
Zidovudine[b] (AZT)	1800	n.d.	1
Stavudine[b] (d4T)	1000	1	1
Zalcitabine	>1000	1	n.d.

Source: From ref. *20*.
n.d., no data.
[a]From ref. *17*, using CEMr1-TK cells transfected with a herpes virus thymidine kinase gene construct.
[b]Some resistance found by Schuetz et al. *(14)* in a PMEA-selected.
MRP4 overproducing T-lymphoid cell line.

The degree of resistance is strictly dependent on the degree of overexpression of the transporter studied in the transfected cell. An additional complication is that not all the transporter molecules made in the transfected cell reach the plasma membrane where they can contribute to resistance. The fraction trapped in the cell can be high and is difficult to quantitate *(23)*. This may explain why groups at Eli Lilly *(24,25)* have observed higher levels of resistance in the *MRP5*-transfected HEK293 cells produced in their laboratory than we have seen in our laboratory. For 6-thioguanine, they find a resistance factor of 9, whereas we obtain 2 (Table 1). They also find resistance for gemcitabine, cytarabine, cladribine, and 5-fluorouracil (5-FU). The 5-FU resistance appears to be caused by transport of 5-fluoro-2′-deoxyuridinemonophosphate (5-F-2′-dUMP). In vesicular transport experiments, the authors found an affinity constant K_m

for this substrate of 1.3 m*M*, confirming that MRP5 is a poor nucleotide analog transporter. The details of these results remain to be published.

HEK293 cells are highly resistant to the cytostatic effect of 3′-deoxy-2′3′-didehydrothymidine (d4T), and they also are not inhibited by So324, the recently synthesized arylphosphoramidate precursors of d4 T-5′-monophosphate (d4TMP). We have shown, however, that both d4TMP, and especially alaninyl-d4TMP, which is released from the prodrug prior to d4TMP formation *(26)*, are transported by MRP5, albeit with low affinity *(20)*.

3. TRANSPORT OF PHYSIOLOGICAL SUBSTRATES BY MRP4 AND MRP5

MRP4 *(15)* and MRP5 *(22)* are able to transport cyclic guanosine 5′-monophosphate and cyclic adenosine 5′-monophosphate, but whether the affinity of these transporters for cyclic nucleotides is high enough to make an impact on the cellular disposition of these compounds remains controversial *(20,27)*. However, we have found a range of other physiological substrates for MRP4 that are transported with micromolar affinity. The list now includes steroid-glucuronides and steroid-sulfates *(28)*, hydroxylated bile salts conjugated to glucuronide (unpublished results), and prostaglandins E_1 and E_2 *(29)*. There is clearly no lack of high-affinity substrates for MRP4. Whether this transporter is normally not too occupied with physiological substrates to transport nucleotide analogs or cyclic nucleotides remains to be seen. For MRP5, no high-affinity substrate is known yet.

4. TRANSPORT OF NUCLEOTIDE ANALOGS BY MRP8 AND MRP9

The two latest additions to the MRP family, MRP8 *(30–32)* and MRP9 *(30,31,33,34)*, resemble MRP4 and MRP5 most *(30)*. They might therefore also be able to transport nucleotide analogs. It has been difficult to obtain high-level expression of MRP8 in the standard cell lines used in our laboratory, but Guo et al. *(35)* managed to obtain expression of MRP8 in a pig LLC-PK1 kidney cell line. As shown in Table 2, these cells are resistant to PMEA as well as zalcitabine, 5-FU, and 5-fluoro-2′-deoxyuridine, but not to 6-thioguanine, cladribine, deoxycoformycin, 2′3′-dideoxy-3′-thial-cytidine, or zidovudine. Membrane vesicles from MRP8-overproducing cells transport 5-F-2′-dUMP, suggesting that MRP8 causes resistance by transporting nucleotide analogs out of the cell, like MRP4 and MRP5. Whether MRP8 also has a low affinity for these substrates remains to be determined. The substrate specificity of MRP9 has not yet been determined.

Table 2
Drug Sensitivity of MRP8-Transfected Pig Kidney Cells (LLC-PK1)

Drug	*IC_{50} of parental cells (μM)*	*Fold resistance in MRP8 transfectant*
PMEA	0.5	5.4
6-TG	0.5	1.1
Zalcitabine	0.4	6.1
5-FU	0.01	2.9
5-Fluoro-2′-deoxyuridine	0.02	5.2

Source: Modified from ref. *35*.

No resistance was found for cladribine, deoxycoformycin (DCF), 3TC, or zidovudine.

5. CONCLUDING REMARKS

The precise role of MRP4, MRP5, MRP8, and possibly MRP9 in limiting cellular accumulation of base, nucleoside, and nucleotide analogs remains to be established. As pointed out, the low affinity of MRP4 and MRP5 for the nucleotide analogs studied thus far makes a prominent role of these transporters in drug resistance less likely at present. As it stands, there is no evidence that anticancer chemotherapy in patients is adversely affected by any of these MRPs.

The knockout mice lacking one or more of these transporters could, in the long run, provide more solid information on the ability of MRPs to contribute to resistance to base, nucleoside, and nucleotide analogs. We have not observed altered pharmacokinetics of PMEA in the Mrp5(–/–) mice (unpublished results), but it is possible that this negative result is caused by compensatory activity of related transporters. In collaboration with Dr. John Schuetz (Memphis, TN), we have generated an *Mrp5*/*Mrp4* double-knockout mouse to investigate this issue.

ACKNOWLEDGMENTS

Our experimental work on which this review is based was supported by grants NKI 2001-2473 to P. B. and J. Wijnholds and NKI 2001-2474 to P. B.

REFERENCES

1. Borst P, Balzarini J, Ono N, et al. The potential impact of drug transporters on nucleoside-analog-based antiviral chemotherapy. Antiviral Res 2004;62:1–7.
2. Holland IB, Kuchler, K, Higgins C, Cole S, eds. ABC Proteins: From Bacteria to Man. London: Academic Press;2003.
3. Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annu Rev Biochem 2002;71:537–592.

4. Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev 2003;55:3–29.
5. Smith AJ, van Helvoort A, van Meer G, et al. MDR3 P-glycoprotein, a phosphatidylcholine translocase, transports several cytotoxic drugs and directly interacts with drugs as judged by interference with nucleotide trapping. J Biol Chem 2000;275:23,530–23,539.
6. Childs S, Ych RL, Georges E, Ling V. Identification of a sister gene to P-glycoprotein. Cancer Res 1995;55:2029–2034.
7. Gerloff T, Stieger B, Hagenbuch B, et al. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem 1998;273:10,046–10,050.
8. Childs S, Yeh RL, Hui D, Ling V. Taxol resistance mediated by transfection of the liver specific sister gene of P-glycoprotein. Cancer Res 1998;58:4160–4167.
9. Frank NY, Pendse SS, Lapchak PH, et al. Regulation of progenitor cell fusion by ABCB5 P-glycoprotein, a novel human ATP-binding cassette transporter. J Biol Chem 2003;278:47,156–47,165.
10. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters, the MRPs. J Natl Cancer Inst 2000;92:1295–1302.
11. Kruh GD, Zeng H, Rea PA, et al. MRP subfamily transporters and resistance to anticancer agents. J Bioenerg Biomembr 2001;33:493–501.
12. Jedlitschky G, Keppler D. Transport of Leukotriene C_4 and structurally related conjugates. Vitam Horm 2002;64:153–184.
13. Wijnholds J, Mol CAAM, Scheffer GL, Scheper RJ, Borst P. Multidrug resistance protein 5, a candidate multispecific organic anion transporter. Proc Am Assoc Cancer Res 1999;40:2088.
14. Schuetz JD, Connelly MC, Sun D, et al. MRP4: A previously unidentified factor in resistance to nucleoside-based antiviral drugs. Nat Med 1999;5:1048–1051.
15. Chen Z-S, Lee K, Kruh GD. Transport of cyclic nucleotides and estradiol 17-β-D-glucuronide by multidrug resistance protein 4: resistance to 6-mercaptopurine and 6-thioguanine. J Biol Chem 2001;276:33,747–33,754.
16. Adachi M, Reid G, Schuetz JD. Therapeutic and biological importance of getting nucleotides out of cells: a case for the ABC transporters, MRP4 and 5. Adv Drug Deliv Rev 2002;54:1333–1342.
17. Adachi M, Sampath J, Lan L-B, et al. Expression of MRP4 confers resistance to ganciclovir and compromises bystander cell killing. J Biol Chem 2002;277: 38,998–39,004.
18. Lai L, Tan TMC. Role of glutathione in the multidrug resistance protein 4 (MRP4/ABCC4)-mediated efflux of cAMP and resistance to purine analogues. Biochem J 2002;361:497–503.
19. Wielinga PR, Reid G, Challa EE, et al. Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. Mol Pharmacol 2002;62:1321–1332.
20. Reid G, Wielinga P, Zelcer N, et al. Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. Mol Pharmacol 2003;63:1094–1103.
21. Wijnholds J, Mol CAAM, Van Deemter L, et al. Multidrug-resistance protein 5 is a multispecific organic anion transporter able to transport nucleoside analogs. Proc Natl Acad Sci USA2000;97:7476–7481.

22. Jedlitschky G, Burchell B, Keppler D. The multidrug resistance protein 5 (MRP5) functions as an ATP-dependent export pump for cyclic nucleotides. J Biol Chem 2000;275:30,069–30,074.
23. Borst P, Evers R, Kool M, Wijnholds J. The multidrug resistance protein family. Biochim Biophys Acta 1999;1461:347–357.
24. Davidson JD, Ma L, Iverson PW, et al. Human multi-drug resistance protein 5 (MRP5) confers resistance to gemcitabine. Proc Am Assoc Cancer Res 2000;43:3868.
25. Dantzig AH, Pratt SE, Shepard RL. MRP5 confers resistance to 5-FU and transports a phosphorylated metabolite of 5-FU. Proc Am Assoc Cancer Res 2003;44:3696.
26. Balzarini J, Karlsson A, Aquaro S, al. Mechanism of anti-HIV action of masked alaninyl D4T-MP derivatives. Proc Natl Acad Sci USA1996;93:7295–7299.
27. Wielinga PR, Van der Heijden I, Reid G, et al. Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells. J Biol Chem 2003;278:17,664–17,671.
28. Zelcer N, Reid G, Wielinga P, et al. Steroid and bile acid conjugates are substrates of human multidrug-resistance protein (MRP)4 (ATP-binding cassette C4). Biochem J 2003;371:361–367.
29. Reid G, Wielinga P, Zelcer N, et al. The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. Proc Natl Acad Sci USA2003;100:9244–9249.
30. Tammur J, Prades C, Arnould I, et al. Two new genes from the human ATP-binding cassette transporter superfamily, *ABCC11* and *ABCC12*, tandemly duplicated on chromosome 16q12. Gene 2001;273:89–96.
31. Yabuuchi H, Shimizu H, Takayanagi S, Ishikawa T. Multiple splicing variants of two new human ATP-binding cassette transporters, ABCC11 and ABCC12. Biochem Biophys Res Commun 2001;288:933–939.
32. Bera TK, Lee S, Salvatore G, et al. *MRP8*, a new member of ABC transporter superfamily, identified by EST database mining and gene prediction program, is highly expressed in breast cancer. Mol Med 2001;7:509–516.
33. Shimizu H, Taniguchi K, Hippo Y, et al. Characterization of the mouse ABcc12 gene and its transcript encoding an ATP-binding transporter, an orthologue of human ABCC12. Gene 2003;310:17–28.
34. Bera TK, Iavarone C, Kumar V, et al. *MRP9*, an unusual truncated member of the ABC transporter superfamily, is highly expressed in breast cancer. Proc Natl Acad Sci USA2002;99:6997–7002.
35. Guo Y, Kotova E, Chen Z-S, et al. MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2′,3′-dideoxycytidine and 9′-(2′-phosphonylmethoxyethyl)adenine. J Biol Chem 2003;278:29,509–29,514.

6 Cytosine Arabinoside

Metabolism, Mechanisms of Resistance, and Clinical Pharmacology

Isabelle Hubeek, PhD,
Gert-Jan L. Kaspers, MD, PhD,
Gert J. Ossenkoppele, MD, PhD,
and Godefridus J. Peters, PhD

CONTENTS

SUMMARY

The deoxynucleoside analog cytarabine (ara-C) remains one of the most effective drugs used in the treatment of acute leukemia as well as other hematopoietic malignancies. The activity of ara-C depends on the conversion to its cytotoxic triphosphate derivative, ara-CTP. This process is influenced by multiple factors, including transport, phosphorylation, deamination, and levels of competing metabolites, deoxycytidine triphosphate in particular.

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

Furthermore, the efficacy of ara-C is determined by the ability of ara-CTP to interfere with deoxyribonucleic acid (DNA) polymerases in the extent of incorporation into the DNA, leading to chain termination. Finally, several factors in the apoptotic pathway also determine sensitivity to ara-C. Ara-C has been given intravenously over a wide range of doses. The standard or conventional dose varies from 100 to 200 mg/m^2 daily and is given by intermittent injection or by continuous infusion over 5–10 d. The presence of drug refractoriness and relapsing leukemia together with insights into the mechanisms of ara-C resistance led to the development of high-dose (1–3 g/m^2) ara-C treatment. A number of different strategies have been developed to increase the efficacy of ara-C. First, biochemical modulation of ara-C-mediated cytotoxicity, in which ara-C is combined with compounds that enhance its metabolism or interfere with its catabolism, has been successful. Second, ara-C has been encapsulated into multivesicular liposomes, and several ara-C prodrugs containing lipophilic side chains in the base or in the sugar moiety have been designed to increase cellular uptake of ara-C and delay its deamination and clearance. Greater understanding of the metabolism and mechanisms of action of ara-C could contribute to the development of novel therapeutic strategies capable of overcoming ara-C resistance and is essential to improve therapeutic efficacy.

Key Words: Acute myeloid leukemia; biochemical modulation; cytarabine; cytosine arabinoside; deoxycytidine kinase; high-dose ara-C; FLAG; fludarabine.

1. INTRODUCTION

Cytosine arabinoside (1-β-D-arabinofuranosylcytosine, cytarabine, ara-C) is a classical deoxynucleoside analog and is the most effective agent in the treatment of acute myeloid leukemia (AML). It is used as part of first-line treatment in all AML treatment protocols *(1)* and is active in other hematological malignancies, including acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma *(2–5)*. The administration of high-dose ara-C has become firmly established over the past decades. These schedules achieve considerably higher ara-C plasma levels than those obtained with standard-dose therapy. Moreover, a subset of refractory and relapsed AML patients responds to high-dose ara-C therapy *(6)*. Unfortunately, remissions obtained with high-dose ara-C are short-lived, and patients ultimately relapse with highly resistant disease *(7)*. Therefore, a clear rationale exists for efforts to understand the mechanisms of action of ara-C. Knowledge of the mechanisms that can cause resistance to this drug (Table 1) can be used to design strategies capable of circumventing resistance. Leukemic cells can be obtained relatively easy, and therefore several ex vivo pharmacological studies have been conducted with these cells in relation to the antileukemic effect, but studies with solid tumors are limited *(3,4)*.

Table 1
Mechanisms Implicated in Resistance to Ara-C

Resistance mechanism
Reduced transport (human equilibrative nucleoside transporter 1)
Decreased activation (deoxycytidine kinase)
Enhanced degradation
Deamination (cytidine deaminase)
Dephosphorylation (5′-nucleotidase)
Increased rate of ara-CTP dephosphorylation
Increased dCTP pools
Cell death pathways
p53
Bcl-2

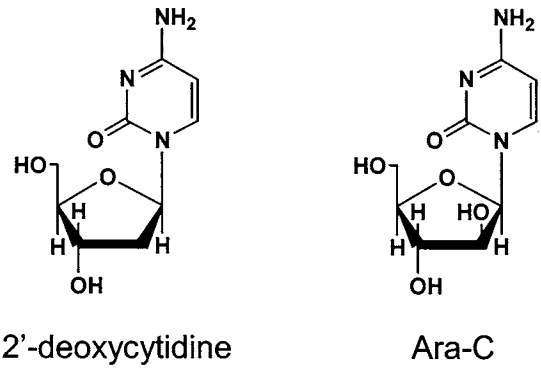


Fig. 1. Ara-C is a structural analog of 2′-deoxycytidine and differs by the presence of a hydroxyl group in the β-configuration at the 2′ position of the sugar moiety.

2. MECHANISMS OF ACTION AND RESISTANCE

2.1. Structure and Metabolism

Ara-C is a structural analog of 2′-deoxycytidine and differs by the presence of a hydroxyl group in the β-configuration at the 2′ position of the sugar moiety (Fig. 1). Ara-C is a prodrug that has to be converted to the active triphosphate derivative (ara-CTP) to exert its cytotoxic effect *(5)*. As an analog of deoxycytidine, ara-C shares the same metabolic pathways with this deoxynucleoside. Ara-C is transported across the cell membrane by a facilitated nucleoside diffusion mechanism, after which it is converted to its nucleotide derivative by the pyrimidine salvage pathway enzyme deoxycytidine kinase (dCK).

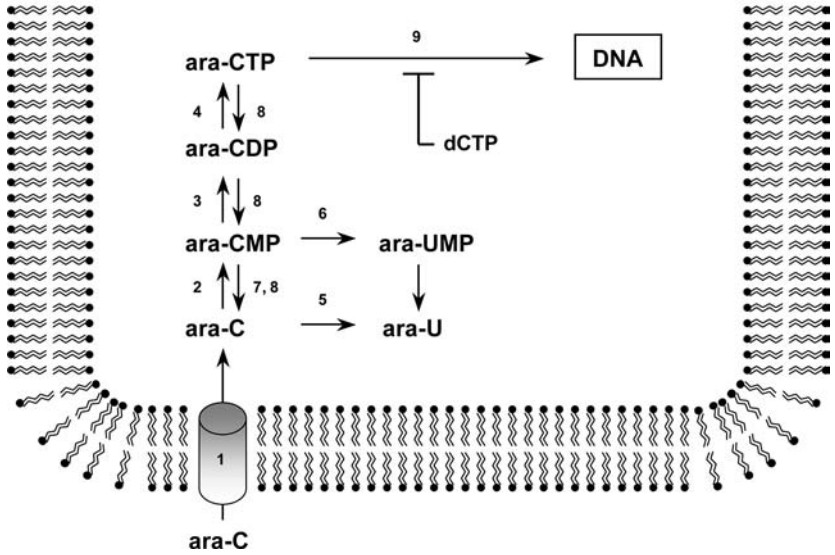


Fig. 2. Activation of ara-C and, ultimately, ara-C-mediated cytotoxicity depend on a complex interplay among ara-C anabolism, catabolism, and intracellular endogenous purine and pyrimidine levels.

After subsequent phosphorylation by monophosphate and diphosphate kinases, ara-C is converted to its active form, ara-CTP. Ara-CTP inhibits deoxyribonucleic acid (DNA) polymerase-α and is incorporated into elongating DNA strands, causing chain termination. These anabolic processes are opposed by the degradative enzymes cytidine deaminase (CDA) and deoxycytidylate deaminase, which convert ara-C and ara-CMP to the inactive derivatives ara-U and ara-UMP, respectively. Finally, the intracellular metabolism of ara-C can be influenced by several feedback mechanisms. Deoxycytidine triphosphate (dCTP), for instance, is a potent feedback inhibitor of dCK. In summary, the activation of ara-C and, ultimately, ara-C-mediated cytotoxicity depend on a complex interplay among ara-C anabolism, catabolism, and intracellular endogenous purine and pyrimidine levels (Fig. 2).

2.2. Transport

To be activated, ara-C first has to be transported into the cell. Cellular uptake of ara-C is mediated by facilitated diffusion via nucleoside-specific membrane transport carriers *(8,9)*. The human equilibrative nucleoside transporter 1 (hENT1) is responsible for 80% of ara-C influx in human leukemic blast cells *(10,11)*. At "standard-dose" ara-C (100–200 mg/m^2), plasma levels of 0.5–1 μ*M* are reached, and the drug has to compete with

other nucleosides for the transport carrier. At these concentrations, transport is the rate-limiting factor in the intracellular accumulation of ara-C. In *MLL* gene-rearranged infant, ALL-elevated expression of hENT1 contributed to ara-C sensitivity *(12)*. Reduced hENT1 expression correlated with clinical outcome in adult AML, suggesting that hENT1 deficiency may be involved in clinical resistance to ara-C in AML *(13,14)*. Impaired transport may be overcome by the use of high-dose (1–3 g/m^2) ara-C, by which high plasma levels (>50 μ*M*) can be achieved. At these concentrations, passive diffusion rates exceed those of pump-mediated transport *(2,15)*.

Efflux of ara-C may also limit its cytotoxic effect, and a useful approach to increase ara-C retention within the cell, thereby enhancing its cytotoxic effects, may be to combine it with a nucleoside transport inhibitor *(16)*. However, this has not led to useful clinical application. Drug efflux may also be mediated by the multidrug resistance-associated proteins (MRPs), which belong to the adenosine triphosphate (ATP)-binding cassette superfamily of transporters and have the ability to function as outward pumps for chemotherapeutic drugs. Several MRPs have been linked to drug resistance; in childhood ALL, for instance, MRP3 has been related to worse prognosis *(17)*. Also, in AML MRP3 gene expression was higher in patients who did not achieve remission, and expression of MRP2 or MRP3 was associated with a lower rate of survival; patients who expressed high levels of both genes had a particularly poor prognosis *(18)*. No direct link with ara-C could be established, however. Recent reports have demonstrated that MRP4 and MRP5 are able to efflux monophosphorylated forms of several nucleoside analogs, thereby potentially limiting their efficacy *(19)*. Reid et al., however, showed no MRP4- and MRP5-mediated resistance against ara-C in HEK293 cells overexpressing these transporters *(19)*. It therefore seems unlikely that MRP-mediated efflux plays a role in ara-C resistance.

2.3. Phosphorylation

Ara-C is dependent on phosphorylation catalyzed by dCK for its biochemical activation. Conversion of ara-C into ara-CMP by dCK is the rate-limiting step in the formation of ara-CTP *(20,21)*. Conversion of ara-CMP into the active metabolite ara-CTP involves phosphorylation by dCMP kinase and dCDP, respectively. Both these enzymes are expressed abundantly and are therefore not rate limiting in the accumulation of ara-CTP *(22)*. dCK activity has been shown to be decreased or absent in different cell lines resistant to ara-C *(23,24)*. Moreover, transfection of the dCK gene in dCK-deficient cell lines restores in vitro sensitivity to ara-C *(25,26)*. Furthermore, in vitro models have shown crossresistance between ara-C and the deoxynucleosides 2-chlorodeoxyadenosine (2-CdA, cladribine), gemcitabine, and fludarabine (F-ara-A), with reduced dCK activity as the underlying determinant of resistance *(27–29)*.

The relevance of dCK in clinical resistance to ara-C, however, remains controversial. In AML *(30,31)*, ALL *(32,33)*, and lymphoproliferative disorders *(34)*, some authors have observed decreased expression of the dCK gene or a significant decrease in activity of the enzyme as one of the mechanisms responsible for clinical resistance to ara-C or other deoxynucleoside analogs such as 2-CdA and F-ara-A. Conversely, other authors reported no significant correlation between dCK activity and clinical response to deoxynucleoside analogs in these malignancies *(35,36)*.

In vitro structural analyses of the dCK gene have revealed inactivating mutations and deletions causing dCK deficiency *(32,33,37–39)*. These mutations rarely occur in vivo, however, and therefore do not constitute a major clinical resistance mechanism. Alternatively, spliced forms of dCK messenger ribonucleic acid (mRNA) have been detected in vitro as well as in leukemic blasts from patients with clinically resistant AML. These splice variants were shown to be inactive, indicating that the presence of dCK splice products may contribute to clinical ara-C resistance *(40)*.

Structural alterations of the dCK gene other than mutations may also play a role in ara-C resistance in vivo. Hypermethylation of the promoter region of the gene might result in downregulation of dCK activity *(41,42)*. The precise role that promoter methylation plays in the regulation of dCK activity remains controversial, however. Despite reports that promoter hypermethylation resulted in downregulation of dCK activity *(43)*, Leegwater et al. failed to demonstrate any methylation of the dCK promoter in the leukemic CCRF-CEM cell line *(44)*. Another possible regulation mechanism of dCK involves phosphorylation of the enzyme itself. This issue is still under debate (*see* Chapter 2).

Finally, dCK activity is feedback regulated by dCTP, which is a potent inhibitor of dCK. Intracellular dCTP pools potently antagonize the formation of ara-CTP *(20)*. dCTP pools may be modulated by inhibition of ribonucleotide reductase, which catalyzes the reduction of CDP to dCDP *(45)*. CDP production may be mediated by alteration of CTP synthetase. Several ara-C-resistant Chinese hamster ovary cell lines displayed base-pair substitutions, rendering CTP synthetase unresponsive to allosteric regulation, leading to high dCTP pools *(46)*. However, this mechanism of ara-C resistance could not be identified in primary human leukemic blasts *(47)*.

2.4. Drug Degradation

Degradation of ara-C by deamination may decrease its antitumor activity *(48,49)*. The catabolism of ara-C results from rapid deamination by CDA to the nontoxic metabolite arabinoside uracil (ara-U). Forced expression of CDA by transfection of the gene resulted in resistance to ara-C and other deoxycytidine analogs *(50–52)*, and it was postulated that the ratio between

the deaminase and the kinase might determine the response to ara-C in leukemic cells *(48)*. Patients who responded to treatment had a low deaminase/kinase ratio; in nonresponders, the ratio was high. Although there was a large variation in enzyme activities, Jahns-Streubel et al. *(53)* demonstrated that patients who responded to treatment had a lower median deaminase activity compared to nonresponding patients. Ara-U itself can have pronounced effects on the metabolism and cytotoxicity of ara-C through a self-potentiating mechanism *(54)*. Preincubation of cells with ara-U enhanced the cytotoxicity of ara-C by an increased phosphorylation to ara-CTP and incorporation into DNA *(55)*.

Ara-CMP can be converted to the inactive ara-UMP form by deoxycytidylate deaminase (dCMPD), abrogating ara-C toxicity *(56)*. Studies have shown that tetrahydrodeoxyuridine (dTHU), which inhibits dCMPD, potentiated the metabolism of ara-C in lymphoblastic leukemic cell lines *(49)*. Furthermore, ara-CMP can be dephosphorylated by high-K_m cytoplasmic 5′-nucleotidase (5′-NT). Galmarini et al. reported that patients with AML whose blasts expressed high 5′-NT mRNA had shorter time to relapse and overall survival than patients with no or low expression *(57)*.

Once formed, ara-CTP may be dephosphorylated, which limits the intracellular accumulation of this metabolite *(58)*.

3. Ara-C-MEDIATED CYTOTOXICITY

3.1. Inhibition of DNA Polymerases and DNA Incorporation

The active metabolite of ara-C, the triphosphate ara-CTP, acts in two ways: (1) as an inhibitor of DNA polymerases and (2) by incorporation of ara-CTP into the DNA, causing chain termination, resulting in a block of DNA synthesis. DNA polymerase-α has a comparable affinity for ara-CTP and dCTP *(59)*. Ara-CTP can therefore act as a weak competitive inhibitor of this enzyme *(3,60)*. When present at high intracellular concentrations, ara-CTP also inhibits DNA polymerase-β and consequently DNA repair *(61–64)*. Ara-CTP is also a substrate for DNA polymerase-α and is incorporated into replicating DNA strands, behaving as a relative chain terminator *(65)*. This incorporation disrupts further DNA elongation and ultimately triggers apoptosis *(66)*. Accumulation and retention of ara-CTP and its incorporation into DNA have been shown by several authors to be the main factors determining its cytotoxic effect *(3,49,67–71)*.

3.2. Ara-CTP Accumulation and Retention

In vitro studies have clearly shown that an initially high accumulation of ara-CTP, long retention, and high incorporation into DNA determine the sensitivity of cells in culture. In addition, Rustum et al. *(70,72)* showed a relationship between initially high accumulation of ara-CTP and long

retention in leukemic lymphoblasts and the response rate and duration of response. Plasma ara-C levels and ara-CTP formation are not related, which implies that there is great variability in levels of activating enzymes. In addition, ara-CTP formation was observed to continue after the end of the infusion in several patients and declined thereafter with a $t_{1/2}$ of 3–4 *(73,74)*. The retention of ara-CTP appears to be a critical factor in the response of patients to ara-C treatment. Preisler et al. observed a correlation between ara-CTP retention and the duration of complete remission in acute myelogenous leukemia *(75)*.

One of the reasons for giving high-dose ara-C treatment is to enhance ara-CTP formation. However, Plunkett et al. showed that ara-CTP formation in leukemic blasts during ara-C treatment is saturated at plasma levels reached at a dose of 0.5–1 g/m^2 (plasma concentrations >10 μ*M*), which is considerably lower than the 1–3 g/m^2 of high-dose ara-C often given *(76)*.

3.3. S Phase

Cells are maximally sensitive to ara-C during the S phase, in which DNA synthesis is active. Ara-C can therefore generally be considered as an S-phase-specific drug *(77)*, and the cytotoxicity of ara-C will therefore depend on cell cycle kinetics. Preisler et al. showed that the outcome of high-dose ara-C treatment was highly dependent on the percentage of cells in S phase before the start of treatment *(78)*. Several strategies (e.g., drug scheduling, pharmacological means, and the use of hematopoietic growth factors) have been designed to recruit cells into S phase, making them more susceptible to ara-C-mediated cytotoxicity *(79)*.

3.4. Intracellular Signaling and Cell Death Pathways

Incorporation of ara-C into DNA causes localized alterations in the DNA duplex and stabilizes covalent topoisomerase I-DNA complexes, converting the enzyme into a cellular poison *(80)*. Ara-C-induced DNA damage triggers a wide spectrum of intracellular signaling elements that may contribute to ara-C-mediated cytotoxicity *(5)*, including the lipid second messengers diglyceride *(81)* and ceramide *(82)*, activation of protein kinase C (PKC) *(83)*, and the MAPK and SAPK cascades *(84,85)*. However, the molecules that detect and respond to the DNA damage caused by incorporated nucleoside analogs remain to be characterized fully.

It has been suggested that the DNA protein kinase and p53 may form a sensor complex that detects the disruption in DNA replication caused by incorporated nucleoside analogs and may subsequently signal for apoptosis *(86)*. Enforced expression of p53 rendered U937 cells more sensitive to ara-C-induced apoptosis *(87)*. Kanno et al. recently reported that leukemic cell lines with a high expression level of p53 were more susceptible to

ara-C-mediated cytotoxicity compared to p53-null leukemic cell lines *(88)*. Exposure to ara-C also results in upregulation of various transregulatory factors (e.g., activator protein-1, NF-κB) and the induction of the proto-oncogene *c-jun* in human myeloid leukemia cells *(89,90)*. Screenivasan et al. reported that ara-C was able to induce apoptosis in NF-κB-expressing cells by dephosphorylating the p65 subunit of NF-κB *(91)*.

Ara-C-induced inhibition of colony formation could be enhanced by a combination of granulocyte-macrophage colony-stimulating factor and interleukin 3, which was associated with an increase in the degree of apoptosis and correlated with the enhanced expression of the *c-jun* transcriptional activator *(92)*. This effect is mediated by an interaction of an activated *c-jun* protein with its own promoter *(93)*. In the National Cancer Institute's in vitro screening panel, an increased sensitivity to ara-C was observed in cells with an activated *ras* oncogene compared to cells with wild-type *ras* alleles; in cells with activated *ras* oncogenes, induction of apoptotic cell death was efficient, and S-phase arrest was prevented *(94,95)*.

Defects in distal cell death pathways may confer resistance to a broad range of cytotoxic compounds. Ara-C-mediated JNK signaling can directly regulate the intrinsic pathway for apoptosis by translocating to the mitochondria and interacting with Bcl-X_L or by promoting the release of SMAC into the cytosol *(80)*. An increased expression of the antiapoptotic protein bcl-2 protects leukemic cells from ara-C-induced cell death *(96–99)*. Increased expression of bcl-2 in acute leukemia was associated with a decrease in therapy-induced apoptosis, reduced patient survival, and in vitro autonomous growth of leukemic cells *(100–103)*. Inhibition of bcl-2 with antisense oligonucleotides induced apoptosis and increased the sensitivity of AML blasts to ara-C *(104)*. In bcl-2-transfected cells, a decreased sensitivity to ara-C and a lower percentage of apoptosis was observed. Ara-C treatment did not change bcl-2 levels; the accumulation of ara-CTP and its incorporation into DNA were not changed *(100)*. This indicates that high intracellular levels of bcl-2 operate distally to inhibit the final apoptotic pathway.

Various kinases may be involved in this process; Chelliah et al. observed an increase in ara-C-induced apoptosis by the PKC activator bryostatin in human leukemia HL-60 cells *(105)*. In contrast, dysregulation of the cyclin-dependent kinase inhibitor p21 increased susceptibility of U937 cells to ara-C-induced apoptosis associated with mitochondrial perturbations implicated in the activation of the apoptotic protease cascade, such as caspase-3 activation *(106)*. Members of the inhibitors of apoptosis family, survivin and XIAP, did not affect the survival of primary cells in response to ara-C *(107)*. It is clear from these data that not only factors involved in drug activation and degradation, but also several factors in the apoptotic pathway determine sensitivity to ara-C.

4. CLINICAL PHARMACOLOGY OF Ara-C

4.1. Pharmacokinetics

Measurement of ara-C is done either by a simple, rapid and specific immunoassay *(108)* or by high-performance liquid chromatography, which has the advantage of determining both ara-C and its deaminated product ara-U *(109)*. Ara-C is not administered orally because of high CDA levels in the liver and the high first-pass elimination. Ara-C has been given intravenously over a wide range of doses *(2,110,111)*. The standard or conventional dose varies from 100 to 200 mg/m^2 daily and is given by intermittent injection or by continuous infusion over 5–10 d. The steady-state plasma concentration generally is in the range of 0.1–0.5 μ*M*, and the clearance is about 134 L/m^2 *(112)*. Males have a significantly faster clearance than females. Clearance was correlated with the pretreatment white blood cell blast count *(112)*.

In the "high-dose" protocols, the drug is administered at 1–3 g/m^2, resulting in peak concentrations in the range of 100 μ*M* ara-C, which fall rapidly with a $t_{1/2,\alpha}$ of 7–20 min and a $t_{1/2,\beta}$ of 0.5–2 h. Ara-U is rapidly formed from ara-C, and peak levels are found within 15 min after ara-C administration and are about 100 μ*M*. The half-life of ara-U is much longer than that of ara-C, on the order of 0.5–6 h. The parent drug is rapidly distributed into total body water; levels in the cerebrospinal fluid reach about 50% of that in plasma. About 70% of the ara-C is excreted in the urine in the form of ara-U. In children, the rate of conversion of ara-C to ara-U increases with age *(113)* (*see* Chapter 14).

High-dose ara-C compared to standard doses appears to give comparable or greater efficacy in remission induction in AML, but some individuals are resistant to conventional dosing and may obtain remission with higher doses *(2,114)*. Because high-dose ara-C treatment leads to high drug levels in the cerebrospinal fluid, this regimen may provide an adequate prophylaxis or treatment for central nervous system leukemia. The rationale behind high-dose ara-C is that it can overcome impaired transport and may enhance intracellular ara-CTP pools, and cells at the boundary of G_1-S may be more sensitive to high-dose ara-C.

Ara-C has also been given in very low doses (20 mg/m^2/d) *(115,116)*. Very low steady-state plasma levels were observed, below the level (100 n*M* ara-C) assumed to be required for cytotoxicity. There is still the question whether ara-C in these dosages may act as a differentiation-inducing agent *(117)*.

4.2. Toxicity

The main side effects (Table 2) of standard-dose ara-C consist of nausea, vomiting, mucositis, hair loss, and pancytopenia. In varying degrees,

Table 2
Main Adverse Effects Associated With Ara-C Treatment

Standard-dose ara-C (100–200 mg/m^2)	*High-dose ara-C (1–3 g/m^2)*
Diarrhea	Higher incidence of standard-dose toxicities
Mucositis	Neurotoxicity (especially cerebellar dysfunction)
Myelosuppression	
Nausea	
Vomiting	
"Ara-C syndrome" (fever, myalgia, bone pain, chest pain, maculopapular rash, conjunctivitis, and malaise)	

the so-called ara-C syndrome is observed and consists of fever, myalgia, joint and bone pain, chest pain, a maculopapular rash, and keratoconjunctivitis, although the symptoms can also occur separately *(6,114)*. Occasionally, standard-dose ara-C causes "ara-C lung," acute pancreatitis, and peritonitis. An ara-C lung is characterized by (sub)acute respiratory failure accompanied by diffuse changes on chest films, and the diagnosis is usually made when other explanations (such as infection) can be excluded. With high-dose ara-C, the incidence and severity of these toxicities increases, as, for instance, clearly shown for the severity of skin problems, gastrointestinal toxicity, and ocular signs *(6)*. Dose-limiting toxicity of high-dose ara-C consists of central nervous system dysfunction (especially cerebellar). Pericarditis and peripheral neuropathies have also been reported with high-dose ara-C. The pathogenesis of the pulmonary side effects is not clear, although some evidence has been presented that the incidence and severity are more pronounced with high-dose ara-C.

5. ANTILEUKEMIC ACTIVITY OF Ara-C

The introduction of ara-C in the late 1960s was the single most important development in the therapy of AML. Ara-C is the most important drug used in the treatment of AML and is given in induction, maintenance, and consolidation treatment. In addition, ara-C is also used for the treatment of other hematological malignancies, such as ALL and non-Hodgkin's lymphoma (Table 3). In general, ara-C has low activity against solid tumors. Intraperitoneal ara-C in combination with cisplatin has been used for the treatment of ovarian carcinoma with a high surgically defined response in patients with small-volume residual ovarian cancer *(118,119)*.

Table 3
Treatment Regimens Containing Cytarabine Used in Other Malignancies

Disease	*Acronym*	*Regimen*				*Reference*
Pediatric ALL						
Induction		Regimen	Age 1 yr	Age 2 yr	Age ≥ 3yr	
	TIT (CNS prophylaxis)	Methotrexate	10 mg	12.5 mg	15 mg	Lauer et al., 1993
		Cytarabine	20 mg	25 mg	30 mg	
		Hydrocortisone	10 mg	12.5 mg	15 mg	
		Administer intrathecally on d 1, 2, 3, 6, 11, 16, 21, 26, 31, then every 12 wk				
Consolidation/ intensification	6-MP/CMP/ara-C	Cyclophosfamide 1 mg/m^2 iv, d 0 and d 14				Lauer et al., 1993
		Cytarabine 75 mg/m^2/d sq or iv, d 1–4, 8–11, 15–18, and 22–25				
		Mercaptopurine 60 mg/m^2/d po for 28 d				
Non-Hodgkin's lymphoma	DHAP	Dexamethasone 40 mg/d po or iv, d 1–4				Velasquez et al., 1988
		Cisplatin 100 mg/m^2, iv CI, d 1				
		Cytarabine 2 g/m^2 iv over 3 h every 12 h for two doses, d 2				
	ESHAP	Etoposide 40 mg/m^2/d iv, d 1–4				Velasquez et al.,1994
		Methylprednisolone 500 mg/day iv, d 1–5				
		Cytarabine 2 g/m^2 iv over 2–3 h, d 5				
		Cisplatin 25 mg/m^2/day iv CI, d 1–4				

CI, continuous infusion.

5.1. Standard Dose Ara-C

As a single agent (standard dose), ara-C induced complete remissions in 25% of patients *(120)*. Initial randomized phase II studies with ara-C were fashioned after laboratory studies regarding the treatment of L1210 leukemia in mice *(121)*. The results of these trials were reported in 1974, 1981, 1987, and 1991 (Table 4). The Southwest Oncology Group compared an administration schedule of 400 mg/m^2/d ara-C for 2 d with the administration of 200 mg/m^2/d for 5 d and found a superior complete response (CR) rate with the 5-d regimen (24% vs 38% with the 2- and 5-d regimens, respectively) *(122)*.

These observations were extended by the Cancer and Leukemia Group B, who compared a 5-d vs a 7-d treatment regimen using 100 mg/m^2/d ara-C. The outcome of the 7-d regimen was reported to be superior (39% vs 55% CR for 5- and 7-d treatments, respectively). Although results from this study were confounded because of the administration of either two or three doses of daunorubicin along with the 5- or 7-d ara-C treatment, respectively, combined treatment appeared additive *(123,124)*. Additional phase II trials studied extension of the duration of exposure to 10 d, adding 6-thioguanine to the regimen *(125)* or doubling the dose of ara-C to 200 mg/m^2/d *(126)*, but no additional therapeutic benefit was observed. Increased doses of ara-C have been explored in induction.

Schiller et al. compared intermediate-dose ara-C (at 500 mg/m^2 given with daunorubicin at 60 mg/m^2) with conventional-dose ara-C (200 mg/m^2) and observed similar results with respect to CR and DFS rates *(127)*.

On the basis of these trials, the administration of a 7-d continuous infusion of ara-C along with three daily doses of daunorubicin (the so-called 7 + 3 regimen) became standard practice. In general, 65% of patients will enter CR with this regimen; approx 20% will have drug-resistant progressive leukemia *(128)*.

5.2. High-Dose Ara-C

The presence of drug refractoriness and relapsing leukemia together with insights into the mechanisms of ara-C resistance led to the development of high-dose ara-C treatment. Detailed pharmacokinetic studies with human leukemic cells showed that at standard-dose ara-C conditions transport is rate limiting; if transport is in excess, then the carrier will equilibrate ara-C nucleoside concentrations on both sides of the membrane, and the anabolic rate depends on the capacity for phosphorylation.

This prompted investigators to explore gram doses of ara-C in patients refractory to daily doses of 100–200 mg/m^2/d ara-C, given in combination with other drugs *(128)*. The first evidence demonstrating the superior antileukemic activity of high-dose ara-C was presented in 1977, when

Table 4
Development of Ara-C-Containing Treatment Regimens in AML

Southwest Oncology Group (122)			
	n = 67		*n = 70*
Ara-C CI	400 mg/m^2		200 mg/m^2
Duration	2 d		5 d
DNR	None		None
6-TG	None		None
CR %	24		38
Cancer and Leukemia Group B (123)			
	n = 75		*n = 104*
Ara-C CI	100 mg/m^2		100 mg/m^2
Duration	5 d		7 d
DNR	45 mg/m^2		45 mg/m^2
6-TG	None		None
CR %	39		55
Cancer and Leukemia Group B (125)			
	n = 211	*n = 216*	*n = 241*
Ara-C CI	100 mg/m^2	100 mg/m^2	100 mg/m^2
Duration	7 d	7 d	10 d
DNR	30–45 mg/m^2	30–45 mg/m^2	30–45 mg/m^2
6-TG	None	100 mg/m^2	None
CR %	53	57	57
Cancer and Leukemia Group B (126)			
	n = 160		*n = 166*
Ara-C CI	100 mg/m^2		200 mg/m^2
Duration	7 d		7 d
DNR	30–45 mg/m^2		30–45 mg/m^2
6-TG	None		None
CR %	57		63

CI, continuous infusion; DNR, daunorubicin; 6-TG, – thioguanine.

investigators showed that a 15- to 30-fold increase in dose to 3 g/m^2 had additional efficacy despite the fact that the leukemia was refractory to standard dose ara-C. This trial explored one to four doses as high as 7.5 g/m^2 *(129)* and stimulated a number of phase II trials.

Capizzi and Powell evaluated the efficacy of high-dose ara-C (3 g/m^2 every 12 h for 6–12 doses) in 400 relapsed or refractory AML patients and

Table 5
Randomized Studies of High-Dose Ara-C vs Standard-Dose Ara-C in Induction in AML

Australia Leukemia Study Group (Bishop et al., 1996)			
	n = 149	*n = 152*	*p value*
Ara-C	3 g/m^2 every 12 h on d 1, 3, 5, and 7	100 mg/m^2 CI for 7 d	
Daunorubicin	50 mg/m^2 d 1–3	50 mg/m^2 d 1–3	
Etoposide	75 mg/m^2 d 1–7	75 mg/m^2 d 1–7	
CR (%)	71	74	0.70
5-year DFS (%)	41	23	0.007
5-year OS (%)	31	25	0.44
The Southwest Oncology Group (Weick et al., 1996)			
	n = 172	*n = 493*	
Ara-C	2 g/m^2 every 12 h for 6 d	200 mg/m^2 CI for 7 d	
Daunorubicin	45 mg/m^2 for 3 d	45 mg/m^2 for 3 d	
CR (%)	55	58	0.96
5-year DFS (%)	33	21	0.049
5-year OS (%)	32	22	0.41

CI, continuous infusion.

observed a 23–27% CR rate. The combination of high-dose ara-C with other active drugs in the same disease setting resulted in a 42–62% CR rate *(130)*. The high response rate achieved with high-dose ara-C in patients with recurrent or refractory leukemia would indicate that the initial therapy with standard-dose ara-C in newly diagnosed patients utilized a suboptimal dose.

High-dose ara-C has also been used for remission induction in patients with newly diagnosed AML. Two prospectively randomized trials that accrued large numbers of patients compared high-dose ara-C with standard induction while employing the same postremission therapy in both arms (Table 5). Both trials failed to identify a higher CR rate with high-dose ara-C compared with standard induction, but the high-dose regimen was associated with increased toxicity *(131)*. High-dose ara-C has also been used to intensify induction by adding a course of high-dose ara-C during the 3 d following standard ara-C plus daunorubicin induction *(132)*.

Both the ECOG and Southwest Oncology Group have completed phase II studies exploring this strategy but observed no difference in the CR rate among patients given the intensified induction compared to historical results with standard induction *(131)*. The German AML Cooperative Group randomized newly diagnosed patients to either two courses of standard dose

ara-C with daunorubicin and 6-thioguanine or one course of the same chemotherapy followed by high-dose ara-C with mitoxantrone on d 21. There was no difference in CR; however, a subgroup of high-risk patients had a higher CR rate, a superior event-free survival rate at 5 yr, and a median OS at 5 yr with the high-dose ara-C and mitoxantrone arm *(133)*.

Several groups have prospectively evaluated the role of intensive postremission consolidation with high-dose ara-C. The CALGB conducted a prospectively randomized clinical trial in which AML patients in CR were randomly assigned to receive either a 5-d continuous infusion of 100 or 400 mg/m^2/d or six 3-h infusions of high-dose ara-C (3 g/m^2) administered on d 1, 3, and 5 at a 12-h interval *(128)*. High rates of central nervous system toxicity were observed in patients older than 60 yr randomly assigned to the high-dose regimen, and randomization was subsequently limited to patients 60 yr or younger. The disease-free survival at 4 yr was significantly higher following treatment with high-dose ara-C in patients under 60 yr of age. These results were supported by phase II trials that utilized high-dose ara-C for postremission intensification and are consistent with survival rates associated with the use of bone marrow transplantation *(124)*. In addition, this trial demonstrated a dose–response effect for ara-C in patients undergoing postremission therapy. Although the high-dose regimen used in this trial has become widely adopted, the number of high-dose ara-C courses required for optimal postremission therapy remains uncertain *(131)*.

6. Ara-C COMBINATIONS

6.1. Modulation of Ara-C-Mediated Cytotoxicity

A number of different strategies have been developed to increase the efficacy of ara-C. One approach has been biochemical modulation of ara-C-mediated cytotoxicity, in which ara-C is combined with compounds that enhance its metabolism or interfere with its catabolism. Reduction of dCTP levels enhances ara-CTP formation through the allosteric activation of dCK and reduced competition for incorporation of ara-CTP into the DNA *(5)*. Ara-C has therefore been combined with several inhibitors of the *de novo* pyrimidine biosynthesis, which results in the depletion of intracellular dCTP pools. Favorable interactions between ara-C and several of these agents have been reported, such as thymidine *(134,135)*, hydroxyurea *(136,137)*, 3-deazauridine *(138)*, PALA *(139,140)*, deoxyguanosine *(141)*, and imidazole pyrazole *(142)*.

6.2. Ara-C in Combination With Purine Analogs

Several in vitro and ex vivo studies have confirmed the ability of purine analogs to enhance the cytotoxicity of ara-C through stimulation of dCK

(143,144). An interesting combination is that of F-ara-A and ara-C; both compounds require activation catalyzed by dCK. The triphosphate of F-ara-A is a potent inhibitor of ribonucleotide reductase, which results in depletion of deoxynucleotide pools. In leukemic lymphocytes from patients with chronic lymphocytic leukemia, the F-ara-A-induced increase in ara-CTP was 1.5-fold *(145)*, and it was and 1.8-fold in ALL blasts *(146)*, with no effect on plasma ara-C concentration, deamination, or elimination of cellular ara-CTP. The maximum F-ara-ATP concentration is achieved within 4 h of administration and correlates with potentiation of ara-CTP formation; therefore, a protocol was derived using the sequential administration of F-ara-A and ara-C after a 4-h interval *(147)*.

Pharmacokinetic studies in a small group of patients with AML revealed a median 1.8-fold increase in the ara-CTP AUC *(146)*. Clinical studies of this schedule (ara-C 1 g/m^2 d 1–6 plus fludarabine 30 mg/m^2 d 2–6) in patients newly diagnosed with AML found a CR rate of 36%. These results were not significantly better than results previously achieved with high- or intermediate-dose ara-C, although subgroup analysis showed that the schedule was associated with significantly higher remission rates in patients whose initial remission duration was longer than 1 yr *(148)*.

Another purine analog that has been combined with ara-C is 2-CdA. In vitro and ex vivo pharmacologic studies have demonstrated a 50–65% increase in the rate of ara-CTP accumulation in leukemic blasts after pretreatment with 2-CdA *(143,149,150)*. A clinical study of the combination (2-CdA 12 mg/m^2/d × 5 d by CI and ara-C 1 g/m^2/d over 2 h) in adult refractory/relapsed AML showed an increase in ara-CTP uptake in these heavily treated patients, but the regimen did not appear to be an improvement over existing modalities *(150)*. Crews et al. reported that the combination of 2-CdA and ara-C seemed to be effective therapy for newly diagnosed pediatric AML. Intracellular accumulation of ara-CTP was increased when 2-CdA was given with ara-C, but no schedule-dependent differences in this effect were seen *(151)*. This combination was evaluated in a phase II trial in pediatric relapsed/refractory AML and was not effective *(152)*.

6.3. Ara-C in Combination With Growth Factors

In addition to the potentiating effects of purine analogs, it has also been demonstrated in vitro that growth factors can enhance the cytotoxicity to ara-C *(153–155)*. Lowenberg et al. evaluated the addition of granulocyte colony-stimulating factor (G-CSF) to induction chemotherapy (ara-C plus idarubicin for cycle 1 and ara-C plus amsacrine for cycle 2, with or without G-CSF) in patients with newly diagnosed AML. After a median follow-up of 55 mo, patients in CR after induction chemotherapy with G-CSF had a higher rate of disease-free survival

than patients who did not receive G-CSF, owing to a reduced probability of relapse. Although G-CSF did not improve overall survival, patients with standard-risk AML benefited from treatment with G-CSF *(79)*.

6.4. FLAG

Further enhancement of the efficacy of the sequential administration of fludarabine and ara-C in acute leukemias has been achieved by the addition of G-CSF, resulting in the FLAG (fludarabine, high-dose cytarabine, and G-CSF) regimen. The prior administration of G-CSF results in increased recruitment of cells into S phase, making them more susceptible to ara-C-mediated cytotoxicity. G-CSF has also been shown to enhance the cytotoxicity of ara-C with increased [^{3}H]-ara-C DNA incorporation and apoptosis *(156)*. Clinically, FLAG is now one of the most active regimens in AML *(144)* and has been used successfully in the treatment of those with poor prognosis and relapsed AML (Table 6). The efficacy of the FLAG regimen in inducing remissions in heavily pretreated children relapsing with both acute lymphoblastic and myeloid leukemias has been established with a CR rate of 70% *(157)*. The advantage of the FLAG regimen in these patients is that, although it is intensive, it is also potentially less cardiotoxic because of the avoidance of anthracyclines.

The precise role of G-CSF in the FLAG combination has not been fully elucidated *(158)*. Colony-stimulating factors (G-CSF and GM-CSF) have been used for the management of neutropenia in cancer patients *(159)*. But, for this prophylactic effect, G-CSF is administered after chemotherapy, which accelerates neutrophil recovery, resulting in a reduction in the number of documented infections *(160,161)*. In the FLAG regimen, however, G-CSF is given prior to chemotherapy to sensitize leukemic cells to the action of cytotoxic compounds (priming). AML is a heterogeneous disease, and some subsets of AML patients (e.g., unfavorable cytogenetics) might benefit from G-CSF priming *(79,162–164)*. Besides the benefit of G-CSF, it should be considered that addition of a growth factor may also increase tumor load in some cases (e.g., patients with a high white blood cell count).

Unfortunately, there are no randomized studies comparing FLAG with fludarabine and ara-C (FLA). Estey et al. reported similar survival rates, when comparing a cohort of those newly diagnosed with AML and myelodysplastic syndrome (MDS) patients treated with either FLA or FLAG *(165)*. In a prospective randomized study, Ossenkoppele et al. evaluated FLAG in comparison with G-CSF/ara-C in high-risk MDS and elderly individuals with AML *(166)*. Although ara-CTP accumulation was enhanced after FLAG in leukemic blasts, clinical outcome was not significantly improved. In vitro studies have also shown that the addition of G-CSF did not result in increased cytotoxicity of FLA *(167)*.

Table 6
Efficacy of the FLAG Regimen in Adult Acute Myeloid Leukemia/MDS

Reference	*Schedule*	*Patients*	*n*	*CR rate (%)*
Estey et al. (1994)	Fludarabine 30 mg/m^2 d 1–5	Newly diagnosed AML	69	65
	Ara-C 2 g/m^2 d 1–5	MDS	43	60
	G-CSF 400 g/m^2 d 0–CR			
Visani et al. (1994)	Fludarabine 30 mg/m^2 d 1–5	Refractory AML	8	75
	Ara-C 2 g/m^2 d 1–5	Relapsed AML	10	10
	G-CSF 5 mg/kg d 0–CR	Secondary AML	10	80
Nokes et al. (1997)	Fludarabine 30 mg/m^2 d 1–5	Newly diagnosed AML	1	
	Ara-C (variable doses)	Refractory AML	4	50
	G-CSF 400 g/m^2 d 1–CR	Relapsed AML	19	67
		MDS (RAEB-t)	8	75
Huhnman et al. (1996)	Fludarabine 25 mg/m^2 d 1–5	Refractory AML	8	50
	Ara-C 2 g/m^2 d 1–5	Relapsed AML	14	
	G-CSF 400 g/m^2 d 1–CR			
Montillo et al. (1998)	Fludarabine 30 mg/m^2 d 1–5	Refractory AML	16	44
	Ara-C 2 g/m^2 d 1–5	Relapsed AML	22	64
	G-CSF 5 g/kg d 0–CR			
Jackson et al. (2001)	Fludarabine 30 mg/m^2 d 1–5	Refractory AML[a]	45	30
	Ara-C 2 g/m^2 d 1–5	Relapsed AML	21	81
	G-CSF 30 MU d 0–6	MDS (RAEB-t)	23	56

[a]Includes early relapses of AML (<6 mo).

The Medical Research Council is conducting a high-risk AML study that randomizes patients to therapy with ADE (ara-C, daunorubicin, and etoposide) with/without G-CSF with/without ATRA or FLA (fludarabine and ara-C) with/without G-CSF with/without ATRA. This trial will provide the first direct comparison of FLAG against other conventional combination chemotherapy regimens *(143)*.

6.5. Other Approaches to Modulate Ara-C-Induced Cytotoxicity

Another approach to enhance the cytotoxicity of ara-C has been to combine ara-C with agents that interfere with its degradation. Tetrahydrouridine effectively inhibits CDA and has been shown to potentiate ara-C in vivo *(168,169)*. Galmarini et al. showed that high expression levels of high K_m 5′-NT in leukemic blasts at diagnosis correlated with adverse outcome in AML patients treated with ara-C *(13,14,57)*. Combinations of ara-C with an appropriate 5′-NT inhibitor might therefore also potentiate ara-C-induced cytotoxicity.

dCTP inhibits the phosphorylation of ara-C and the incorporation of ara-CTP into DNA. dCTP can be synthesized indirectly from CTP by CTP synthetase. Cyclopentenyl cytosine is a nucleoside analog that inhibits CTP synthetase, inducing a depletion of CTP and dCTP *(170,171)*. Cyclopentenyl cytosine has increased the phosphorylation and incorporation into DNA of ara-C, as well as enhanced ara-C-mediated apoptosis in acute leukemic cell lines and neuroblastoma cells *(172–175)*.

Ara-CTP has to be incorporated into the DNA to induce cytotoxicity. Inhibitors of DNA repair may sensitize cancer cells to DNA-damaging agents *(176)*. Aphidicolin inhibits DNA polymerases-α, -δ, and -ε *(177)*. Aphidicolin has been shown to potentiate ara-C-induced apoptosis in human myeloid leukemia cell lines *(178)*.

Sargent et al. demonstrated that aphidicolin can decrease resistance to ara-C in vitro in blasts from adult and childhood AML *(177,179)*. In the blasts of a small group of AML patients, aphidicolin significantly increased the in vitro sensitivity to ara-C, especially in resistant patients, suggesting that aphidicolin targeted the population most requiring rescue and implicated increased DNA repair as a major resistance mechanism to ara-C.

A recent approach has been to combine ara-C with agents that act through cell signal transduction pathways. These agents do not influence the metabolism of ara-C directly but may increase the susceptibility of cells to activation of the apoptotic cascade by drug-mediated cytotoxicity *(5)*. Inhibitors of PKC are potent inducers of apoptosis in human leukemic cells *(180,181)*. The macrocyclic lactone bryostatin 1 alters the threshold for cytotoxic drug-induced apoptosis by downregulation of PKC activity. Preincubation of human leukemic blasts with bryostatin 1 facilitated ara-C-induced apoptosis and resulted in a synergistic antileukemic effect *(182)*. A phase I trial of bryostatin 1 (24-h continuous infusion, d 1 and 11) and high-dose ara-C (1.5 g/m^2, every 12 h × 4 on d 2, 3, 9, and 10) showed activity in refractory acute leukemia *(183)*. Direct inhibition of PKC by staurosporine and its derivative 7-hydroxystaurosporine has also been shown to augment ara-C-mediated cytotoxicity *(184)* and circumvented

resistance to ara-C-induced apoptosis in human leukemic cells that overexpress bcl-2 *(185,186)*. These agents may be able to overcome drug resistance that arises from distal defects in the apoptosis pathway.

7. Ara-C PRODRUGS

Several characteristics of ara-C, such as metabolic deamination, low affinity for dCK, and rapid elimination of ara-CTP, limit its cytotoxic activity *(111)*. To overcome these problems, several chemical modifications of ara-C have been synthesized. Ara-C has been encapsulated into multivesicular liposomes *(187–189)*, and several conjugates have been designed *(190)*. Ara-C prodrugs containing lipophilic side chains in the base or in the sugar moiety have been designed with the aim to increase cellular uptake of ara-C and delay its deamination and clearance *(191–193)*. Such molecules can enter cells by a transporter-independent mechanism and possibly increase the intracellular half-life of ara-C *(193,194)*.

These prodrugs have activity against solid tumor models, in contrast to ara-C itself. This is possibly to a better penetration and subsequent retention of these drugs. One of these prodrugs, CP-4055, is undergoing phase I clinical evaluation in which 6 of 17 pretreated patients with solid tumors showed stable disease *(195)*. The pronucleotide analog UA911, a precursor of 5′-ara-CMP, was capable of overcoming dCK-mediated ara-C resistance in human follicular lymphoma RL-G cells *(196)*.

The application of an ara-C prodrug may enable the administration of these drugs as an oral formulation; the nature of several of these compounds will result in longer retention of ara-C in body fluids. These properties might make these analogs suitable for treatment of solid tumors, but the compounds still require further clinical investigation.

8. CONCLUSIONS AND FUTURE PERSPECTIVES

Ara-C remains the most important drug in the treatment of AML. The aim is therefore to optimize the efficacy of this compound and develop more potent ara-C-derived compounds that are less sensitive to resistance mechanisms. Greater understanding of the metabolism and mechanisms of action of ara-C have already created opportunities for improving its antitumor effect and will continue to do so in the future. Understanding the factors that contribute to the emergence of resistance is essential to improve patient outcome.

Over the past decades, much has been learned about the mechanisms responsible for ara-C-induced cytotoxicity as well as about the factors that determine the response of cells to this drug. It has become clear that the response of cells to ara-C depends on drug-specific mechanisms (e.g.,

transport, phosphorylation and dephosphorylation, and deamination), as well as less-specific downstream pathways (DNA repair mechanisms and cell death pathways).

Identifying the cellular mechanisms of resistance also provides clues to circumvent this resistance. Metabolic modulation of the cytotoxicity of ara-C has been approached with the aim of reducing intracellular pools of nucleotides. Inhibition of ribonucleotide reductase by fludarabine followed by treatment with ara-C, for instance, has achieved responses in AML patients, although no clear additional benefit. Deoxynucleoside analog combinations have proven successful in the clinic. Combining ara-C with novel deoxynucleoside analogs (e.g., clofarabine) could further increase its cytotoxicity. Ara-C combined with agents modulating apoptotic responses or novel "targeted" therapies is expected to provide additional benefit. A multifactorial approach to ara-C resistance should allow progress in the treatment of resistant disease.

REFERENCES

1. Lister TA, Rohatiner AZ, Bassan R, et al. Conventional dose cytosine arabinoside in combination chemotherapy for acute myelogenous leukemia. Semin Oncol 1987;14(2 suppl 1):53–54.
2. Bolwell BJ, Cassileth PA, Gale RP. High dose cytarabine: a review. Leukemia 1988;2:253–260.
3. Plunkett W, Gandhi V. Cellular pharmacodynamics of anticancer drugs. Semin Oncol 1993;20:50–63.
4. Rustum YM, Raymakers RA. 1-β-Arabinofuranosylcytosine in therapy of leukemia: preclinical and clinical overview. Pharmacol Ther 1992;56:307–321.
5. Grant S. Ara-C: cellular and molecular pharmacology. Adv Cancer Res 1998;72: 197–233.
6. Herzig RH, Wolff SN, Lazarus HM, Phillips GL, Karanes C, Herzig GP. High-dose cytosine arabinoside therapy for refractory leukemia. Blood 1983;62:361–369.
7. Estey E. Treatment of refractory AML. Leukemia 1996;10:932–936.
8. Paterson AR, Kolassa N, Cass CE. Transport of nucleoside drugs in animal cells. Pharmacol Ther 1981;12:515–536.
9. Sirotnak FM, Barrueco JR. Membrane transport and the antineoplastic action of nucleoside analogues. Cancer Metastasis Rev 1987;6:459–480.
10. Sundaram M, Yao SY, Ingram JC, et al. Topology of a human equilibrative, nitrobenzylthioinosine (NBMPR)-sensitive nucleoside transporter (hENT1) implicated in the cellular uptake of adenosine and anti-cancer drugs. J Biol Chem 2001;276:45,270–45,275.
11. Clarke ML, Mackey JR, Baldwin SA, Young JD, Cass CE. The role of membrane transporters in cellular resistance to anticancer nucleoside drugs. Cancer Treat Res 2002;112:27–47.
12. Stam RW, Den Boer ML, Meijerink JP, et al. Differential mRNA expression of ara-C-metabolizing enzymes explains ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood 2003;101:1270–1276.

13. Galmarini CM, Thomas X, Calvo F, et al. In vivo mechanisms of resistance to cytarabine in acute myeloid leukaemia. Br J Haematol 2002;117:860–868.
14. Galmarini CM, Thomas X, Calvo F, Rousselot P, Jafaari AE, Cros E et al. Potential mechanisms of resistance to cytarabine in AML patients. Leuk Res 2002;26: 621–629.
15. Capizzi RL, Yang JL, Rathmell JP, et al. Dose-related pharmacologic effects of high-dose ara-C and its self-potentiation. Semin Oncol 1985;12(2 suppl 3):65–74.
16. Chan TC. Augmentation of 1-β-D-arabinofuranosylcytosine cytotoxicity in human tumor cells by inhibiting drug efflux. Cancer Res 1989;49:2656–2660.
17. Steinbach D, Wittig S, Cario G, et al. The multidrug resistance-associated protein 3 (MRP3) is associated with a poor outcome in childhood ALL and may account for the worse prognosis in male patients and T-cell immunophenotype. Blood 2003;102:4493–4498.
18. Steinbach D, Lengemann J, Voigt A, Hermann J, Zintl F, Sauerbrey A. Response to chemotherapy and expression of the genes encoding the multidrug resistance-associated proteins MRP2, MRP3, MRP4, MRP5, and SMRP in childhood acute myeloid leukemia. Clin Cancer Res 2003;9:1083–1086.
19. Reid G, Wielinga P, Zelcer N, et al. Characterization of the transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. Mol Pharmacol 2003;63:1094–1103.
20. Liliemark JO, Plunkett W. Regulation of 1-β-D-arabinofuranosylcytosine 5′-triphosphate accumulation in human leukemia cells by deoxycytidine 5′-triphosphate. Cancer Res 1986;46:1079–1083.
21. Kufe DW, Spriggs DR. Biochemical and cellular pharmacology of cytosine arabinoside. Semin Oncol 1985;12(2 suppl 3):34–48.
22. Hande KR, Chabner BA. Pyrimidine nucleoside monophosphate kinase from human leukemic blast cells. Cancer Res 1978;38:579–585.
23. Bergman AM, Pinedo HM, Jongsma AP, et al. Decreased resistance to gemcitabine (2′,2′-difluorodeoxycitidine) of cytosine arabinoside-resistant myeloblastic murine and rat leukemia cell lines: role of altered activity and substrate specificity of deoxycytidine kinase. Biochem Pharmacol 1999;57:397–406.
24. Bhalla K, Nayak R, Grant S. Isolation and characterization of a deoxycytidine kinase-deficient human promyelocytic leukemic cell line highly resistant to 1-β-D-arabinofuranosylcytosine. Cancer Res 1984;44:5029–5037.
25. Stegmann AP, Honders WH, Willemze R, Ruiz van Haperen V, Landegent JE. Transfection of wild-type deoxycytidine kinase (dck) cDNA into an ara C- and DAC-resistant rat leukemic cell line of clonal origin fully restores drug sensitivity. Blood 1995;85:1188–1194.
26. Hapke DM, Stegmann AP, Mitchell BS. Retroviral transfer of deoxycytidine kinase into tumor cell lines enhances nucleoside toxicity. Cancer Res 1996;56:2343–2347.
27. Ruiz van Haperen V, Veerman G, Eriksson S, et al. Development and molecular characterization of a 2′,2′-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. Cancer Res 1994;54:4138–4143.
28. Bergman AM, Giaccone G, Van Moorsel CJ, et al. Cross-resistance in the 2′,2′-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cancer cell line AG6000 to standard and investigational drugs. Eur J Cancer 2000;36:1974–1983.
29. Dumontet C, Fabianowska-Majewska K, Mantincic D, et al. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol 1999;106:78–85.

30. Tattersall MH, Ganeshaguru K, Hoffbrand AV. Mechanisms of resistance of human acute leukaemia cells to cytosine arabinoside. Br J Haematol 1974;27:39–46.
31. Colly LP, Peters WG, Richel D, Arentsen-Honders MW, Starrenburg CW, Willemze R. Deoxycytidine kinase and deoxycytidine deaminase values correspond closely to clinical response to cytosine arabinoside remission induction therapy in patients with acute myelogenous leukemia. Semin Oncol 1987;14(2 suppl 1):257–261.
32. Kakihara T, Fukuda T, Tanaka A, et al. Expression of deoxycytidine kinase (dCK) gene in leukemic cells in childhood: decreased expression of dCK gene in relapsed leukemia. Leuk Lymphoma 1998;31(3–4):405–409.
33. Stammler G, Zintl F, Sauerbrey A, Volm M. Deoxycytidine kinase mRNA expression in childhood acute lymphoblastic leukemia. Anticancer Drugs 1997;8:517–521.
34. Kawasaki H, Carrera CJ, Piro LD, Saven A, Kipps TJ, Carson DA. Relationship of deoxycytidine kinase and cytoplasmic 5′-nucleotidase to the chemotherapeutic efficacy of 2-chlorodeoxyadenosine. Blood 1993;81:597–601.
35. Albertioni F, Lindemalm S, Eriksson S, Juliusson G, Liliemark J. Relationship between cladribine (CdA) plasma, intracellular CdA-5′-triphosphate (CdATP) concentration, deoxycytidine kinase (dCK), and chemotherapeutic activity in chronic lymphocytic leukemia (CLL). Adv Exp Med Biol 1998;431:693–697.
36. Leiby JM, Snider KM, Kraut EH, Metz EN, Malspeis L, Grever MR. Phase II trial of 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-monophosphate in non-Hodgkin(s lymphoma: prospective comparison of response with deoxycytidine kinase activity. Cancer Res 1987;47:2719–2722.
37. Owens JK, Shewach DS, Ullman B, Mitchell BS. Resistance to 1-β-D-arabinofuranosylcytosine in human T-lymphoblasts mediated by mutations within the deoxycytidine kinase gene. Cancer Res 1992;52:2389–2393.
38. Flasshove M, Strumberg D, Ayscue L, et al. Structural analysis of the deoxycytidine kinase gene in patients with acute myeloid leukemia and resistance to cytosine arabinoside. Leukemia 1994;8:780–785.
39. Al Madhoun AS, van der Wilt CL, Loves WJ, et al. Detection of an alternatively spliced form of deoxycytidine kinase mRNA in the 2′-2′-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cancer cell line AG6000. Biochem Pharmacol 2004;68:601–609.
40. Veuger MJ, Honders MW, Landegent JE, Willemze R, Barge RM. High incidence of alternatively spliced forms of deoxycytidine kinase in patients with resistant acute myeloid leukemia. Blood 2000;96:1517–1524.
41. Nyce J, Liu L, Jones PA. Variable effects of DNA-synthesis inhibitors upon DNA methylation in mammalian cells. Nucleic Acids Res 1986;14:4353–4367.
42. Chottiner EG, Shewach DS, Datta NS, et al. Cloning and expression of human deoxycytidine kinase cDNA. Proc Natl Acad Sci USA 1991;88:1531–1535.
43. Antonsson BE, Avramis VI, Nyce J, Holcenberg JS. Effect of 5-azacytidine and congeners on DNA methylation and expression of deoxycytidine kinase in the human lymphoid cell lines CCRF/CEM/0 and CCRF/CEM/dCk-1. Cancer Res 1987;47:3672–3678.
44. Leegwater PA, De Abreu RA, Albertioni F. Analysis of DNA methylation of the 5′ region of the deoxycytidine kinase gene in CCRF-CEM-sensitive and cladribine (CdA)- and 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (CAFdA)-resistant cells. Cancer Lett 1998;130:169–173.
45. Xie KC, Plunkett W. Deoxynucleotide pool depletion and sustained inhibition of ribonucleotide reductase and DNA synthesis after treatment of human lymphobla-

stoid cells with 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine. Cancer Res 1996;56:3030–3037.
46. Whelan J, Phear G, Yamauchi M, Meuth M. Clustered base substitutions in CTP synthetase conferring drug resistance in Chinese hamster ovary cells. Nat Genet 1993;3:317–322.
47. Whelan J, Smith T, Phear G, Rohatiner A, Lister A, Meuth M. Resistance to cytosine arabinoside in acute leukemia: the significance of mutations in CTP synthetase. Leukemia 1994;8:264–265.
48. Colly LP, Peters WG, Richel D, Arentsen-Honders MW, Starrenburg CW, Willemze R. Deoxycytidine kinase and deoxycytidine deaminase values correspond closely to clinical response to cytosine arabinoside remission induction therapy in patients with acute myelogenous leukemia. Semin Oncol 1987;14(2 suppl 1):257–261.
49. Fridland A, Verhoef V. Mechanism for ara-CTP catabolism in human leukemic cells and effect of deaminase inhibitors on this process. Semin Oncol 1987;14(2 suppl 1):262–268.
50. Schroder JK, Kirch C, Flasshove M, et al. Constitutive overexpression of the cytidine deaminase gene confers resistance to cytosine arabinoside in vitro. Leukemia 1996;10:1919–1924.
51. Neff T, Blau CA. Forced expression of cytidine deaminase confers resistance to cytosine arabinoside and gemcitabine. Exp Hematol 1996;24:1340–1346.
52. Eliopoulos N, Bovenzi V, Le NL, et al. Retroviral transfer and long-term expression of human cytidine deaminase cDNA in hematopoietic cells following transplantation in mice. Gene Ther 1998;5:1545–1551.
53. Jahns-Streubel G, Reuter C, Auf der LU, et al. Activity of thymidine kinase and of polymerase alpha as well as activity and gene expression of deoxycytidine deaminase in leukemic blasts are correlated with clinical response in the setting of granulocyte-macrophage colony-stimulating factor-based priming before and during TAD-9 induction therapy in acute myeloid leukemia. Blood 1997;90:1968–1976.
54. Yang JL, Cheng EH, Capizzi RL, Cheng YC, Kute T. Effect of uracil arabinoside on metabolism and cytotoxicity of cytosine arabinoside in L5178Y murine leukemia. J Clin Invest 1985;75:141–146.
55. Capizzi RL, Yang JL, Rathmell JP, et al. Dose-related pharmacologic effects of high-dose ara-C and its self-potentiation. Semin Oncol 1985;12(2 suppl 3):65–74.
56. Mancini WR, Cheng YC. Human deoxycytidylate deaminase. Substrate and regulator specificities and their chemotherapeutic implications. Mol Pharmacol 1983;23:159–164.
57. Galmarini CM, Graham K, Thomas X, et al. Expression of high K_m 5′-nucleotidase in leukemic blasts is an independent prognostic factor in adults with acute myeloid leukemia. Blood 2001;98:1922–1926.
58. Jamieson GP, Finch LR, Snook M, Wiley JS. Degradation of 1-β-D-arabinofuranosylcytosine 5′-triphosphate in human leukemic myeloblasts and lymphoblasts. Cancer Res 1987;47:3130–3135.
59. Graham FL, Whitmore GF. Studies in mouse L-cells on the incorporation of 1-β-D-arabinofuranosylcytosine into DNA and on inhibition of DNA polymerase by 1-β-D-arabinofuranosylcytosine 5′-triphosphate. Cancer Res 1970;30: 2636–2644.
60. Momparler RL, Momparler LF. Chemotherapy of L1210 and L1210/ARA-C leukemia with 5-aza-2′-deoxycytidine and 3-deazauridine. Cancer Chemother Pharmacol 1989;25:51–54.

61. Dunn WC, Regan JD. Inhibition of DNA excision repair in human cells by arabinofuranosyl cytosine: effect on normal and xeroderma pigmentosum cells. Mol Pharmacol 1979;15:367–374.
62. Hiss EA, Preston RJ. The effect of cytosine arabinoside on the frequency of single-strand breaks in DNA of mammalian cells following irradiation or chemical treatment. Biochim Biophys Acta 1977;478:1–8.
63. Fram RJ, Kufe DW. DNA strand breaks caused by inhibitors of DNA synthesis: 1-β-D-arabinofuranosylcytosine and aphidicolin. Cancer Res 1982;42:4050–4053.
64. Miller MR, Chinault DN. The roles of DNA polymerases α, β, and γ in DNA repair synthesis induced in hamster and human cells by different DNA damaging agents. J Biol Chem 1982;257:10,204–10,209.
65. Townsend AJ, Cheng YC. Sequence-specific effects of ara-5-aza-CTP and ara-CTP on DNA synthesis by purified human DNA polymerases in vitro: visualization of chain elongation on a defined template. Mol Pharmacol 1987;32:330–339.
66. Ross DD, Cuddy DP, Cohen N, Hensley DR. Mechanistic implications of alterations in HL-60 cell nascent DNA after exposure to 1-β-D-arabinofuranosylcytosine. Cancer Chemother Pharmacol 1992;31:61–70.
67. Kufe D, Spriggs D, Egan EM, Munroe D. Relationships among ara-CTP pools, formation of (ara-C)DNA, and cytotoxicity of human leukemic cells. Blood 1984;64:54–58.
68. Momparler RL, Onetto-Pothier N, Bouffard DY, Momparler LF. Cellular pharmacology of 1-β-D-arabinofuranosylcytosine in human myeloid, B-lymphoid and T-lymphoid leukemic cells. Cancer Chemother Pharmacol 1990;27:141–146.
69. Plunkett W, Hug V, Keating MJ, Chubb S. Quantitation of 1-β-D-arabinofuranosylcytosine 5′-triphosphate in the leukemic cells from bone marrow and peripheral blood of patients receiving 1-β-D-arabinofuranosylcytosine therapy. Cancer Res 1980;40:588–591.
70. Rustum YM, Preisler HD. Correlation between leukemic cell retention of 1-β-D-arabinofuranosylcytosine 5′-triphosphate and response to therapy. Cancer Res 1979;39:42–49.
71. Colly LP, Richel DJ, Arentsen-Honders W, Starrenburg IW, Edelbroek PM, Willemze R. A simplified assay for measurement of cytosine arabinoside incorporation into DNA in ara-C-sensitive and -resistant leukemic cells. Cancer Chemother Pharmacol 1990;27:151–156.
72. Rustum YM, Danhauser L, Luccioni C, Au JL. Determinants of response to antimetabolites and their modulation by normal purine and pyrimidine metabolites. Cancer Treat Rep 1981;65(suppl 3):73–82.
73. Hiddemann W, Schleyer E, Unterhalt M, Kern W, Buchner T. Optimizing therapy for acute myeloid leukemia based on differences in intracellular metabolism of cytosine arabinoside between leukemic blasts and normal mononuclear blood cells. Ther Drug Monit 1996;18:341–349.
74. Liliemark JO, Plunkett W, Dixon DO. Relationship of 1-β-D-arabinofuranosylcytosine in plasma to 1-β-D-arabinofuranosylcytosine 5′-triphosphate levels in leukemic cells during treatment with high-dose 1-β-D-arabinofuranosylcytosine. Cancer Res 1985;45(11 pt 2):5952–5957.
75. Preisler HD, Rustum Y, Priore RL. Relationship between leukemic cell retention of cytosine arabinoside triphosphate and the duration of remission in patients with acute non-lymphocytic leukemia. Eur J Cancer Clin Oncol 1985;21:23–30.

76. Plunkett W, Liliemark JO, Adams TM, et al. Saturation of 1-β-D-arabinofuranosylcytosine 5′-triphosphate accumulation in leukemia cells during high-dose 1-β-D-arabinofuranosylcytosine therapy. Cancer Res 1987;47:3005–3011.
77. Karon M, Shirakawa S. The locus of action of 1-β-D-arabinofuranosylcytosine in the cell cycle. Cancer Res 1969;29:687–696.
78. Preisler HD, Azarnia N, Raza A, et al. Relationship between the per cent of marrow cells in S phase and the outcome of remission-induction therapy for acute nonlymphocytic leukaemia. Br J Haematol 1984;56:399–407.
79. Lowenberg B, van Putten W, Theobald M, et al. Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. N Engl J Med 2003;349:743–752.
80. Sampath D, Rao VA, Plunkett W. Mechanisms of apoptosis induction by nucleoside analogs. Oncogene 2003;22:9063–9074.
81. Kucera GL, Capizzi RL. 1-β-D-Arabinofuranosylcytosine-diphosphate-choline is formed by the reversal of cholinephosphotransferase and not via cytidylyltransferase. Cancer Res 1992;52:3886–3891.
82. Strum JC, Small GW, Pauig SB, Daniel LW. 1-β-D-Arabinofuranosylcytosine stimulates ceramide and diglyceride formation in HL-60 cells. J Biol Chem 1994;269:15493–15497.
83. Kharbanda S, Datta R, Kufe D. Regulation of c-jun gene expression in HL-60 leukemia cells by 1-β-D-arabinofuranosylcytosine. Potential involvement of a protein kinase C dependent mechanism. Biochemistry 1991;30:7947–7952.
84. Kharbanda S, Emoto Y, Kisaki H, Saleem A, Kufe D. 1-β-D-Arabinofuranosylcytosine activates serine/threonine protein kinases and c-jun gene expression in phorbol ester-resistant myeloid leukemia cells. Mol Pharmacol 1994;46: 67–72.
85. Saleem A, Datta R, Yuan ZM, Kharbanda S, Kufe D. Involvement of stress-activated protein kinase in the cellular response to 1-β-D-arabinofuranosylcytosine and other DNA-damaging agents. Cell Growth Differ 1995;6:1651–1658.
86. Achanta G, Pelicano H, Feng L, Plunkett W, Huang P. Interaction of p53 and DNA-PK in response to nucleoside analogues: potential role as a sensor complex for DNA damage. Cancer Res 2001;61:8723–8729.
87. Decker RH, Levin J, Kramer LB, Dai Y, Grant S. Enforced expression of the tumor suppressor p53 renders human leukemia cells (U937) more sensitive to 1-[β-D-arabinofuranosyl]cytosine (ara-C)-induced apoptosis. Biochem Pharmacol 2003;65:1997–2008.
88. Kanno S, Higurashi A, Watanabe Y, Shouji A, Asou K, Ishikawa M. Susceptibility to cytosine arabinoside (ara-C)-induced cytotoxicity in human leukemia cell lines. Toxicol Lett 2004;152:149–158.
89. Brach MA, Herrmann F, Kufe DW. Activation of the AP-1 transcription factor by arabinofuranosylcytosine in myeloid leukemia cells. Blood 1992;79:728–734.
90. Brach MA, Kharbanda SM, Herrmann F, Kufe DW. Activation of the transcription factor kappa B in human KG-1 myeloid leukemia cells treated with 1-β-D-arabinofuranosylcytosine. Mol Pharmacol 1992;41:60–63.
91. Sreenivasan Y, Sarkar A, Manna SK. Mechanism of cytosine arabinoside-mediated apoptosis: role of Rel A (p65) dephosphorylation. Oncogene 2003;22:4356–4369.
92. Bhalla K, Holladay C, Arlin Z, Grant S, Ibrado AM, Jasiok M. Treatment with interleukin-3 plus granulocyte-macrophage colony-stimulating factors improves

the selectivity of ara-C in vitro against acute myeloid leukemia blasts. Blood 1991;78:2674–2679.
93. Brach MA, Herrmann F, Kufe DW. Activation of the AP-1 transcription factor by arabinofuranosylcytosine in myeloid leukemia cells. Blood 1992;79:728–734.
94. Koo HM, Monks A, Mikheev A, et al. Enhanced sensitivity to 1-β-D-arabinofuranosylcytosine and topoisomerase II inhibitors in tumor cell lines harboring activated ras oncogenes. Cancer Res 1996;56:5211–5216.
95. Koo HM, McWilliams MJ, Alvord WG, Vande Woude GF. Ras oncogene-induced sensitization to 1-β-D-arabinofuranosylcytosine. Cancer Res 1999;59:6057–6062.
96. Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. Blood 1993;81:151–157.
97. Hu ZB, Minden MD, McCulloch EA. Post-transcriptional regulation of bcl-2 in acute myeloblastic leukemia: significance for response to chemotherapy. Leukemia 1996;10:410–416.
98. Ibrado AM, Huang Y, Fang G, Liu L, Bhalla K. Overexpression of Bcl-2 or Bcl-xL inhibits ara-C-induced CPP32/Yama protease activity and apoptosis of human acute myelogenous leukemia HL-60 cells. Cancer Res 1996;56:4743–4748.
99. Manome Y, Weichselbaum RR, Kufe DW, Fine HA. Effect of Bcl-2 on ionizing radiation and 1-β-D-arabinofuranosylcytosine-induced internucleosomal DNA fragmentation and cell survival in human myeloid leukemia cells. Oncol Res 1993;5:139–144.
100. Bullock G, Ray S, Reed JC, et al. Intracellular metabolism of ara-C and resulting DNA fragmentation and apoptosis of human AML HL-60 cells possessing disparate levels of Bcl-2 protein. Leukemia 1996;10:1731–1740.
101. Guedez L, Suresh A, Tung F, Zucali J. Quantitation of resistance to cytosine arabinoside by myeloid leukemic cells expressing bcl-2. Eur J Haematol 1996;57: 149–156.
102. Haarman EG, Kaspers GJ, Pieters R, et al. BCL-2 expression in childhood leukemia vs spontaneous apoptosis, drug induced apoptosis, and in vitro drug resistance. Adv Exp Med Biol 1999;457:325–333.
103. Salomons GS, Smets LA, Verwijs-Janssen M, et al. Bcl-2 family members in childhood acute lymphoblastic leukemia: relationships with features at presentation, in vitro and in vivo drug response and long-term clinical outcome. Leukemia 1999;13:1574–1580.
104. Keith FJ, Bradbury DA, Zhu YM, Russell NH. Inhibition of bcl-2 with antisense oligonucleotides induces apoptosis and increases the sensitivity of AML blasts to ara-C. Leukemia 1995;9:131–138.
105. Chelliah J, Freemerman AJ, Wu-Pong S, Jarvis WD, Grant S. Potentiation of ara-C-induced apoptosis by the protein kinase C activator bryostatin 1 in human leukemia cells (HL-60) involves a process dependent upon c-Myc. Biochem Pharmacol 1997;54:563–573.
106. Wang Z, Van Tuyle G, Conrad D, Fisher PB, Dent P, Grant S. Dysregulation of the cyclin-dependent kinase inhibitor p21WAF1/CIP1/MDA6 increases the susceptibility of human leukemia cells (U937) to 1-β-D-arabinofuranosylcytosine-mediated mitochondrial dysfunction and apoptosis. Cancer Res 1999;59: 1259–1267.
107. Carter BZ, Kornblau SM, Tsao T, et al. Caspase-independent cell death in AML: caspase inhibition in vitro with pan-caspase inhibitors or in vivo by XIAP or Survivin does not affect cell survival or prognosis. Blood 2003;102:4179–4186.

108. Piall EM, Aherne GW, Marks VM. A radioimmunoassay for cytosine arabinoside. Br J Cancer 1979;40:548–556.
109. Linssen P, Drenthe-Schonk A, Wessels H, Haanen C. Determination of 1-β-D-arabinofuranosylcytosine and 1-β-D-arabinofuranosyluracil in human plasma by high-performance liquid chromatography. J Chromatogr 1981;223:371–378.
110. Peters GJ, Schornagel JH, Milano GA. Clinical pharmacokinetics of anti-metabolites. Cancer Surv 1993;17:123–156.
111. Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues and nucleobases in cancer treatment. Lancet Oncol 2002;3:415–424.
112. Fleming RA, Capizzi RL, Rosner GL, et al. Clinical pharmacology of cytarabine in patients with acute myeloid leukemia: a cancer and leukemia group B study. Cancer Chemother Pharmacol 1995;36:425–430.
113. Periclou AP, Avramis VI. NONMEM population pharmacokinetic studies of cytosine arabinoside after high-dose and after loading bolus followed by continuous infusion of the drug in pediatric patients with leukemias. Cancer Chemother Pharmacol 1996;39:42–50.
114. Peters WG, Colly LP, Willemze R. High-dose cytosine arabinoside: pharmacological and clinical aspects. Blut 1988;56:1–11.
115. Kreis W, Chaudhri F, Chan K, et al. Pharmacokinetics of low-dose 1-β-D-arabinofuranosylcytosine given by continuous intravenous infusion over 21 days. Cancer Res 1985;45(12 pt 1):6498–6501.
116. Spriggs D, Griffin J, Wisch J, Kufe D. Clinical pharmacology of low-dose cytosine arabinoside. Blood 1985;65:1087–1089.
117. Papayannopoulou T, Torrealba dR, Veith R, Knitter G, Stamatoyannopoulos G. Arabinosylcytosine induces fetal hemoglobin in baboons by perturbing erythroid cell differentiation kinetics. Science 1984;224:617–619.
118. King ME, Pfeifle CE, Howell SB. Intraperitoneal cytosine arabinoside therapy in ovarian carcinoma. J Clin Oncol 1984;2:662–669.
119. Markman M, Hakes T, Reichman B, et al. Intraperitoneal cisplatin and cytarabine in the treatment of refractory or recurrent ovarian carcinoma. J Clin Oncol 1991;9: 204–210.
120. Gale RP. Advances in the treatment of acute myelogenous leukemia. N Engl J Med 1979;300:1189–1199.
121. Skipper HE, Schabel FM Jr, Wilcox WS. Experimental evaluation of potential anticancer agents. XXI. Scheduling of arabinosylcytosine to take advantage of its S-phase specificity against leukemia cells. Cancer Chemother Rep 1967;51:125–165.
122. Bickers JN, Gehan EA, Freireich EJ, et al. Cytarabine for acute leukemia in adults. Effect of schedule on therapeutic response. Arch Intern Med 1974;133:251–259.
123. Rai KR, Holland JF, Glidewell OJ, et al. Treatment of acute myelocytic leukemia: a study by cancer and leukemia group B. Blood 1981;58:1203–1212.
124. Capizzi RL. Curative chemotherapy for acute myeloid leukemia: the development of high-dose ara-C from the laboratory to bedside. Invest New Drugs 1996;14:249–256.
125. Preisler H, Davis RB, Kirshner J, et al. Comparison of three remission induction regimens and two postinduction strategies for the treatment of acute nonlymphocytic leukemia: a cancer and leukemia group B study. Blood 1987;69:1441–1449.
126. Dillman RO, Davis RB, Green MR, et al. A comparative study of two different doses of cytarabine for acute myeloid leukemia: a phase III trial of Cancer and Leukemia Group B. Blood 1991;78:2520–2526.

127. Schiller G, Gajewski J, Nimer S, et al. A randomized study of intermediate vs conventional-dose cytarabine as intensive induction for acute myelogenous leukaemia. Br J Haematol 1992;81:170–177.
128. Mayer RJ, Davis RB, Schiffer CA, et al. Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia Group B. N Engl J Med 1994;331:896–903.
129. Rudnick SA, Cadman EC, Capizzi RL, Skeel RT, Bertino JR, McIntosh S. High dose cytosine arabinoside (HDARAC) in refractory acute leukemia. Cancer 1979;44:1189–1193.
130. Capizzi RL, Powell BL. Sequential high-dose ara-C and asparaginase vs high-dose ara-C alone in the treatment of patients with relapsed and refractory acute leukemias. Semin Oncol 1987;14(2 suppl 1):40–50.
131. Tallman MS. Therapy of acute myeloid leukemia. Cancer Control 2001;8:62–78.
132. Mitus AJ, Miller KB, Schenkein DP, et al. Improved survival for patients with acute myelogenous leukemia. J Clin Oncol 1995;13:560–569.
133. Buchner T, Hiddemann W, Wormann B, et al. Double induction strategy for acute myeloid leukemia: the effect of high-dose cytarabine with mitoxantrone instead of standard-dose cytarabine with daunorubicin and 6-thioguanine: a randomized trial by the German AML Cooperative Group. Blood 1999;93: 4116–4124.
134. Cohen A, Ullman B. Analysis of the drug synergism between thymidine and arabinosyl cytosine using mouse S49 T lymphoma mutants. Cancer Chemother Pharmacol 1985;14:70–73.
135. Grant S, Lehman C, Cadman E. Enhancement of 1-β-D-arabinofuranosylcytosine accumulation within L1210 cells and increased cytotoxicity following thymidine exposure. Cancer Res 1980;40:1525–1531.
136. Streifel JA, Howell SB. Synergistic interaction between 1-β-D-arabinofuranosylcytosine, thymidine, and hydroxyurea against human B cells and leukemic blasts in vitro. Proc Natl Acad Sci USA 1981;78:5132–5136.
137. Colly LP, van Bekkum DW. A recommendation for high-dose ara-C interval treatment based on studies in a slow-growing leukemia model (BNML). Med Pediatr Oncol 1982;10(suppl 1):209–219.
138. Mills-Yamamoto C, Lauzon GJ, Paterson AR. Toxicity of combinations of arabinosylcytosine and 3-deazauridine toward neoplastic cells in culture. Biochem Pharmacol 1978;27:181–186.
139. Noordhuis P, Kazemier KM, Kasperrs GJ, Peters GJ. Modulation of metabolism and cytotoxicity of cytosine arabinoside with *N*-(phosphon)-acetyl-L-aspartate in human leukemic blast cells and cell lines. Leuk Res 1996;20:127–134.
140. Grant S, Rauscher F, III, Cadman E. Differential effect of *N*-(phosphonacetyl)-L-aspartate on 1-β-D-arabinofuranosylcytosine metabolism and cytotoxicity in human leukemia and normal bone marrow progenitors. Cancer Res 1982;42:4007–4013.
141. Ross DD, Akman SA, Joneckis CC, Yang E, Bachur NR. Schedule-dependent enhancement of 1-β-D-arabinofuranosylcytosine incorporation into HL-60 DNA by deoxyguanosine. Cancer Res 1984;44:1530–1535.
142. Grant S, Bhalla K, Rauscher F, III, Cadman E. Potentiation of 1-β-D-arabinofuranosylcytosine metabolism and cytotoxicity by 2,3-dihydro-1H-imidazolo[1,2-b]pyrazole in the human promyelocytic leukemic cell, HL-60. Cancer Res 1983; 43:5093–5100.
143. Frewin RJ, Johnson SA. The role of purine analogue combinations in the management of acute leukemias. Hematol Oncol 2001;19:151–157.

144. Robak T. Purine nucleoside analogues in the treatment of myeloid leukemias. Leuk Lymphoma 2003;44:391–409.
145. Gandhi V, Kemena A, Keating MJ, Plunkett W. Fludarabine infusion potentiates arabinosylcytosine metabolism in lymphocytes of patients with chronic lymphocytic leukemia. Cancer Res 1992;52:897–903.
146. Gandhi V, Estey E, Keating MJ, Plunkett W. Fludarabine potentiates metabolism of cytarabine in patients with acute myelogenous leukemia during therapy. J Clin Oncol 1993;11:116–124.
147. Danhauser L, Plunkett W, Keating M, Cabanillas F. 9-β-D-Arabinofuranosyl-2-fluoroadenine 5′-monophosphate pharmacokinetics in plasma and tumor cells of patients with relapsed leukemia and lymphoma. Cancer Chemother Pharmacol 1986;18:145–152.
148. Estey E, Plunkett W, Gandhi V, Rios MB, Kantarjian H, Keating MJ. Fludarabine and arabinosylcytosine therapy of refractory and relapsed acute myelogenous leukemia. Leuk Lymphoma 1993;9:343–350.
149. Gandhi V, Estey E, Keating MJ, Chucrallah A, Plunkett W. Chlorodeoxyadenosine and arabinosylcytosine in patients with acute myelogenous leukemia: pharmacokinetic, pharmacodynamic, and molecular interactions. Blood 1996;87:256–264.
150. Kornblau SM, Gandhi V, Andreeff HM, et al. Clinical and laboratory studies of 2-chlorodeoxyadenosine ± cytosine arabinoside for relapsed or refractory acute myelogenous leukemia in adults. Leukemia 1996;10:1563–1569.
151. Crews KR, Gandhi V, Srivastava DK, et al. Interim comparison of a continuous infusion vs a short daily infusion of cytarabine given in combination with cladribine for pediatric acute myeloid leukemia. J Clin Oncol 2002;20:4217–4224.
152. Rubnitz JE, Razzouk BI, Srivastava DK, Pui CH, Ribeiro RC, Santana VM. Phase II trial of cladribine and cytarabine in relapsed or refractory myeloid malignancies. Leuk Res 2004;28:349–352.
153. Smith MA, Singer CR, Pallister CJ, Smith JG. The effect of haemopoietic growth factors on the cell cycle of AML progenitors and their sensitivity to cytosine arabinoside in vitro. Br J Haematol 1995;90:767–773.
154. Powell BL, Wang LM, Gregory BW, Case LD, Kucera GL. GM-CSF and asparaginase potentiate ara-C cytotoxicity in HL-60 cells. Leukemia 1995;9:405–409.
155. Reuter C, Auf der LU, Schleyer E, et al. Modulation of intracellular metabolism of cytosine arabinoside in acute myeloid leukemia by granulocyte-macrophage colony-stimulating factor. Leukemia 1994;8:217–225.
156. Tosi P, Visani G, Ottaviani E, Manfori S, Zinzani PL, Tura S. Fludarabine + Ara-C + G-CSF: cytotoxic effect and induction of apoptosis on fresh acute myeloid leukemia cells. Leukemia 1994;8:2076–2082.
157. McCarthy AJ, Pitcher LA, Hann IM, Oakhill A. FLAG (fludarabine, high-dose cytarabine, and G-CSF) for refractory and high-risk relapsed acute leukemia in children. Med Pediatr Oncol 1999;32:411–415.
158. Estey EH. Growth factors in acute myeloid leukaemia. Best Pract Res Clin Haematol 2001;14:175–187.
159. Dale DC. Colony-stimulating factors for the management of neutropenia in cancer patients. Drugs 2002;62 Suppl 1:1–15.
160. Ohno R, Tomonaga M, Kobayashi T, et al. Effect of granulocyte colony-stimulating factor after intensive induction therapy in relapsed or refractory acute leukemia. N Engl J Med 1990;323:871–877.
161. Bradstock KF. The use of hematopoietic growth factors in the treatment of acute leukemia. Curr Pharm Des 2002;8:343–355.

162. Russo D, Candoni A, Grattoni R, Bertone A, Zaja F. Fludarabine and cytosine-arabinoside for poor-risk acute myeloid leukemia. Haematologica 1998;83: 281–282.
163. Strickland AH, Seymour C, Prince HM, Wolf M, Juneja S, Januszewicz EH. Fludarabine and high dose cytarabine (FLA): a well tolerated salvage regimen in acute myeloid leukaemia. Aust N Z J Med 1999;29:556–558.
164. Dombret H, Chastang C, Fenaux P, et al. A controlled study of recombinant human granulocyte colony-stimulating factor in elderly patients after treatment for acute myelogenous leukemia. AML Cooperative Study Group. N Engl J Med 1995;332:1678–1683.
165. Estey E, Thall P, Andreeff M, et al. Use of granulocyte colony-stimulating factor before, during, and after fludarabine plus cytarabine induction therapy of newly diagnosed acute myelogenous leukemia or myelodysplastic syndromes: comparison with fludarabine plus cytarabine without granulocyte colony-stimulating factor. J Clin Oncol 1994;12:671–678.
166. Ossenkoppele GJ, Graveland WJ, Sonneveld P, et al. The value of fludarabine in addition to ara-C and G-CSF in the treatment of patients with high-risk myelodysplastic syndromes and AML in elderly patients. Blood 2004;103:2908–2913.
167. Hubeek I, Litvinova E, Peters GJ, et al. The effect of G-CSF on the in vitro cytotoxicity of cytarabine and fludarabine in the FLAG combination in pediatric acute myeloid leukemia. Int J Oncol 2004;25:1823–1829.
168. Kreis W, Budman DR, Chan K, et al. Therapy of refractory/relapsed acute leukemia with cytosine arabinoside plus tetrahydrouridine (an inhibitor of cytidine deaminase)—a pilot study. Leukemia 1991;5:991–998.
169. Kreis W, Chan K, Budman DR, et al. Effect of tetrahydrouridine on the clinical pharmacology of 1-β-D-arabinofuranosylcytosine when both drugs are coinfused over 3 h. Cancer Res 1988;48:1337–1342.
170. Verschuur AC, Van Gennip AH, Leen R, Meinsma R, Voute PA, Van Kuilenburg AB. In vitro inhibition of cytidine triphosphate synthetase activity by cyclopentenyl cytosine in paediatric acute lymphocytic leukaemia. Br J Haematol 2000;110:161–169.
171. Verschuur AC, Van Gennip AH, Leen R, et al. Cyclopentenyl cytosine inhibits cytidine triphosphate synthetase in paediatric acute non-lymphocytic leukaemia: a promising target for chemotherapy. Eur J Cancer 2000;36:627–635.
172. Verschuur AC, Van Gennip AH, Leen R, Voute PA, Brinkman J, Van Kuilenburg AB. Cyclopentenyl cytosine increases the phosphorylation and incorporation into DNA of 1-β-D-arabinofuranosyl cytosine in a human T-lymphoblastic cell line. Int J Cancer 2002;98:616–623.
173. Verschuur AC, Van Gennip AH, Brinkman J, Voute PA, Van Kuilenburg AB. Cyclopentenyl cytosine induces apoptosis and secondary necrosis in a T-lymphoblastic leukemic cell-line. Adv Exp Med Biol 2000;486:319–325.
174. Verschuur AC, Van Gennip AH, Leen R, Voute PA, Van Kuilenburg AB. Cyclopentenyl cytosine increases the phosphorylation and incorporation into DNA of arabinofu-ranosyl cytosine in a myeloid leukemic cell-line. Adv Exp Med Biol 2000;486:311–317.
175. Bierau J, Van Gennip AH, Leen R, Helleman J, Caron HN, Van Kuilenburg AB. Cyclopentenyl cytosine primes SK-N-BE*(2)*c neuroblastoma cells for cytarabine toxicity. Int J Cancer 2003;103:387–392.

176. Barret JM, Hill BT. DNA repair mechanisms associated with cellular resistance to antitumor drugs: potential novel targets. Anticancer Drugs 1998;9: 105–123.
177. Sargent JM, Elgie AW, Williamson CJ, Lewandowicz GM, Taylor CG. Circumvention of ara-C resistance by aphidicolin in blast cells from patients with AML. Br J Cancer 2001;84:680–685.
178. Kuwakado K, Kubota M, Hirota H, et al. Aphidicolin potentiates apoptosis induced by arabinosyl nucleosides in human myeloid leukemia cell lines. Biochem Pharmacol 1993;46:1909–1916.
179. Sargent JM, Williamson CJ, Hubeek I, et al. Aphidicolin decreases ex vivo resistance to cytosine arabinoside in childhood acute leukaemia. Oncol Rep 2003;10:2027–2031.
180. Jarvis WD, Turner AJ, Povirk LF, Traylor RS, Grant S. Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. Cancer Res 1994;54:1707–1714.
181. Bertrand R, Solary E, O'Connor P, Kohn KW, Pommier Y. Induction of a common pathway of apoptosis by staurosporine. Exp Cell Res 1994;211:314–321.
182. Grant S, Jarvis WD, Swerdlow PS, et al. Potentiation of the activity of 1-β-D-arabinofuranosylcytosine by the protein kinase C activator bryostatin 1 in HL-60 cells: association with enhanced fragmentation of mature DNA. Cancer Res 1992;52:6270–6278.
183. Cragg LH, Andreeff M, Feldman E, et al. Phase I trial and correlative laboratory studies of bryostatin 1 (NSC 339555) and high-dose 1-B-D-arabinofuranosylcytosine in patients with refractory acute leukemia. Clin Cancer Res 2002;8: 2123–2133.
184. Grant S, Turner AJ, Bartimole TM, Nelms PA, Joe VC, Jarvis WD. Modulation of 1-[β-D-arabinofuranosyl] cytosine-induced apoptosis in human myeloid leukemia cells by staurosporine and other pharmacological inhibitors of protein kinase C. Oncol Res 1994;6:87–99.
185. Wang S, Vrana JA, Bartimole TM, et al. Agents that down-regulate or inhibit protein kinase C circumvent resistance to 1-β-D-arabinofuranosylcytosine-induced apoptosis in human leukemia cells that overexpress Bcl-2. Mol Pharmacol 1997;52:1000–1009.
186. Tang L, Boise LH, Dent P, Grant S. Potentiation of 1-β-D-Arabinofuranosylcytosine-mediated mitochondrial damage and apoptosis in human leukemia cells (U937) overexpressing bcl-2 by the kinase inhibitor 7-hydroxystaurosporine (UCN-01). Biochem Pharmacol 2000;60:1445–1456.
187. Koller-Lucae SK, Suter MJ, Rentsch KM, Schott H, Schwendener RA. Metabolism of the new liposomal anticancer drug N4-octadecyl-1-β-D-arabinofuranosylcytosine in mice. Drug Metab Dispos 1999;27:342–350.
188. Murry DJ, Blaney SM. Clinical pharmacology of encapsulated sustained-release cytarabine. Ann Pharmacother 2000;34:1173–1178.
189. Hamada A, Kawaguchi T, Nakano M. Clinical pharmacokinetics of cytarabine formulations. Clin Pharmacokinet 2002;41:705–718.
190. Hong CI, Bernacki RJ, Hui SW, Rustum Y, West CR. Formulation, stability, and antitumor activity of 1-β-D-arabinofuranosylcytosine conjugate of thioether phospholipid. Cancer Res 1990;50:4401–4406.
191. Breistol K, Balzarini J, Sandvold ML, et al. Antitumor activity of P-4055 (elaidic acid-cytarabine) compared to cytarabine in metastatic and s.c. human tumor xenograft models. Cancer Res 1999;59:2944–2949.

192. Peters GJ, Voorn DA, Kuiper CM, et al. Cell specific cytotoxicity and structure-activity relationship of lipophilic 1-B-D-arabinofuranosylcytosine (ara-C) derivatives. Nucleosides Nucleotides 1999;18(4–5):877, 878.
193. Bergman AM, Kuiper CM, Voorn DA, et al. Antiproliferative activity and mechanism of action of fatty acid derivatives of arabinofuranosylcytosine in leukemia and solid tumor cell lines. Biochem Pharmacol 2004;67:503–511.
194. Horber DH, Schott H, Schwendener RA. Cellular pharmacology of a liposomal preparation of N4-hexadecyl-1-β-D-arabinofuranosylcytosine, a lipophilic derivative of 1-β-D-arabinofuranosylcytosine. Br J Cancer 1995;71:957–962.
195. Aamdal S. Phase I trial of a nucleoside analog CP-4055 given daily for 5 days every 3 wk in patients (pts) with advanced solid tumors—preliminary results. J Clin Oncol 2004;22:2049.
196. Galmarini CM, Clarke ML, Santos CL, et al. Sensitization of ara-C-resistant lymphoma cells by a pronucleotide analogue. Int J Cancer 2003;107:149–154.

7 Clofarabine

Mechanisms of Action, Pharmacology, and Clinical Investigations

Varsha Gandhi, PhD
and William Plunkett, PhD

CONTENTS

SUMMARY

Clofarabine is a new dAdo analog that shows effectiveness in both adult and pediatric ALL and AML. The pharmacokinetic and pharmacodynamic profile of the drug in acute leukemias is favorable relative to fludarabine or cladribine and may have prognostic value for its clinical activity. The toxicity profile of this agent is different from other nucleoside analogs in that it clearly has clinical activity when used as a single agent against adult acute leukemias at tolerable doses. Further, therapeutic strategies combining clofarabine with DNA-damaging agents are likely to allow mechanism-based rationales to be evaluated with minimal likelihood of untoward toxicity. Exploration of additional schedules and combinations along with availability of an oral formulation strongly indicate promising possibilities with this agent.

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

Key Words: Acute leukemias; cellular pharmacology; cladribine; clofarabine; DNA polymerase; DNA repair; fludarabine.

1. INTRODUCTION TO THE STRUCTURE

As a class of therapeutic agents, nucleoside analogs are more prevalent in the clinical treatment of cancer and viral diseases than other mechanistically similar groups of compounds. Among the medley of these antimetabolites, congeners of deoxycytidine (dCyd) or deoxyadenosine (dAdo) are the most effective agents in the clinic. Minor variations in the sugar moiety of the structures have resulted in dCyd analogs with a wide spectrum of clinical applications. Cytarabine (ara-C), distinguished by an arabinose sugar, is the most active drug in acute myeloid leukemia (AML) *(1)*. The success of ara-C generated enthusiasm for the development of dCyd analogs such as gemcitabine, troxacitabine, and decitabine *(2–4)*. Although these analogs showed some efficacy in acute leukemias, none was as effective as ara-C, and most needed to be used in combinations *(5–7)*.

Purine nucleoside analogs used in acute leukemias include cladribine and fludarabine. Each is derivatized with a halogen at the 2-position of the adenine to prevent deamination and hence deactivation of the compound (Fig. 1). Cladribine has been efficacious for pediatric acute leukemias *(8–10)* but has only marginal activity in adult disease *(11)*. Fludarabine is ineffective at the tolerable doses but has been used effectively in combinations with ara-C in both pediatric and adult acute leukemias *(12,13)*.

Nonetheless, each has shown different degrees of activity against indolent lymphoproliferative disorders. Cladribine, which has the natural deoxyribosyl sugar, is the drug of choice for hairy cell leukemia *(14)* and has proven activity in Waldenstrom's macroglobulinemia *(15)*. Fludarabine, an arabinosyl nucleoside, is widely used in combinations for chronic lymphocytic leukemia (CLL) *(16)* and for indolent lymphomas *(17)*. Activity in these diseases may be attributed not only to the nature of their biologies, but also to the various modifications of the carbohydrate moieties of these nucleosides, which undoubtedly affects their metabolism and pharmacodynamics.

Although both these dAdo analogs are resistant to deamination, they are susceptible to phosphorolysis and are labile in an acidic environment. These shortcomings were addressed by the conceptualization of a new purine nucleoside analog, clofarabine *(18)*. Clofarabine (2-chloro-2′-fluorodeoxy-9-β-D-arabinofuranosyladenine) was synthesized as a rational extension of the dAdo analog experiences (Fig. 1). This hybrid drug retains the 2-chloroadenine aglycone of cladribine. Reminiscent of fludarabine, clofarabine is further derivatized with a fluorine molecule in the *arabino* configuration at the critical 2′-position of the carbohydrate *(18)*. The last

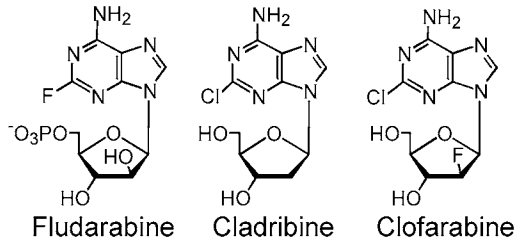


Fig. 1. Structure of dAdo analogs including clofarabine.

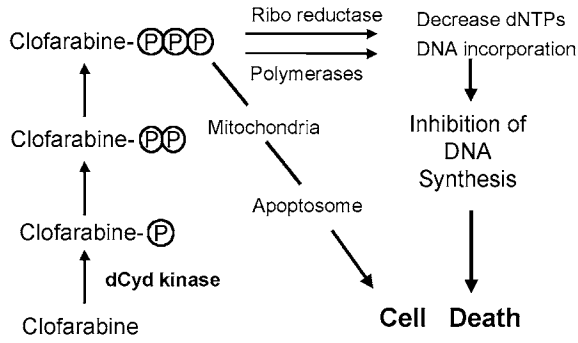


Fig. 2. Metabolism of clofarabine to its triphosphate and actions of analog triphosphate that are DNA-directed and non-DNA-directed.

substitution stabilizes the glycosidic bond, rendering this nucleoside analog highly resistant to bacterial purine nucleoside phosphorylase and to acid hydrolysis *(18–21)*.

Structure–activity relationship investigations demonstrated that clofarabine incorporates the most favorable antimetabolic properties of fludarabine and cladribine (Fig. 2). Similar to these purine congeners, clofarabine requires intracellular phosphorylation by deoxycytidine kinase to be metabolized to the triphosphate form necessary for its cytotoxic effect *(22)*. The triphosphate of fludarabine (F-ara-A triphosphate) primarily inhibits deoxyribonucleic acid (DNA) polymerization after incorporation into DNA; cladribine triphosphate is particularly inhibitory to ribonucleotide reductase. Clofarabine triphosphate has the mechanistically favorable properties of both agents regarding DNA chain termination *(23–25)* and inhibition of ribonucleotide reductase *(19,25)*.

2. CATABOLISM

Structure–activity studies comparing the 2′, 2′-difluoro-, and 2′-deoxy-2′-ribo- derivatives with clofarabine demonstrated that the placement of the fluorine in the arabinosyl configuration was essential for the DNA-directed activity of the compound *(22)*. Furthermore, substitution of a fluorine at the C-2′ position, while retaining the *arabino* configuration, decreased the susceptibility of clofarabine to phosphorolytic cleavage by bacterial purine nucleoside phosphorylase to roughly one-third that of cladribine and of F-ara-A, the nucleoside of fludarabine *(18)* (Table 1). The 2′-*arabino*-fluoro substitution also resulted in an increased acid stability of clofarabine relative to dAdo and cladribine *(18)*.

Comparison of the metabolism of clofarabine and cladribine in isolated perfused rat livers demonstrated that 2-chloroadenine was liberated from each nucleoside *(26)* to a similar extent (50%). The fact that the total recovery of clofarabine from the perfusate was incomplete compared to cladribine remains unexplained, and the enzyme responsible for this first-pass metabolism has not been identified. Nevertheless, the stability of clofarabine to low pH and its relative resistance to phosphorolysis by *Escherichia coli* purine nucleoside phosphorylase, in contrast to fludarabine *(27)*, are attributes that have stimulated investigations for the development of clofarabine as an orally administered drug.

3. ANABOLISM

Early studies indicated that clofarabine was phosphorylated to the monophosphate by deoxycytidine kinase *(19)*. In addition, deoxyguanosine kinase is able to phosphorylate clofarabine and cladribine, although the activity of the enzyme in cells is only a fraction of that of deoxycytidine kinase *(28)*. However, the finding that cells that lack deoxycytidine kinase are resistant to clofarabine *(19,20)* emphasizes its relative importance to cytotoxicity.

Two studies using purified deoxycytidine kinase have detailed the kinetic characteristics of this reaction *(22,29)*. The biochemically purified enzyme from human Molt-4 lymphoblasts exhibited an efficiency (V_{max}/K_m) at phosphorylating clofarabine that was 87% of that of dCyd as the substrate *(22)*. By comparison, phosphorylation of cladribine and fludarabine was 23% and 3.4% as efficient, respectively. Somewhat in contrast, phosphorylation of clofarabine and cladribine by the recombinant human enzyme was 7.6-fold and 2.3-fold more efficient, respectively, than was that of dCyd *(29)*. The reasons for the differences in these values are not obvious but may be caused by the methods used to prepare the enzymes (Table 1; Fig. 2).

Table 1
Comparison of Metabolism of dAdo Analogs

	Fludarabine	*Cladribine*	*Clofarabine*
Glycosidic bond cleavage (pH < 3.0)	Stable	Labile	Stable
Enzymatic phosphorolysis (relative rate)	1	1	0.3
dCyd kinase (K_m, μ*M*)	1600	5	14
Major metabolites	TP	MP + TP	MP + TP
Triphosphate $t_{1/2}$ (h, in CLL cells)	>24	13	>24

TP, 5′-triphosphate; MP, 5′-monophosphate.

Using radiolabeled clofarabine, the metabolism and inhibitory actions of this compound were examined in human CEM lymphoblastoid cells *(24)*. These studies demonstrated that the major metabolite was clofarabine monophosphate, although the triphosphate accumulated to significant concentrations. This investigation was extended in primary CLL cells in vitro *(30)*. As similar characteristics were observed for cladribine, it appears that nucleosides bearing a 2-chloroadenine nucleobase are relatively poor substrates for the monophosphate kinase responsible for their phosphorylation to the respective diphosphates (Table 1). Clofarabine triphosphate was eliminated slowly after washing CEM cells free of exogenous nucleoside *(24,25)*. The observations noted during studies with cell lines were also apparent when these investigations were done using primary leukemia cells *(30,31)*. The differences in pharmacology of fludarabine, cladribine, and clofarabine triphosphate accumulation in primary leukemia cells suggest that this may be a basis for the spectrum of clinical activities generated with these agents.

4. MECHANISMS OF ACTION

DNA-directed and also non-DNA-directed actions have been reported for clofarabine-mediated cytotoxic effects (Fig. 2). Incorporation of clofarabine monophosphate into DNA was strongly correlated with its cytotoxicity in CEM cells *(24)*, as had previously been demonstrated for fludarabine *(32)*, and suggests DNA-directed actions as the primary mechanisms. Clofarabine triphosphate accumulation in K562 cells *(19)* was associated with specific inhibition of DNA synthesis; uptake of radioactive precursors into ribonucleic acid (RNA) and protein was not affected. Although clofarabine was most inhibitory to DNA synthesis in whole cells *(19,24,25)*, a small but reproducible amount of the analog was incorpo-

Table 2
Comparison of Actions of dAdo Analogs

	Fludarabine	*Cladribine*	*Clofarabine*
Ribonucleotide reductase (IC_{50}, μ*M*)	650	65	65
DNA polymerase alpha (dATP)			
K_m incorp, μ*M* (3.7)	7.4	2.8	1.9
K_m extend, μ*M* (0.3)	36	1.4	27
Apoptosome (ATP = 1)	39	8	26
RNA incorporation (% of DNA incorporation)	20	none	1

rated into RNA *(24)*, although less than for F-ara-A triphosphate *(33)*. The last studies demonstrated a strong correlation between incorporation of the analog into DNA, the clonogenic viability of CEM cells, and cells in S phase.

The inhibition of DNA synthesis by clofarabine in K562 cells was associated with a decrease in the concentrations of cellular deoxynucleotides *(22)*, a finding that was later confirmed in CEM cells *(25)*. Cell-free assays demonstrated that clofarabine triphosphate was a potent inhibitor of ribonucleotide reduction *(19,23)*. This was explained by the demonstration that the triphosphate of clofarabine inhibited ribonucleotide reductase. In this respect, clofarabine triphosphate was as inhibitory as cladribine triphosphate, but 10 times more potent than F-ara-A triphosphate (Table 2). In addition to the potential direct effect of reduced concentrations of deoxyribonucleotide triphosphates (dNTPs) on DNA synthesis, these analog triphosphates compete with deoxyadenosine 5′-triphosphate (dATP) for incorporation into DNA. An action such as reductase inhibition that decreases the cellular concentration of the normal nucleotide may enhance the incorporation of the analog triphosphate, an interaction known as self-potentiation *(34)*.

Primer extension assays demonstrated that clofarabine triphosphate is a potent inhibitor of DNA polymerases-α and -ε, each of which is involved in nuclear DNA replication *(19,25)*. However, the activity of DNA polymerase-β, which fills small gaps during base excision repair, and the mitochondrial DNA polymerase-γ are much less sensitive to clofarabine triphosphate *(19)*. As the triphosphates of fludarabine and cladribine also have different activities against these polymerases *(19)*, this may be a factor that influences the differences in clinical responses to these structurally similar nucleosides (Table 2).

The observation that the cytochrome c-induced apoptosis cascade requires binding of dATP in the apoptosome complex *(35)* motivated stud-

ies to determine if triphosphate analogs of dAdo could substitute for dATP. Stimulation of caspase activation by cladribine triphosphate suggested that analog triphosphates could effectively substitute for normal nucleotides in apoptosome functions *(36)*. This was true for several nucleoside analogs, including clofarabine triphosphate *(37)*, which was equally active as dATP (Table 2). Although this appears to be a DNA-independent effect of clofarabine, the trigger to release cytochrome c from mitochondria in whole cells probably occurs after actions of the analog on genomic DNA, further indicating the importance of DNA-directed actions of the agent. At higher concentrations of clofarabine, however, disruption of mitochondrial membrane integrity and loss of mitochondrial functions suggest a direct action on mitochondria *(38)* (Fig. 2). This needs to be evaluated in clinical samples to establish this action, especially in nondividing leukemic lymphocytes. Similarly, other non-DNA-directed actions *(39)* require testing in primary leukemia cells in vitro and during therapy with clofarabine.

5. PRECLINICAL ACTIVITY

Preclinical biological experiments showed that clofarabine is a potent cytotoxic to human cell lines such as CEM, K562, HEp2, and murine leukemia L1210 *(20,40)*. Clofarabine exhibited in vivo antitumor activity against three murine tumors (P388 leukemia, colon 36, and mammary 16/c) and was curative against both early- and advanced-stage colon 36 *(40)*. Other investigations demonstrated impressive activity against a variety of human tumor xenografts from colon, lung, breast, and stomach *(41)*. Its activity in experimental therapeutic investigations was largely independent of route of administration and was favored by a prolonged treatment schedule.

The oral bioavailability of clofarabine in rats was 50%, further suggesting the feasibility of oral regimens *(42)*. In evaluating the therapeutic efficacy of this route of administration, mice that were given oral doses of either clofarabine or cladribine achieved 10-fold greater concentrations of clofarabine in the plasma than those of cladribine *(20)*. This was associated with a significantly greater efficacy of clofarabine against xenografts of human chronic lymphoid leukemia cells in severe combined immunodeficient mice *(20)*. The therapeutic activity of an aqueous solution of clofarabine was efficacious on several schedules in mice bearing a variety of human colon xenografts *(41)*. Thus, there is adequate evidence to support the development of an oral formulation of clofarabine, especially now that the clinical activity of the intravenous administration has been established for the drug.

6. CLINICAL INVESTIGATIONS

Based on its favorable metabolic properties and actions, investigators at M. D. Anderson Cancer Center in Houston, Texas, decided to move the drug

in the clinic. Hence, Investigational New Drug (IND)-directed toxicology and pharmacological investigations were conducted in animal model systems. This has been formulated for clinical trials. Phase I studies have been completed in patients with refractory solid tumors and hematologic malignancies *(43)*; some phase II investigations have been completed, and evaluations have moved to combination clinical trials.

6.1. Clinical Trials in Adults

6.1.1. Phase I Studies

Based on animal toxicology data, a starting dose of clofarabine of 15 mg/m^2/d as a 1-h intravenous infusion daily for 5 d was chosen for the phase I study for patients with advanced solid tumors and hematologic malignancies *(43)*. Grade 3 and 4 myelosuppression in patients with solid tumors forced dose reductions to 7.5 mg/m^2, 4 mg/m^2, and finally 2 mg/m^2/d. The trial eventually established a dose of 2 mg/m^2 intravenously daily for 5 d as the maximum tolerated dose (MTD) in solid tumors and a dose of 4 mg/m^2/d using the same schedule in CLL. The dose-limiting toxicities (DLTs) were defined as myelosuppression and thrombocytopenia, respectively.

In patients with acute leukemias, the dose of 15 mg/m^2/d was escalated stepwise to 52 mg/m^2/d for 5 d. The MTD was established as 40 mg/m^2/d for 5 d, 10- to 20-fold greater than that tolerated by patients with indolent leukemias or solid tumors, respectively. The DLT was hepatotoxicity, which is different from other clinically effective anticancer nucleoside analogs *(44)*. Whereas no significant responses occurred in patients with solid tumors, antitumor activity has been noted in patients with acute leukemias and lymphoproliferative disorders. In short, of 32 patients with acute leukemias, 5 showed response (16%), including complete remissions (CRs) in 2 patients.

6.1.1.1. Pharmacokinetics and Pharmacodynamics

Investigations of the plasma pharmacology of clofarabine, of the pharmacokinetics of its triphosphate in leukemia cells, and of the pharmacodynamic actions in circulating blasts obtained from patients with acute leukemia were conducted during this phase I trial to understand the actions of the drug and to develop a rationale for use of clofarabine in subsequent phase II investigations *(45)*. Adults with refractory acute leukemias, including lymphoblastic (acute lymphocytic leukemia, ALL), AML, and chronic myelogenous leukemia in blastic phase (CML-BP) received clofarabine from 4 to 55 mg/m^2/d for 5 d as a 1-h intravenous infusion. Plasma pharmacology studies done in 25 of the 32 patients indicated a linear increase in the plasma clofarabine concentration with increasing doses. At 40 mg/m^2, the MTD, the median plasma clofarabine level was 1.5 μ*M* (range 0.42–3.2 μ*M*; $n = 7$). Cellular pharmacokinetic studies done at the

end of the first clofarabine infusion in 26 patients appeared dose proportional but showed a wide variation in the concentrations of clofarabine triphosphate. At the MTD, the clofarabine triphosphate concentration was a median 19 μ*M* (range 3–52 μ*M*). In the majority of cases, more than 50% of the analog triphosphate was present 24 h after the end of the infusion.

The mechanistic investigations with clofarabine have demonstrated that this dAdo analog has dual actions on inhibition of DNA synthesis: first, by direct incorporation into DNA followed by inhibition of further DNA chain elongation by DNA polymerases and second by inhibiting ribonucleotide reductase, the enzyme responsible for the supply of dNTPs needed for replicative DNA synthesis *(19,24,25)*. The ratio of the concentrations of clofarabine triphosphate to dATP in cells would be a primary determinant for analog incorporation into DNA. Data from acute leukemia blasts from 9 patients studied during the phase I investigation suggested that the intracellular concentration of dATP was relatively low prior to treatment (median 1.8 μ*M*). Furthermore, after clofarabine infusion, this concentration declined further in the blasts of 3 patients; in 2 patients, it remained unchanged. Hence, it would be predicted that the clofarabine triphosphate concentrations achieved in the leukemia blasts after the first infusion of clofarabine (median 19 μ*M* at the MTD) should provide a ratio of clofarabine triphosphate concentration to that of the normal dNTP that favors the incorporation of the analog into DNA, resulting in inhibition of DNA synthesis.

The dose-dependent pharmacodynamic actions of clofarabine triphosphate were evident regarding the duration of DNA synthesis inhibition in blasts following a single clofarabine infusion. Although doses between 22.5 and 55 mg/m^2 reduced DNA synthesis by 75–95%, this nearly recovered to pretreatment levels in blasts from patients who received 22.5 and 30 mg/m^2 of clofarabine (Fig. 3). In contrast, DNA synthesis remained suppressed at 24 h in the blasts of patients treated with 40 and 55 mg/m^2. The extent and duration of inhibition of DNA synthesis is strongly associated with clofarabine killing of cells in culture *(24,25)*. As with other nucleoside analogs *(46,47)*, it is likely that timing of recovery of DNA synthesis is related to the cellular concentration of triphosphate accumulated and its rate of elimination. Because inhibition of DNA synthesis and its effect on leukemia cells are related to clinical response to other nucleoside analogs *(46)*, it will be important to develop a thorough understanding of this relationship to facilitate the design of optimal schedules and combinations with clofarabine.

6.1.2. Phase II Studies

6.1.2.1. Acute Leukemias

The objective activity in acute leukemias during phase I studies and favorable pharmacokinetics and pharmacodynamics at the MTD in these

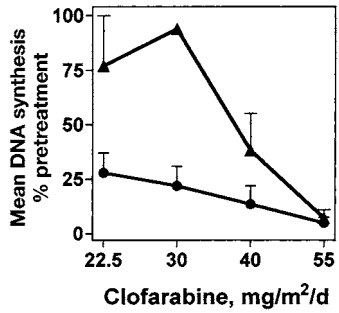


Fig. 3. Inhibition of clofarabine mediated DNA synthesis in leukemia cells during therapy. Inhibition of DNA synthesis was measured either at the end of 1 h infusion (•) or 24 h after infusion (▲). (From ref. *45* with permission.)

patients provided compelling rationales for phase II trials in patients with relapsed acute leukemias and myelodysplastic syndromes (MDSs). Phase II trials in relapsed and refractory acute leukemias were conducted to determine efficacy of clofarabine at a MTD of 40 mg/m^2/d for 5 d and to seek correlations between clofarabine triphosphate cellular pharmacokinetics with clinical response *(48)*. Sixty-two patients with AML, MDS, CML-BP, and ALL were treated with clofarabine 40 mg/m^2 iv over 1 h daily for 5 d every 3–6 wk. Overall, 20 patients (32%) achieved CR, 1 had a partial response, and 9 (15%) had CR but without platelet recovery for an overall response rate of 48%. Overall responses based on diagnosis suggested a preferential response in myeloid disease; however, the number of patients with ALL was small (Table 3).

6.1.2.1.1. Pharmacokinetics. Plasma pharmacokinetic analyses of clofarabine were done in 29 patients at the end of infusion and up to 24 h. There was heterogeneity for accumulation of plasma clofarabine; the median value at the end of infusion was 1.0 μ*M* (range 0.26–1.94 μ*M*), which was similar to that observed during phase I study. To determine the variability in elimination kinetics of the drug from plasma, clofarabine levels were quantified at different time-points after infusion. Clofarabine appeared to be eliminated in a biphasic manner, with more rapid elimination during the first 6 h followed by a slower phase through 24 h. There were insufficient data points for calculation of elimination half-lives. Nonetheless, there was a measurable clofarabine concentration at 24 h. Among the 26 patients, the median concentration of plasma clofarabine at 24 h was 0.038 μ*M* (range 0.013–0.11). When applied to leukemia cell lines in vitro, such concentrations caused greater than 50% loss of clonogenicity *(24)*.

Cellular clofarabine triphosphate concentration investigations in circulating leukemia blasts of these 29 patients suggested peak concentration of

Table 3
Clinical Responses During Phase II Trial in Adult Acute Leukemias ($n = 62$)

		Responders (n)			
Diagnosis	*Total (n)*	*CR*	*CRp*	*PR*	*OR (%)*
AML	31	13	4	—	17 (55)
CML-BP	11	4	2	1	7 (57)
MDS	8	2	2	—	4 (50)
ALL	12	1	1	—	2 (17)
Total	62	20	9	1	30 (48%)

CR, complete remission; CRp, complete remission without platelet recovery; PR, partial remission; OR, overall response. (From ref. *45* with permission.)

20 μ*M* of clofarabine triphosphate. At 24 h, more than 50% of the peak triphosphate concentration was retained, suggesting a long half-life (>24 h) of the triphosphate. Purine nucleoside analogs in general have a prolonged retention of analog triphosphates. For example, the elimination half-lives of nelarabine triphosphate, fludarabine triphosphate, and cladribine triphosphate were >24, >24, and 10 h, respectively *(49–51)*. This positive feature may in part be associated with the favorable response in indolent leukemias. By comparison, the peak levels of fludarabine triphosphate in circulating leukemia blasts of patients with relapsed acute leukemias at the MTD of fludarabine were a median 40 μ*M (52)*. However, the elimination half-life of fludarabine triphosphate was much faster ($t_{1/2} = 7$ h) *(53)*.

With these kinetic profiles, the pharmacodynamic actions likely will be greatly diminished prior to the subsequent fludarabine dose. For cladribine triphosphate, the information in acute leukemia was very limited. Nonetheless, the accumulation of triphosphate in blasts either after continuous infusion or bolus administration was less than 5 μ*M (10,54)*. Thus, the accumulation and retention of clofarabine triphosphate in acute leukemia blasts at the MTD appears somewhat different and has a more favorable profile than does that of either fludarabine or cladribine.

In seeking associations between cellular pharmacokinetics and the clinical response, it was observed that after the first clofarabine infusion responders accumulated a greater clofarabine triphosphate concentration in blasts (median 18 μ*M*) compared to nonresponders (median 10 μ*M*; $p = 0.03$). In contrast to other dAdo analogs, both in responders and nonresponders, the triphosphate of clofarabine was maintained well, and at 24 h 50% of the peak triphosphate concentration was detected (Table 4). Because the starting level of triphosphate was higher in responders, at 24 h higher levels of triphosphate were retained in responders ($n = 15$) than in nonresponders

Table 4
Relationship Between Clofarabine Triphosphate Pharmacokinetics and Clinical Responses

	Median clofarabine triphosphate, μM (range)		
	Responders, n = 19	*Nonresponders, n = 11*	*p value*
d 1 end of infusion	18 μ*M* (5–44)	10 μ*M* (1–23)	0.032
at 24 h	11 μ*M* (1–44)	5 μ*M* (1–20)	0.039
d 2 end of infusion	30 μ*M* (1–67)	9 μ*M* (1–23)	0.015

($n = 10$). Furthermore, there was a trend for an increase in the end of infusion value from d 1 to d 2 in responders compared with nonresponders. Overall, the median concentration of clofarabine triphosphate at the end of second infusion was 30 μ*M* ($n = 15$) for patients who responded to therapy and 9 μ*M* ($n = 10$) for those who failed the treatment ($p = 0.015$). Finally, when compared for the increase in clofarabine triphosphate value after a subsequent dose, responders had a median 1.8-fold increase compared to nonresponders who did not increase the concentration of clofarabine triphosphate from d 1 to d 2 ($p = 0.008$). Taken together, these data suggested an overall greater accumulation, longer duration of analog triphosphate retention, and incremental increase in clofarabine triphosphate during the 5-d therapy in patients who benefited from clofarabine therapy (Table 4).

6.1.2.2. Indolent Leukemias

During the phase I clinical studies, it was observed that clofarabine resulted in cytoreductiveness in indolent leukemias. To explore this possibility further, two phase II studies were initiated using either daily for 5 d or weekly infusion schedules at M. D. Anderson Cancer Center. These studies are ongoing and yet unpublished.

6.1.2.3. Solid Tumors

Similar to indolent leukemias, for patients with solid tumors hematological toxicities resulted in a relatively low MTD (2 mg/m^2/d) on a daily times 5 d schedule. This fact along with the observation that clofarabine triphosphate is long-lived in cells suggested reduction in the frequency of infusions. Hence, a weekly dose schedule is undergoing testing in a phase I setting for patients with solid tumors *(55)*.

6.1.3. Combination Studies

6.1.3.1. Biochemical Modulation

In vitro investigations using human leukemia cell lines demonstrated that cells loaded with clofarabine triphosphate accumulated ara-C triphosphate at higher rate than did cells that were not pretreated with clofarabine *(56,57)*. This may be because of direct and indirect effects of clofarabine triphosphate on the activity of deoxycytidine kinase, the rate-limiting enzyme for the accumulation of ara-C triphosphate *(56,57)*. Based on these observations and the utility of both clofarabine and ara-C in acute leukemias, phase I and II studies were initiated to demonstrate that clofarabine will modulate the accumulation of ara-C triphosphate levels and therefore increase the antileukemic activity of ara-C.

Thirty-two patients with acute leukemias were treated with the combination of clofarabine and ara-C; 12 were entered on the phase I part of the trial with four dose levels of clofarabine (15, 22.5, 30, 40 mg/m^2/d) *(58)*. Clofarabine was given as a 1-h iv infusion daily for 5 d (d 2–6), followed 4 h later by ara-C at 1 g/m^2/d as a 2-h iv infusion daily for 5 d (d 1–5). As no DLTs occurred at any dose level, 40 mg/m^2/d for 5 d was chosen as the phase II dose of clofarabine. Among the 29 patients with AML/MDS, 7 patients (24%) achieved CR, and 5 patients (17%) achieved CR but without platelet recovery, for an overall response rate of 41%. No responses occurred in the 3 patients with ALL and CML, respectively. Adverse events were mainly grade 2 or below and included nausea/vomiting, diarrhea, skin rashes, mucositis, and palmoplantar erythrodysesthesias. Plasma clofarabine levels generated clofarabine triphosphate accumulation, which resulted in an increase in ara-C triphosphate accumulation in the leukemic blasts. The combination regimen of clofarabine with ara-C is safe and active in of patients with acute leukemias with poor prognosis. Cellular pharmacology data support the biochemical modulation strategy. Future studies are needed to investigate the combination in newly diagnosed AML and MDS.

6.1.3.2. Inhibition of DNA Repair

Lymphoid malignancies exhibit strong activation of excision repair processes in response to DNA damage by alkylating agents, platinum derivatives, or ultraviolet light. Studies using CLL lymphocytes demonstrated that clofarabine triphosphate was incorporated into DNA repair patches that were induced following treatment with 4-hydroperoxycyclophophamide, an activated form of cytoxan *(59)*. Comparisons of clofarabine with fludarabine indicated inhibition of such DNA repair activity was qualitatively similar. However, the potency of clofarabine was approx 10 times greater than that of fludarabine. Importantly, the combination of minimally toxic concentrations of clofarabine and the alkylating agent caused significantly more apoptosis than would have been expected from the sum of each agent administered separately. Clinical studies have

stemmed from this observation by combining clofarabine with idarubicin and clofarabine with cytoxan in acute leukemias.

6.2. Clinical Trials in Pediatric Patients

6.2.1. Phase I Studies

Based on the ongoing adult phase I studies in acute leukemias and the encouraging cytoreductiveness, a phase I study of clofarabine in pediatric patients with refractory and relapsed leukemia was initiated at M. D. Anderson Cancer Center *(60)*. Clofarabine was infused intravenously over 1 h daily for 5 d. Six dose levels between 11.25 and 70 mg/m^2/d for 5 d were studied in 25 patients. The MTD was similar to adult acute leukemia population (i.e., 52 mg/m^2/d for 5 d). Pharmacokinetic and pharmacodynamic aspects of investigations were also consistent with observations in adult acute leukemia patients *(60,61)*. At the end of infusion at MTD, clofarabine triphosphate levels in leukemia blasts varied between 6 and 19 μ*M*. Pharmacodynamically, this resulted in complete and sustained inhibition of DNA synthesis. The DLT was reversible hepatotoxicity and skin rash at 70 mg/m^2/day for 5 d. Five patients achieved a CR, and 3 had a partial remission, for an overall response rate of 32%. Taken together, the phase I study defined the dose level of 52 mg/m^2 daily for 5 d as the MTD for clofarabine in children with leukemia. Multicenter phase II trials in pediatric acute lymphoblastic leukemia and AML have been initiated based on these investigations *(62)*.

6.2.2. Phase II Studies

Impressive clinical results, pharmacokinetic profile, and lack of untoward toxicities in heavily pretreated pediatric patients with acute leukemias were the primary reasons to initiate phase II trials. These multicenter phase II studies were initiated to fully determine the efficacy of clofarabine for pediatric patients with either AML or ALL. Clofarabine was administered at 52 mg/m^2/d over 5 consecutive days. Among 69 patients (40 with ALL and 29 with AML), a response rate of 28% in ALL and 24% in AML has been reported *(62)*, which is consistent with the observation during phase I investigations. Considering the multiply prior treatments including transplantation and favorable toxicity profile, and durability of responses suggest major utility of this agent in refractory and relapsed acute leukemias in children.

7. SUMMARY

In conclusion, clofarabine is a new dAdo analog that shows effectiveness in both adult and pediatric ALL and AML. The pharmacokinetic and pharmacodynamic profile of the drug in acute leukemias is favorable relative to fludarabine or cladribine and may have prognostic value for its clinical activity.

The toxicity profile of this agent is different from other nucleoside analogs in that it clearly has clinical activity when used as a single agent against adult acute leukemias at tolerable doses. Further, therapeutic strategies combining clofarabine with DNA-damaging agents are likely to allow mechanism-based rationales to be evaluated with minimal likelihood of untoward toxicity. Exploration of additional schedules and combinations along with availability of an oral formulation strongly indicate promising possibilities with this agent.

ACKNOWLEDGMENT

This work was supported in part by grants CA55164, CA57629, and CA81534 from the National Cancer Institute, Department of Health and Human Services, and grant FD-R-002127 from the Food and Drug Administration.

REFERENCES

1. Schiffer CA. Acute myeloid leukemia in adults: where do we go from here? Cancer Chemother Pharmacol 2001;48(suppl 1):S45–S52.
2. Plunkett W, Gandhi V. Purine and pyrimidine nucleoside analogs. In: Giaccone G, Schilsky R, Sondel P, eds. Cancer Chemotherapy and Biological Response Modifiers. Annual 19. Amsterdam: Elsevier Science; 2001:21–45.
3. Gandhi V, Plunkett W, Du M, Ayres M, Estey E. Prolonged infusion of gemcitabine: clinical and pharmacodynamic studies during a phase I trial in relapsed acute myelogenous leukemia. J Clin Oncol 2002;20:665–673.
4. Wijermans P, Lübbert M, Verhoef G, et al. Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol 2000;18: 956–962.
5. Rizzeri DA, Bass AJ, Rosner GL, et al. Phase I evaluation of prolonged-infusion gemcitabine with mitoxantrone for relapsed or refractory acute leukemia. J Clin Oncol 2002;20:674–679.
6. Silverman LR, Demakos EP, Bercedis L, et al. A randomized controlled trial of azacytidine in patients with myelodysplastic syndrome: a study of the Cancer and Leukemia Group B. J Clin Oncol 2002;20:2429–2440.
7. Giles F, Faderl S, Thomas D, et al. Randomized phase I/II study of troxacitabine combined with cytarabine, idarubicin, or topotecan in patients with refractory myeloid leukemias. J Clin Oncol 2003;21:1050–1056.
8. Krance RA, Hurwitz CA, Head DR, et al. Experience with 2-chlorodeoxyadenosine in previously untreated children with newly diagnosed acute myeloid leukemia and myelodysplastic diseases. J Clin Oncol 2001;19:2804–2811.
9. Rubnitz JE, Razzouk BI, Srivastava DK, Pui CH, Ribeiro RC, Santana VM. Phase II trial of cladribine and cytarabine in relapsed or refractory myeloid malignancies. Leuk Res 2004;28:349–352.

10. Crews KR, Gandhi V, Srivastava DK, et al. An interim analysis of a continuous infusion vs a short daily infusion of cytarabine given in combination with cladribine for pediatric acute myeloid leukemia. J Clin Oncol 2002;20:4217–4224.
11. Kornblau SM, Gandhi V, Andreeff HM, et al. Clinical and laboratory studies of 2-chlorodeoxyadenosine ± cytosine arabinoside for relapsed or refractory acute myelogenous leukemia in adults. Leukemia 1996;10:1563–1569.
12. Avramis VI, Wiersma S, Krailo MD, et al. Pharmacokinetic and pharmacodynamic studies of fludarabine and cytosine arabinoside administered as loading boluses followed by continuous infusions after a phase I/II study in pediatric patients with relapsed leukemias. The Children's Cancer Group. Clin Cancer Res 1998;4:45–52.
13. Marcucci G, Byrd JC, Dai G, et al. Phase 1 and pharmacodynamic studies of G3139, a Bcl-2 antisense oligonucleotide, in combination with chemotherapy in refractory or relapsed acute leukemia. Blood 2003;101:425–432.
14. Jehn U, Bartl R, Dietzfelbinger H, Haferlach T, Heinemann V. An update: 12-yr follow-up of patients with hairy cell leukemia following treatment with 2-chlorodeoxyadenosine. Leukemia 2004;18:1476–1481.
15. Dimopoulos MA, Kantarjian H, Weber D, et al. Primary therapy of Waldenstrom's macroglobulinemia with 2-chlorodeoxyadenosine. J Clin Oncol 1994;12:2694–2698.
16. Rai KR, Peterson BL, Appelbaum FR, et al. Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia. N Engl J Med 2000; 343:1750–1757.
17. Tsimberidou AM, McLaughlin P, Younes A, et al. Fludarabine, mitoxantrone, dexamethasone (FND) compared with an alternating triple therapy (ATT) regimen in patients with stage IV indolent lymphoma. Blood 2002;100:4351–4357.
18. Montgomery JA, Shortnacy-Fowler AT, Clayton SD, Riordan JM, Secrist JA III. Synthesis and biologic activity of 2′-fluoro-2-halo derivatives of 9-β-D-arabinofuranosyladenine. J Med Chem 1992;35:397–401.
19. Parker WB, Shaddix SC, Chang CH, et al. Effects of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) adenine on K562 cellular metabolism and the inhibition of human ribonucleotide reductase and DNA polymerases by its 5′-trisphosphate. Cancer Res 1991;51:2386–2394.
20. Carson DA, Wasson DB, Esparza LM, Carrera CJ, Kipps TJ, Cottam HB. Oral antilymphocyte activity and induction of apoptosis by 2-chloro-2′-arabino-fluoro-2′deoxyadenosine. Proc Natl Acad Sci USA 1992;89:2970–2974.
21. Parker WB, Allan PW, Hassan AE, Secrist JA 3rd, Sorscher EJ, Waud WR. Antitumor activity of 2-fluoro-2′-deoxyadenosine against tumors that express *Escherichia coli* purine nucleoside phosphorylase. Cancer Gene Ther 2003; 10: 23–29.
22. Parker WB, Shaddix SC, Rose LM, et al. Comparison of the mechanism of cytotoxicity of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine, 2-chloro-9-(2-deoxy-2-fluoro-β-D-ribofuranosyl)adenine, 2-chloro-9-(2-deoxy-2,2-difluoro-β-D-ribofuranosyl) adenine in CEM cells. Mol Pharmacol 1999;55: 515–520.
23. Parker WB, Bapat AR, Shen JX, Townsend AJ, Cheng YC. Interaction of 2-halogenated dATP analogs (F, Cl, and Br) with human DNA polymerases, DNA primase, and ribonucleotide reductase. Mol Pharmacol 1988;34:485–491.

24. Xie C, Plunkett W. Metabolism and actions of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine in human lymphoblastoid cells. Cancer Res 1995;55: 2847–2852.
25. Xie KC, Plunkett W. Deoxynucleotide pool deletion and sustained inhibition of ribonucleotide reductase and DNA synthesis after treatment of human lymphoblastoid cells with 2-chloro-(2-deoxy-fluoro-β-D-arabinofuranosyl)adenine. Cancer Res 1996;56:3030–3037.
26. Albertioni F, Hassan M, Silbering J, Liliemark J. Kinetics and metabolism of 2-chloro-2′-deoxyadenosine and 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine in the isolated perfused rat liver. Euro J Drug Metab Pharmcokinet 1995;20: 225–232.
27. Huang P, Plunkett W. Phosphorolytic cleavage of 2-fluoroadenine from 9-β-D-arabinofuranosyl-2-fluoroadenine by *Escherichia coli*. Biochem Pharmacol 1987;36:2945–2950.
28. Sjoberg AH, Wang L, Eriksson S. Substrate specificity of human recombinant mitochondrial deoxyguanosine kinase with cytostatic and antiviral purine and pyrimidine analogs. Mol Pharmacol 1998;53:270–273.
29. Lotfi K, Mansson E, Spasokoukotskaja T, et al. Biochemical pharmacology and resistance to 2-chloro-2′-arabinofluoro-2′-deoxyadenosine, a novel analogue of cladribine in human leukemic cells. Clin Cancer Res 1999;5:2438–2444.
30. Gandhi V, Robertson LE, Plunkett W. 2-Chloro-2′-fluoro-arabinosyladenine: pharmacokinetics and action in chronic lymphocytic leukemia cells. Proc Am Assoc Clin Oncol 1993;34:414.
31. Lindemalm S, Liliemark J, Gruber A, et al. Comparison of cytotoxicity of 2-chloro-2′ -arabino-fluoro-2′-deoxyadenosine (clofarabine) with cladribine in mononuclear cells from patients with acute myeloid and chronic lymphocytic leukemia. Haematologica 2003;88:324–332.
32. Huang, P, Chubb S, Plunkett W. Termination of DNA synthesis by 9-β-D-arabinofuranosyl-2-fluoroadenine: a mechanism for cytotoxicity. J Biol Chem 1990; 265:16,617–16,625.
33. Huang P, Plunkett W. Action of 9-β-D-arabinofuranosyl-2-fluoroadenine on RNA metabolism. Mol Pharmacol 1991;39:449–455.
34. Chang CH, Cheng YC. Effects of deoxyadenosine triphosphate and 9-beta-D-arabinofuranosyl-adenine 5′-triphosphate on human ribonucleotide reductase from Molt-4F cells and the concept of "self-potentiation". Cancer Res. 1980;40:3555–3558.
35. Jiang X, Wang X. Cytochrome C-mediated apoptosis. Annu Rev Biochem 2004; 73:87–106.
36. Leoni LM, Chao Q, Cottam HB, et al. Induction of an apoptotic program in cell-free extracts by 2-chloro-2′-deoxyadenosine 5′-triphosphate and cytochrome c. Proc Natl Acad Sci USA1998;95:9567–9571.
37. Genini D, Budihardjo I, Plunkett W, et al. Nucleotide requirements for the in vitro activation of the apoptosis protein-activating factor-1-mediated caspase pathway. J Biol Chem 2000;275:29–34.
38. Genini D, Adachi S, Chao Q, et al. Deoxyadenosine analogs induce programmed cell death in chronic lymphocytic leukemia cells by damaging the DNA and by directly affecting the mitochondria. Blood 2000;96:3537–3543.
39. Takahashi T, Shimizu M, Akinaga S. Mechanisms of the apoptotic activity of Cl-F-araA in a human T-ALL cell line, CCRF-CEM. Cancer Chemother Pharmacol 2002;50:193–201.

40. Waud WR, Schmid SM, Harrison SD Jr, Secrist JA III, Montgomery JA. Preclinical antitumor activity of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine. (Cl-F-ara-A). Nucleosides Nucleotides Nucleic Acids 2000;19:447–460.
41. Takahashi T, Kanazawa J, Akinaga S, Tamaoki T, Okabe M. Antitumor activity of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine, a novel deoxyadenosine analog, against human colon tumor xenografts by oral administration. Cancer Chemother Pharmacol 1999;43:233–240.
42. Qian M, Wang X, Shanmuganathan K, Chu CK, Gallo JM. Pharmacokinetics of the anticancer agent 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine in rats. Cancer Chemother Pharmacol 1994;33:484–488.
43. Kantarjian H, Gandhi V, Kozuch P, et al. Phase I clinical and pharmacology study of clofarabine (chloro-2′-fluoro-deoxy-9-beta-D-arabinofuranosyladenine) in patients with solid and hematologic cancers. J Clin Oncol 2003;21:1167–1173.
44. Chiao N, Bumgardner A, Duvic M. Clofarabine-induced acral erythema during the treatment of patients with myelodysplasia and acute leukemia: report of two cases. Leuk Lymphoma 2003;44:1405–1407.
45. Gandhi V, Kantarjian H, Faderl S, et al. Pharmacokinetics and pharmacodynamics of plasma clofarabine and cellular clofarabine triphosphate in patients with acute leukemias. Clin Cancer Res 2003;9:6335–6342.
46. Kantarjian HM, Estey E, Plunkett W, et al. Phase I-II and cellular pharmacology study of high dose cytosine arabinoside in refractory leukemia. Am J Med 1986;81:387–394.
47. Gandhi V, Plunkett W, Du M, Ayres M, Estey EH. Prolonged infusion of gemcitabine: clinical and pharmacodynamic studies during a phase I trial in relapsed acute myelogenous leukemia. J Clin Oncol 2002;20:665–673.
48. Kantarjian H, Gandhi V, Cortes J, et al. Phase II clinical and pharmacology study of clofarabine inpatients with refractory or relapsed acute leukemias. Blood 2003;102:2379–2386.
49. Gandhi V, Plunkett W, Weller S, et al. Evaluation of the combination of nelarabine and fludarabine in leukemias: clinical response, pharmacokinetics and pharmacodynamics in leukemia cells. J Clin Oncol 2001;19:2142–2152.
50. Gandhi V, Kemena A, Keating MJ, Plunkett W. Cellular pharmacology of fludarabine triphosphate in chronic lymphocytic leukemia cells during fludarabine therapy. Leukemia Lymphoma 1993;10:49–56.
51. Albertioni F, Lindemalm S, Reichelova V, et al. Pharmacokinetics of cladribine in plasma and its 5′-monophosphate and 5′-triphosphate in leukemic cells of patients with chronic lymphocytic leukemia. Clin Cancer Res 1998;4:653–658.
52. Gandhi V, Estey E, Keating MJ, Plunkett W. Fludarabine potentiates metabolism of arabinosylcytosine in patients with acute myelogenous leukemia during therapy. J Clin Oncol 1993;11:116–124.
53. Kemena A, Gandhi V, Shewach D, Keating MJ, Plunkett W. Inhibition of fludarabine metabolism by arabinosylcytosine during therapy. Cancer Chemother Pharmacol 1992;31:193–199.
54. Gandhi V, Estey E, Keating MJ, Chucrallah A, Plunkett W. Chlorodeoxyadenosine and arabinosylcytosine in patients with acute myelogenous leukemia: Pharmacokinetic, pharmacodynamic, and molecular interactions. Blood 1996;87:156–164.
55. Cunningham CC, Nemunaitis J, Senzer N, et al. A phase I dose-finding and safety study of clofarabine in patients with advanced solid malignancies. Proc Am Soc Clin Oncol 2003;22:151;. Abstract 605.

56. Spasokoukotskaja T, Sasvari-Szekely M, Hullan L, Albertioni F, Eriksson S, Staub M. Activation of deoxycytidine kinase by various nucleoside analogues. Adv Exp Med Biol 1998;431:641–645.
57. Cooper T, Ayres M, Nowak B, Gandhi V. Biochemical modulation of cytarabine triphosphate by clofarabine. Cancer Chemother Pharmacol 2005;55:361–368.
58. Faderl S, Gandhi V, O'Brien S, et al. Results of a phase 1–2 study of clofarabine in combination with cytarabine (ara-C) in relapsed and refractory acute leukemias. Blood 2005;105:940–947.
59. Yamauchi T, Nowak B, Keating MJ, Plunkett W. DNA repair initiated in chronic lymphocytic leukemia lymphocytes by 4-hydroperoxycyclophosphamide is inhibited by fludarabine and clofarabine. Clin Cancer Res 2001;7:3580–3589.
60. Jeha S, Gandhi V, Chan KW, et al. Clofarabine, a novel nucleoside analog, is active in pediatric patients with advanced leukemia. Blood 2004;103:784–789.
61. Bonate PL, Craig A, Gaynon P, et al. Population pharmacokinetics of clofarabine, a second-generation nucleoside analog, in pediatric patients with acute leukemia. J Clin Pharmacol 2004;44:1309–1322.
62. Jeha S, Razzouk B, Gaynon P, et al. Clofarabine therapy for the treatment of relapsed or refractory pediatric acute leukemias. Proc Am Soc Clin Oncol 2004; 22:796.

8 L-Nucleosides as Chemotherapeutic Agents

Giuseppe Gumina, PhD,
Youhoon Chong, PhD,
and Chung K. Chu, PhD

CONTENTS

SUMMARY

Nucleoside analogs have been a major class of chemotherapeutic agents. Currently, more than 30 commercially available antiviral and antitumor nucleosides and nucleoside analogs are available. Among nucleoside analogs, nonnatural L-enantiomers have been particularly interesting. The most notable example is lamivudine, which has been playing an important role in the treatment of human immunodeficiency virus and hepatitis B virus infections. The reason for their high therapeutic potential is that, when biologically active, L-nucleosides have proven to possess favorable toxicological, chemical, and biochemical profiles, such as potency, low toxicity, and high metabolic stability. These characteristics have been the key factors in their success as drugs. Currently, two L-nucleosides, lamivudine

From: *Cancer Drug Discovery and Development:*
Deoxynucleoside Analogs in Cancer Therapy
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

Acronyms and Abbreviations

3TC	Lamivudine	IC_{50}	50% Inhibitory concentration
ACV	Acyclovir		
araA	Vidarabine	IdU	Idoxuridine
AZT	Zidovudine, 3′-azido-3′-deoxythymidine	L-2′-Fd4C	2′,3′-Didehydro-2′,3′-dideoxy-2′-fluoro-L-cytidine
cccDNA	Covalently closed circular DNA	L-d4C	2′,3′-Didehydro-2′,3′-dideoxycytidine
d4T	Stavudine, 3′-deoxy-2′,3′-didehydrothymidine	L-d4FC	2, 3′-Didehydro-2′,3′-dideoxy-5-fluorocytidine
dCK	Deoxycytidine kinase	L-d4T	3′-Deoxy-2′, 3′-didehydrothymidine
dCTP	Deoxycytidine triphosphate	L-dA	L-2′-Deoxyadenosine
ddC	Zalcitabine, 2″,3″-dideoxycytydine	L-dC	L-2′-Deoxycytidine
ddI	Didanosine, 2″,3″-dideoxyadenosine	L-dT	Telbivudine, L-thymidine
DHPG	Ganciclovir	L-FMAU	Clevudine
DNA	Deoxyribonucleic acid	L-I-OddU	5′-Iodo-L-dioxolane uridine
dNTP	Deoxynucleoside triphosphate	L-OddC	Troxacitabine
EBV	Epstein-Barr virus	PBM	Peripheral bone marrow
EC_{50}	Half maximal response	PCV	Penciclovir
EdU	Acedurid	RNA	Ribonucleic acid
FCV	Famciclovir	RT	Reverse transcriptase
FTC	Emtricitabine	TFT	Trifluridine
HBV	Hepatitis B virus	TK	Thymidine kinase
HIV	Human immunodeficiency virus	TPs	Triphosphates
		val-ACV	Valaciclovir
		val-L-dC	Valtorcitabine
HPMPC	Cidofovir	WHV	Woodchuck hepatitis virus

and emtricitabine, are available for the treatment of human immunodeficiency virus and hepatitis B virus infections, and several other analogs, such as clevudine and troxacitabine, are in advanced clinical trial development stages. Among these, troxacitabine, a true chain terminator, is the first L-nucleoside endowed with promising antitumor activity against leukemia as well as solid tumors. This chapter reviews the important L-nucleosides used in therapy as well as compounds in development.

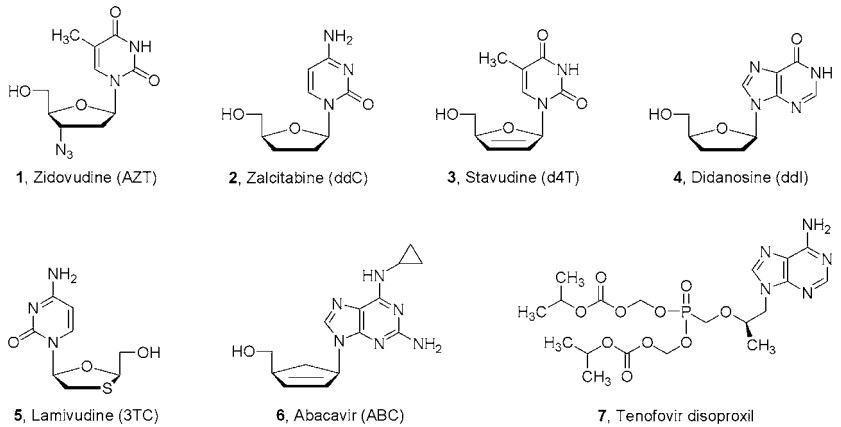


Fig. 1. Anti-HIV nucleoside/nucleotide analogs approved by the Food and Drug Administration.

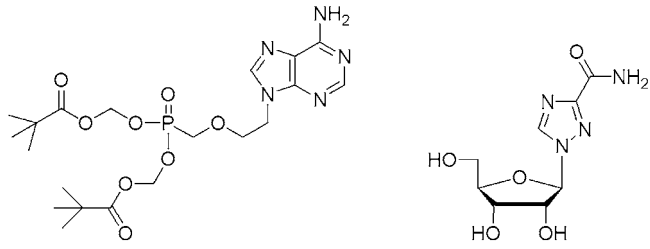
Key Words: Anticancer agents; antiviral agents; deoxycytidine kinase; L-nucleosides; troxacitabine.

1. INTRODUCTION

Nucleoside analogs have been effective weapons in the fight against viral infections and tumors. In the antiviral field, over 20 nucleosides are currently used in therapy against a wide array of viruses. These agents include the nucleoside/nucleotide reverse transcriptase inhibitors used in the treatment of HIV infection: zidovudine or AZT *(1)*, zalcitabine *(2)*, stavudine (d4T) *(3)*, didanosine (ddI) *(2)*, lamivudine (3TC) *(4)*, abacavir *(5,6)*, and the recently approved tenofovir isoproxil *(7)* (Fig. 1). 3TC is also the drug of choice for the treatment of HBV infection, chronically carried by approx 350 million patients worldwide.

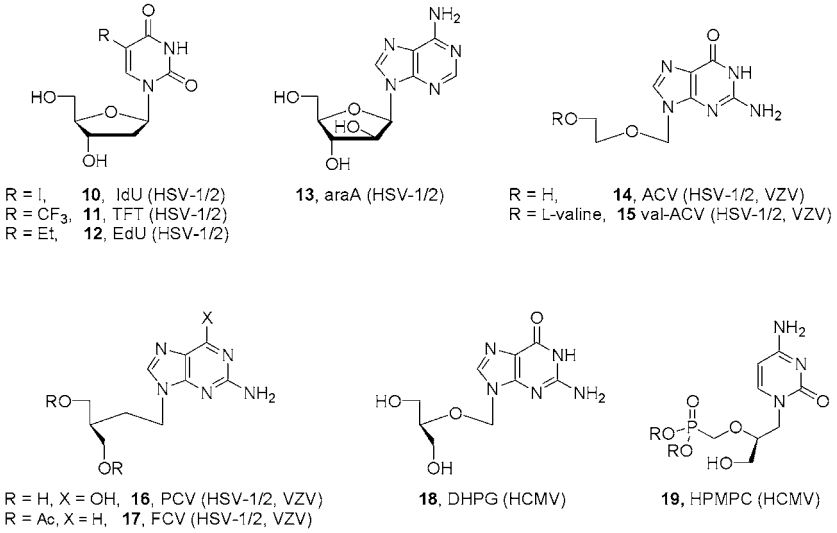
3TC has recently been joined by the acyclic nucleotide analog adefovir dipivoxil (Hepsera™) *(8–10)* (Fig. 2) as an anti-HBV agent. Ribavirin *(11)* (Fig. 2), a nucleoside analog containing a triazole base moiety, is a viral ribonucleic acid (RNA) polymerase inhibitor active against respiratory syncytial virus and, in combination with interferon-α, is the only approved nucleoside for the treatment of hepatitis C infection.

Ten more nucleoside analogs (idoxuridine, trifluridine, acedurid, vidarabine, acyclovir, valaciclovir, penciclovir, famciclovir, ganciclovir, and cidofovir) are currently available for the treatment of diseases caused by viruses belonging to the herpes family, particularly herpes simplex types 1 and 2, cytomegalovirus, and varicella zoster virus (Fig. 3).



8, Adefovir dipivoxil **9**, Ribavirin

Fig. 2. The anti-HBV drug adefovir dipivoxil and the anti-hepatitis C virus drug ribavirin.



R = I, **10**, IdU (HSV-1/2)
R = CF_3, **11**, TFT (HSV-1/2)
R = Et, **12**, EdU (HSV-1/2)

13, araA (HSV-1/2)

R = H, **14**, ACV (HSV-1/2, VZV)
R = L-valine, **15** val-ACV (HSV-1/2, VZV)

R = H, X = OH, **16**, PCV (HSV-1/2, VZV)
R = Ac, X = H, **17**, FCV (HSV-1/2, VZV)

18, DHPG (HCMV)

19, HPMPC (HCMV)

Fig. 3. Nucleoside analogs used in antiherpes therapy.

A number of other nucleoside analogs are currently undergoing preclinical studies or clinical trials. These include an impressive number of L-nucleosides, "unnatural" analogs of those naturally found in nucleic acids. Emtricitabine (FTC), structurally related to 3TC, is in phase III clinical trials as an anti-HBV agent and has recently been approved for anti-HIV therapy, developed by Gilead. L-dT and val-L-dC are promising anti-HBV agents developed by Idenix Pharmaceuticals, currently in phase III and II, respectively. L-FMAU is an anti-HBV agent characterized by extremely low toxicity, currently in phase III clinical trials developed by

Gilead. L-2′-Fd4C is a potent anti-HBV agent developed by Pharmasset Incorporated and is currently in preclinical evaluation. Elvucitabine (L-d4FC), developed by Achillion, is in phase II clinical trials as an anti-HIV as well as anti-HBV agent. L-I-OddU is undergoing preclinical evaluation as an anti-EBV agent by Achillion Pharmaceuticals. L-OddC is the first L-nucleoside endowed with promising selective antitumor activity, currently in phase III clinical trials, and was developed by Shire.

The efficacy of nucleoside analogs depends on their ability to mimic natural nucleosides, thus interacting with viral or cellular enzymes and inhibiting critical processes in the metabolism of nucleic acids. For this reason, it has been believed that only D-analogs, which possess the same stereochemistry as natural nucleosides, could effectively interact and inhibit metabolic enzymes. This paradigm has proven not to be true with the discovery of the antiviral activity of L-oxathiolane nucleosides *(12–14)*, which eventually led to the approval of 3TC as a therapeutic agent and the development of FTC and L-FMAU, among others (Fig. 4). Favorable features of L-nucleosides include an antiviral activity comparable and sometimes greater than their D-counterparts, a more favorable toxicological profile, and greater metabolic stability. The synthesis and biology of L-nucleosides have been the object of previous reviews *(15–17)*. In this chapter, we focus on the most promising L-nucleosides currently in preclinical or clinical studies.

2. LAMIVUDINE

Although the synthesis of the first L-nucleoside was reported by Smejkal and Sorm in 1964 *(18)*, the discovery of the important biological features of this class of compounds began with the discovery of the anti-HIV activity of (±)-BCH-189 and the realization that the antiviral activity resides both at the (–)-L- (3TC) and (+)-D- (BCH-189) enantiomers. More surprisingly, 3TC (**5**, Fig. 1) is more active (EC_{50} 0.002 μ*M* in HIV-1-infected PBM cells and 0.24 μ*M* in HIV-2-infected ROD cells) than its (+)-D counterpart (EC_{50} 0.2 μ*M* in PBM cells). Furthermore, the cytotoxicity resides mainly in (+)-BCH-189 (IC_{50} 2.7 μ*M* in PBM cells), whereas 3TC is virtually nontoxic *(4,19)*. Also, as an anti-HBV agent, 3TC is more potent (EC_{50} 0.01 μ*M* in HepG2 2.2.15 cells) *(20)* than its (+)-enantiomer.

First isolated by resolution of the racemic mixture, the structural characterization of each enantiomer was possible only after the stereospecific synthesis of each enantiomer. The synthesis of the D-isomer **33** has been accomplished starting from D-mannose **28** *(21)* or D-galactose **29** *(22)* via the 1,6-thioanhydro sugars **30** or **31** (Scheme 1). These were converted to the key oxathiolane acetate **32** by a series of reactions, including an oxidative cleavage of the diol functionality. Coupling of intermediate **32** with protected bases

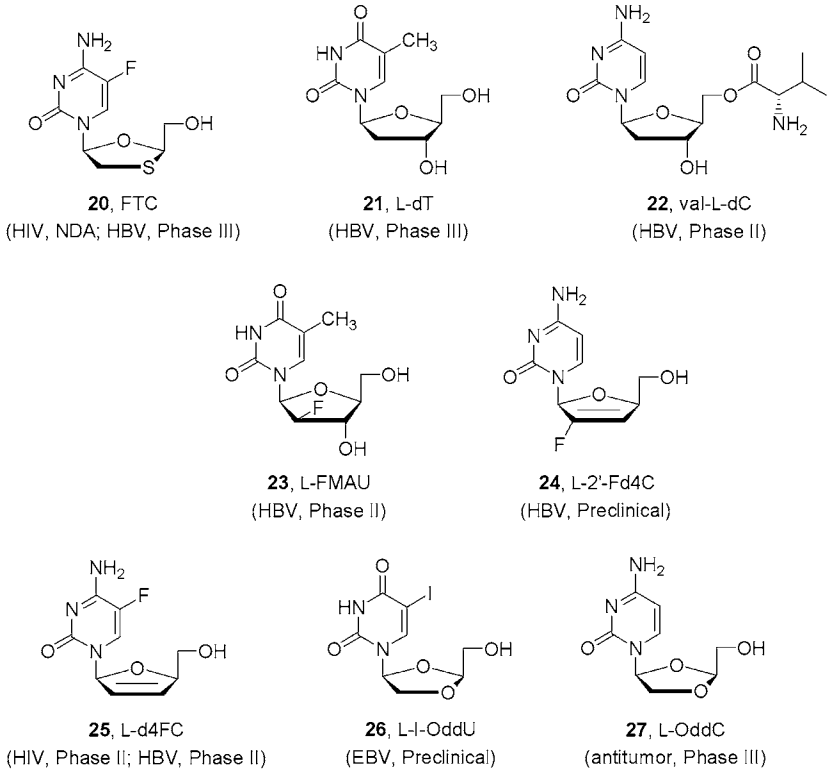
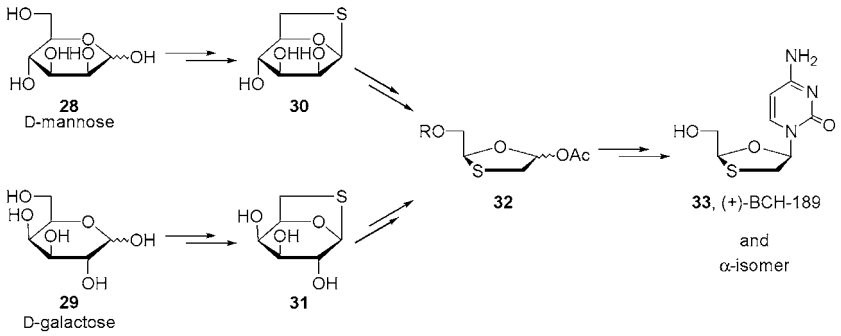


Fig. 4. Nucleoside analogs currently undergoing preclinical or clinical studies.

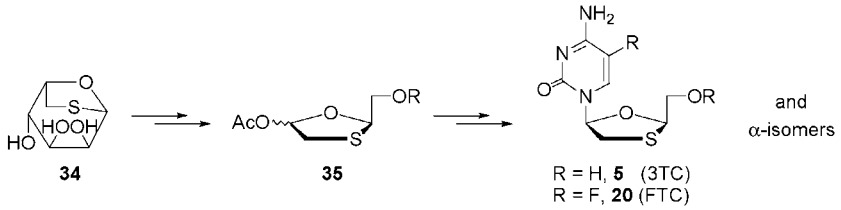
in Vorbrüggen conditions followed by further elaboration/deprotection reactions afforded a series of D-nucleosides, including (+)-BCH-189 **33**, and their α-isomers.

3TC **5** and its congeners, such as the 5-fluoro analog FTC **19**, were synthesized from 1,6-thioanhydro-L-gulose **32** using a similar strategy (Scheme 2) *(23)*.

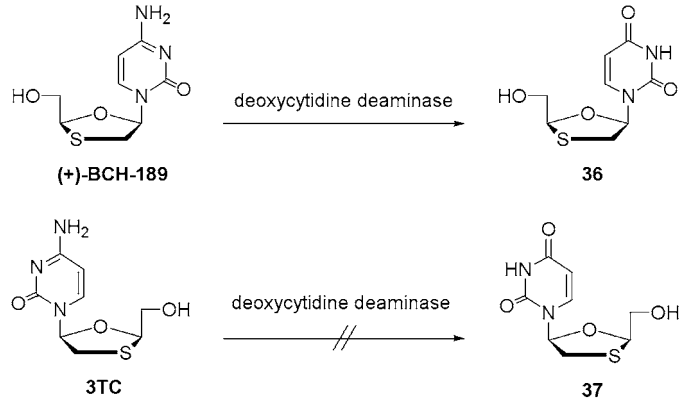
Although the mechanistic basis for the stereochemical selectivity and differential toxicity of the isomeric 3TC compounds is not completely understood, the different antiviral activity and toxicological profiles of (+)-BCH-189 and (–)-BCH-189 (3TC) can be partially explained based on their metabolic and pharmacodynamic characteristics. From a pharmacokinetic point of view, 3TC is resistant to deamination, whereas (+)-BCH-189 is deaminated to inactive 2′-deoxy-3′-thiauridine **34** by deoxycytidine deaminase (Scheme 3) *(24)*.



Scheme 1. Synthesis of (+)-BCH-189, **33**.

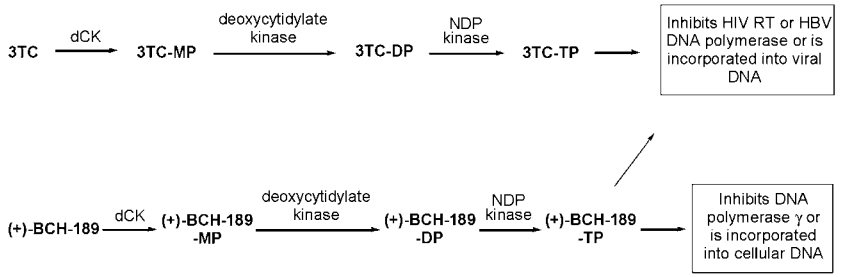


Scheme 2. Synthesis of 3TC **5** and FTC **20**.



Scheme 3. Catabolism of 3TC and (+)-BCH-189.

Both isomers are substrates of dCK, which converts them to the corresponding monophosphates (*25*); further phosphorylations to the di- and triphosphates are accomplished by deoxycytidylate and nucleoside diphosphate kinases, respectively (Scheme 4). Because the first phosphorylation is



Scheme 4. Metabolic activation of 3TC and (+)-BCH-189.

the rate-limiting step in their conversion to the active TPs, the preferential affinity of 3TC to dCK may explain its higher potency over its enantiomer *(26)*. Both TPs act as dCTP analogs, competitively inhibiting HIV RT and HBV DNA polymerase, and both can be incorporated into viral DNA, causing chain termination. However, the TP of (+)-BCH-189 also inhibits cellular DNA polymerases-β and -γ, resulting in toxic effects. In particular, 3TCTP has a high IC_{50} value (43.8 μ*M*) against human DNA polymerase-γ at concentration of dCTP equal to its K_m value, whereas the TP of (+)-BCH-189 has an IC_{50} of 0.005 μ*M* *(27)*.

From a pharmacodynamic point of view, the TPs of both 3TC and (+)-BCH-189 can interact effectively with HIV RT *(28)*. When the base moiety and the 5′-hydroxymethyl group are considered reference points, the furanose rings of each pair of the D- and L-nucleosides are superimposable even though the furanose ring does not superimpose atom by atom. The binding mode of 3TCTP to the dNTP binding site of RT is very similar to those of their natural D-nucleoside TPs except that their β-L-configurations cause the sugar portion to be relatively shifted toward Met184, as suggested by Sarafianos et al. *(29)*.

Treatment with 3TC rapidly induces resistance both in HIV- and HBV-infected individuals. In the case of HIV, this is caused by a single point mutation, with the substitution of valine (or isoleucine) for methionine at position 184 within the YMDD motif of HIV-1 RT. The substitution of valine with a bulkier amino acid prevents the mutant enzyme from accommodating 3TCTP (Fig. 5). The efficacy of 3TC against M184V mutants is reduced over 1000-fold *(30)*.

An analogous point mutation has been detected in position 204 of HBV DNA polymerase. (Position 204 [rtM204V/I], corresponding to the M184V/I mutation of HIV RT, has also been named 552, 550, 539, or 549, depending on different HBV genotypes considered. In this review, we use the recently proposed genotype-independent nomenclature system of ref. *31*.) However, other mutations outside the YMDD motif, such as rtL180M (previously

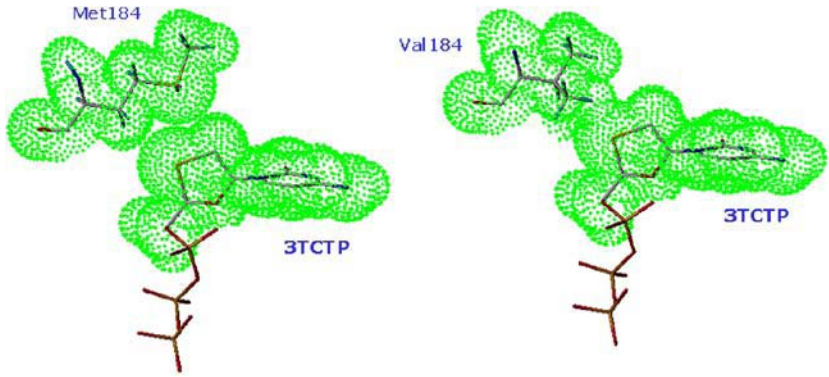


Fig. 5. Interactions between 3TCTP and wt or M184V RT.

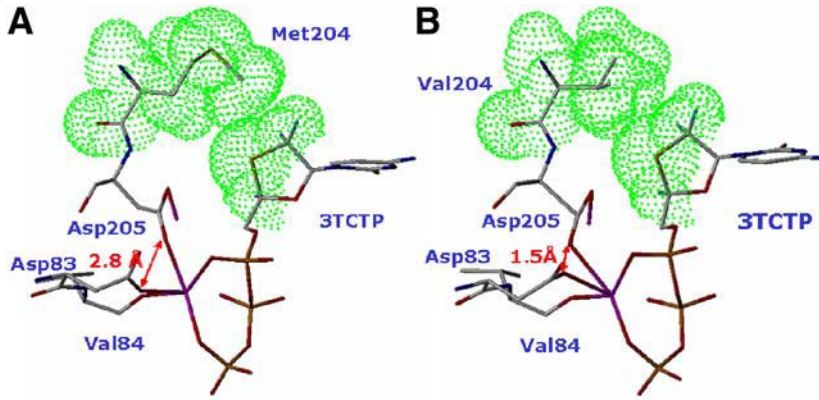
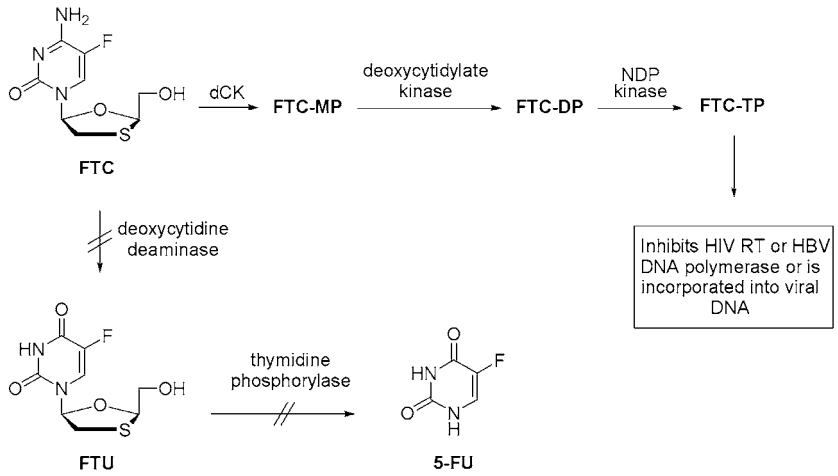


Fig. 6. Interactions between 3TCTP and wt or rtM204V HBV polymerase.

described as L526M), seem to enhance viral replication of the rtM204V-mutated HBV DNA polymerase, which indicates that mutations outside the YMDD motif are an essential part in causing clinical viral resistance *(32)*. These mutations often confer resistance to other anti-HBV nucleoside analogs. For instance, the rtL180M mutation causes resistance to penciclovir and, like the V553I, to L-FMAU *(33)*.

As shown in Fig. 6, the 3TC/wild-type (wt) HBV polymerase complex is stabilized by extensive hydrophobic interactions between Met204 and the sugar moiety of the nucleoside (Fig. 6A) *(34)*. Resistance to 3TC can be explained with a large movement of 3TCTP toward Val204 (Fig. 6B), which causes an unfavorable close contact (1.5 Å) between two negatively



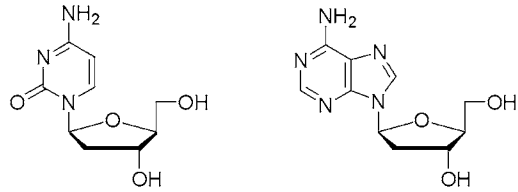
Scheme 5. Metabolism of FTC.

charged aspartic acid residues (Asp83 and Asp205), repulsive electrostatic interactions, and thereby poor binding energy.

Considering the rapid occurrence of resistance on treatment with 3TC, its success as an anti-HIV drug would hardly be explained. However, M184V mutants are characterized by suboptimal fitness, with a much lower replication ability compared to the wt. Besides, the combination of 3TC with other nucleoside RT inhibitors has been shown to reduce the emergence of 3TC-resistant strains *(35)*. This fact is the basis of combination therapy, in which 3TC is used in combination with AZT (Combivir®) *(36)*, and more effective triple combinations with AZT and protease inhibitors such as nelfinavir *(37)*, indinavir *(38)*, and ritonavir *(39)* or AZT and abacavir (Trizivir®) *(40)*.

3. EMTRICITABINE

FTC (**20**, Fig. 4 and Scheme 2), the 5-fluorinated analog of 3TC, resembles the parent compound in antiviral and resistance profile as well as cellular metabolism. Like 3TC, the L-(–)-enantiomer is 100-fold more potent (EC_{50} 0.008 μ*M* in HIV-1-infected PBM cells) than the D-(+)-isomer *(41)* and has the same anti-HBV potency as 3TC (EC_{50} 0.01 μ*M* in HepG2 2.2.15 cells) *(42)*. The metabolic activation of FTC is similar to that of 3TC (Scheme 5). FTC has a bioavailability of 73% with oral administration and penetrates the blood-brain barrier. Pharmacokinetic studies indicated that the renal excretion of the racemic FTC is the major route of elimination *(43)*. Although its D-enantiomer is slowly deaminated to the 5-fluorouracil analog (+)-FTU via cellular cytidine-deoxycytidine deaminase, (–)-FTC is



38, L-dC **39**, L-dA

Fig. 7. Selective anti-HBV agents L-dC and L-dA.

essentially resistant to this enzyme *(43)*. Neither enantiomers of FTU are metabolized by thymidine phosphorylase to cytotoxic 5-fluorouracil *(43)*.

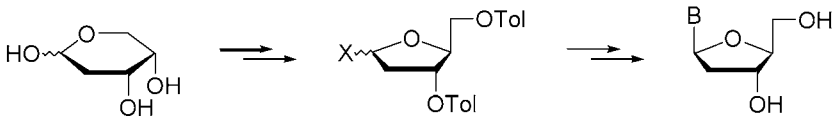
Like 3TC, FTC shows synergistic anti-HIV activity when used in combination with AZT or d4T, but only additive activity when in combination with ddI or ddC *(44)*. None of these combinations shows additive toxicity in cell culture. FTC has recently been approved as an anti-HIV drug and is currently undergoing phase III clinical trials as anti-HBV agent.

4. TELBIVUDINE AND VALTORCITABINE

Among L-2′-deoxynucleosides, L-dT (**21,** Fig. 4), L-dC and L-dA (**38 and 39**, respectively, Fig. 7) showed selective antiviral activity against HBV, with EC_{50} values of 0.24, 0.19, and 0.10–1.9 μ*M*, respectively, but not against HIV-1, herpes simplex virus types 1 and 2, varicella zoster virus, EBV, human cytomegalovirus, adenovirus type 1, influenza A and B viruses, measles virus, parainfluenza virus type 3, rhinovirus type 5, or respiratory syncytial virus at concentrations as high as 200 μ*M (45)*.

In the structure-activity relationship study of L-dC analogs, substitution of a halogen atom at position 5 of the cytosine ring decreased the potency against HBV but retained the specificity for HBV. In contrast, analogs of L-dC lacking the 3′-OH group on the deoxyribose sugar, such as L-ddC, 3TC, and L-d4C, lose antiviral selectivity for HBV and also showed activity against HIV. Similarly, replacement of the 3′-OH group with a 3′-fluoro moiety eliminated the antiviral selectivity, but the antiviral potency against HBV and HIV was retained.

The results of structure-activity relationships for the L-dT and L-dA series are similar to those observed for the L-dC series. The selective anti-HBV activity of L-2′-deoxynucleosides was lost by removal or substitution of the 3′-OH group. L-dT and L-dC were phosphorylated by TK and dCK in human and woodchuck, which means that the activity of a potential combination of the two agents would not be limited by competition for the same metabolic enzyme. L-dTTP and L-dCTP inhibited WHV DNA polymerase (IC_{50} 0.24 and 1.8 μ*M*, respectively) *(46)*.



40 X = Cl, **41** X = OAc, **42** **43**

Scheme 6. Synthesis of L-2′-deoxynucleosides **43**.

In an in vivo study of a chronically WHV-infected woodchuck model, oral administration of L-dT, L-dC, and L-dA at a dose of 10 mg/kg/d significantly inhibited WHV DNA replication. Serum WHV DNA levels (HBV viremia) decreased in the L-dT-treated animals by as much as 8 logs, in which WHV DNA levels of pretreated animals were 10^{11} genome equivalents/milliliter, and during the treatment the levels decreased to 10^3 or detection limit (300 genome equivalents/milliliter) by quantitative polymerase chain reaction method. After drug withdrawal, viral rebound reached near-pretreatment levels between wk 4 and wk 8. During treatment of L-dC and L-dA, serum WHV DNA levels decreased by 6 logs and 1.5 logs, respectively, which was also followed by viral rebound within the first week posttreatment. Currently, L-dT and val-L-dC (valtorcitabine, a prodrug of L-dC) are in phase III and II clinical studies by Novirio Pharmaceuticals as anti-HBV agents.

For the syntheses of L-2′-deoxynucleosides, 2-deoxy-L-ribose **40** *(47)* (Scheme 6) is converted to 1-chloro-2-deoxy-3,3-di-*O*-*p*-toluoyl-α-L-*erythro*-pentofuranose **41** *(48)*or 1-*O*-acetyl-2-deoxy-3,3-di-*O*-p-toluoyl-α-L-L-*erythro*-pentofuranose **42** *(49)*, which can be condensed with appropriate bases to give the corresponding nucleosides **43**.

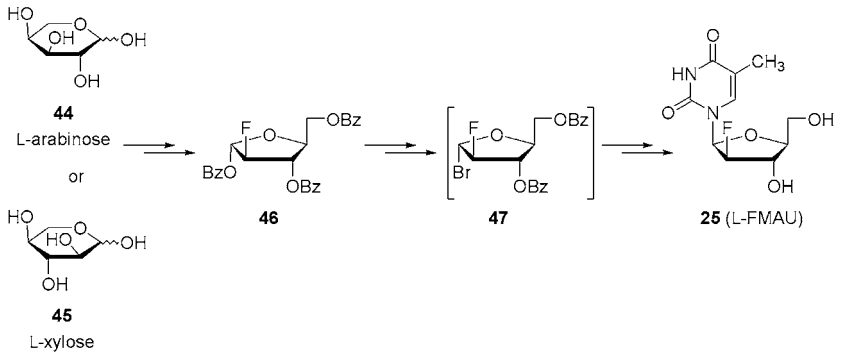
5. CLEVUDINE

Among carbohydrate ring substituents that confer favorable biological features to nucleoside analogs, fluorine has been one of the most useful. 1-(β-D-2-Fluoro-2-deoxyarabinofuranosyl)-5-methyluracil (D-FMAU) showed promising anti-HBV activity, but its development was interrupted in phase I clinical trials because of severe neurological toxicity *(50)*. Its enantiomer L-FMAU (clevudine **25**, Fig. 4), on the other hand, is much more active (EC_{50} 0.1 μ*M* in HepG2 2.2.15 cells) *(51,52)* and nontoxic. Table 1 shows the biological evaluation of the two isomers. The low toxicity of L-FMAU was confirmed by growth inhibition studies in a variety of human cell lines and is clearly shown by its high selectivity index (>2000). In addition, L-FMAU does not inhibit growth of human bone marrow progenitor cells.

Unlike L-ddC analogs, L-FMAU is inactive against HIV. Interestingly, it shows antiviral activity against EBV, with an EC_{90} value of approx 5 μ*M* *(53)*.

Table 1
Anti-HBV and Anti-EBV Activities of L- and D-FMAU

	Anti-HBV activity in 2.2.15 cells (EC_{50}; μM)	*Anti-EBV activity in H1 cells (EC_{90}; μM)*	*Growth inhibition (ID_{50}; μM) in:*				*Selectivity index*	
			MT2	*CEM*	*H1*	*2.2.15*	*2.2.15*	*H1*
L-FMAU	0.1	5 ± 0.8	100	>100	913 ± 70	>200	>2000	183
D-FMAU	2.0	0.1 ± 0.02	8–9	0	<10	50	25	>100

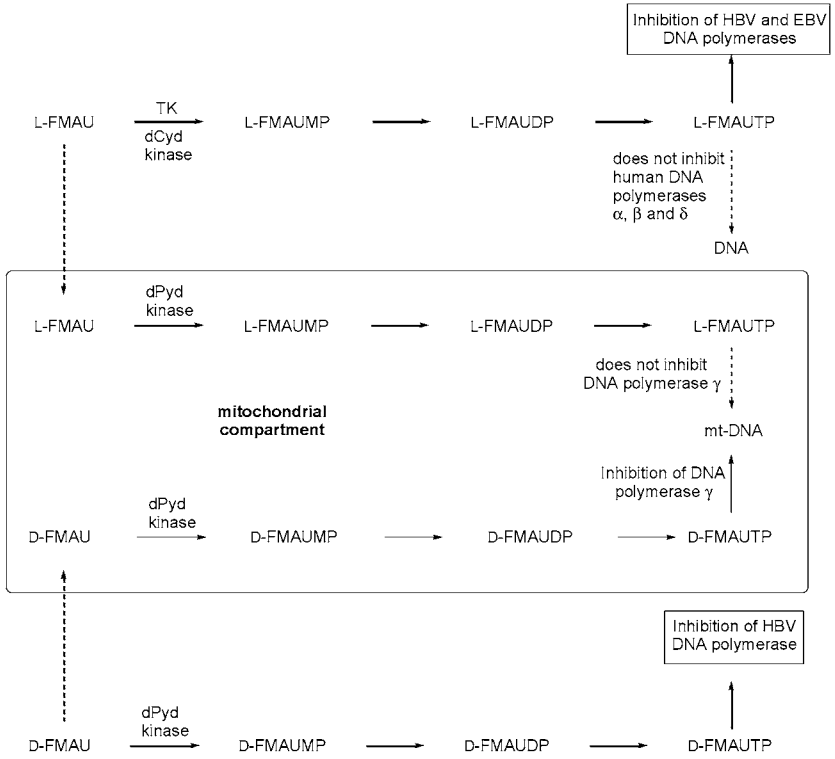


Scheme 7. Synthesis of L-FMAU **25**.

A convenient synthesis of L-FMAU (Scheme 7) and its congeners was accomplished by Chu and coworkers starting from L-arabinose **44** *(54)* or L-xylose **45** *(55)*. Thus, L-arabinose or L-xylose was converted to fluorinated L-arabinose derivative **46**, which was condensed to persilylated thymine via the bromosugar intermediate **47**.

The differential activity and toxicity between D- and L-FMAU have been explained based on the metabolism of the two isomers (Scheme 8). Both isomers are activated intracellularly to their TPs, which are the active species. The first phosphorylation is the rate-limiting step and is catalyzed by TK and dCK for the L-isomer *(56)* and by deoxypyrimidine kinase for the D-isomer. The following phosphorylations can be accomplished by various cytosolic enzymes. Although both TPs inhibit HBV DNA polymerase [L-FMAUTP also inhibits EBV DNA polymerase *(57)*, but it is not incorporated into HBV or cellular DNA *(53)*], D-FMAUTP also inhibits DNA polymerase-γ, causing mitochondrial toxicity *(58)*.

In the in vivo duck model, L-FMAU has proved to decrease the level of viremia effectively, both in short-term and prolonged administration *(59)*. In in vivo studies in the woodchuck model, treatment with L-FMAU for 4 wk significantly reduced viremia, antigenemia, intrahepatic WHV replication, and expression of WHV core antigen in a dose-dependent manner *(60)*. Toxicological studies suggest no apparent toxicity for 30 d at 50 mg/kg/d in mice and for 3 mo in woodchucks at 10 mg/kg/d *(61)*. The development of a new and effective anti-HBV drug is critical because long-term therapy with 3TC causes resistance, treatment with α-interferon is effective in only 20–30% of patients, and there are a number of limitations such as numerous side effects, high cost, and administration by injection *(62)*. In addition, as a reverse transcription inhibitor 3TC inhibits viral replication but not cccDNA, which functions as the viral DNA reservoir as well as template for the synthesis of new DNA *(62)*. At this time, it is not known

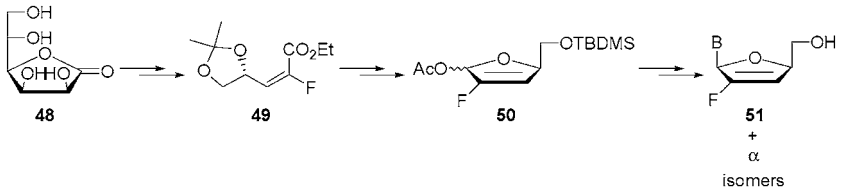


Scheme 8. Cellular metabolism of D- and L-FMAU.

whether cccDNA is lost during mitosis or transmitted to daughter cells along with host chromosomes *(63)*. Treatment of woodchucks infected with WHV with L-FMAU causes a decrease in the average cccDNA copy number in infected cells *(63)*. This fact may support the first hypothesis and suggesting possible use of L-FMAU in combination therapy with 3TC. Currently, L-FMAU is in phase II clinical trials by Triangle Pharmaceuticals and Bukwang Pharmaceuticals as an anti-HBV agent.

6. L-2′-Fd4C

Unsaturated 2′-fluorinated nucleosides (**51**, Scheme 9) revealed potent anti-HIV and anti-HBV activities. Also in this series, L-isomers are more potent or less toxic than their natural counterparts. Thus, L-2′-Fd4C (**24**, Fig. 4) and its 5-fluoro congener L-2′-Fd4FC are potent anti-HIV (EC_{50} 0.51 and 0.17 μ*M* in PBM cells, respectively) *(64)* and highly potent anti-HBV (EC_{50} 2 and 4 n*M*, respectively, in HepG2 cells) *(65,66)* agents with no toxicity up to 100 μ*M* in PBM, Vero, and CEM cells. Their D-counterparts also



Scheme 9. Synthesis of L-2′-fluoro-2′,3′-unsaturated nucleosides **51**.

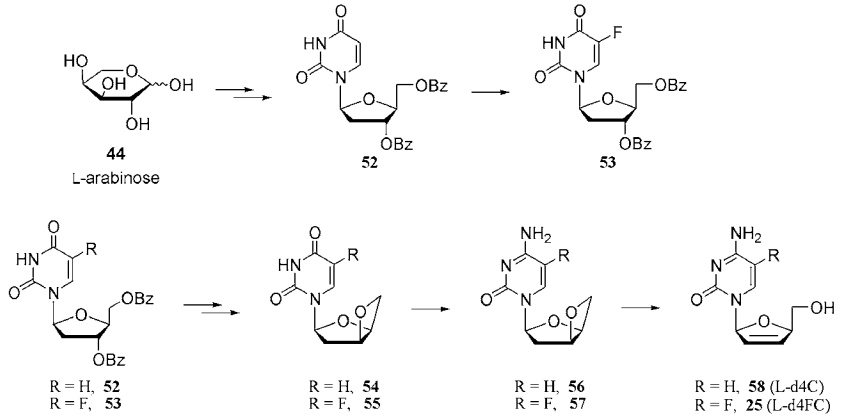
show from moderate to potent anti-HIV (EC_{50} 0.37 and 1.1 μ*M*, respectively) and HBV (EC_{50} 2.0 and 0.05 μ*M*, respectively) activity *(67)*. The adenine derivative L-2′-Fd4A is moderately active against HIV (EC_{50} 1.5 μ*M*) and HBV (EC_{50} 1.7 μ*M*) *(64)*, whereas D-2′-Fd4A displays anti-HIV activity with an EC_{50} of 0.44 μ*M* *(67)*. Interestingly, however, the D-adenine as well as the hypoxanthine (EC_{50} 1.0 μ*M*) analogs did not show crossresistance with 3TC when tested against 3TC-resistant M184V Pitt virus *(67)*. In view of the importance of 3TC in combination therapy for the treatment of HIV infection, it is crucial to develop new anti-HIV compounds active against 3TC-resistant viral strains.

The synthesis of L-L-2′-fluoro-2′,3′-unsaturated nucleosides was accomplished starting from L-gulonic-γ-lactone **48** via the acyclic fluorinated intermediate **49** (Scheme 9), which was cyclized, protected, and acetylated to the key intermediate **50**. Condensation of **68** with various persilylated bases followed by derivatization/deprotection afforded the target nucleosides **51** along with their α-isomers *(64)*.

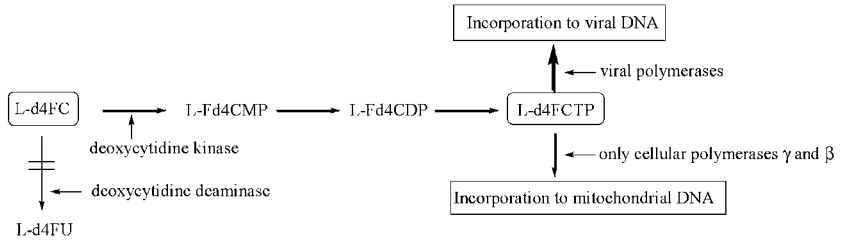
7. ELVUCITABINE

Although L-d4T *(68)*, the unnatural isomer of d4T, shows no activity against HIV and HBV, other "L-d4-type" nucleosides with cytosine and 5-fluorocytosine bases, L-d4C **58** and L-d4FC **22** *(69)*, showed potent activity against HIV (EC_{50} 1 μ*M* and 0.09 μ*M* for L-d4C and L-d4FC, respectively) and HBV (EC_{50} 0.01 μ*M* and 0.002 μ*M* for L-d4C and L-d4FC, respectively). L-d4C and L-d4FC were synthesized by Lin et al. *(69)* from L-arabinose **44** via L-3′,5′-dibenzoyl-2′-deoxyuridine **52**, which was transglycosylated to give L-3′,5′-dibenzoyl-2′-deoxy-5-fluorouridine **53** (Scheme 10). These uridines were converted to the corresponding L-3′,5′-anhydro-derivatives **54** and **55**, which were converted to cytosine derivatives **56** and **57** by the triazole method followed by treatment with a Brønsted base to give L-d4C **5 8** and L-d4FC **25**.

Like 3TC, L-d4FC could be phosphorylated to mono-, di-, and TP metabolites, and L-d4FCTP acts as a competitive inhibitor for viral polymerase and a terminator of viral DNA chain elongation *(70)* (Scheme 11). The enzyme responsible for the phosphorylation of L-d4FC is cytosolic dCK. When L-d4FC is incubated with human dCK, it serves as a substrate, but with



Scheme 10. Synthesis of L-2′,3′-didehydro-2′,3′-dideoxynucleosides **58** and **2**.



Scheme 11. Cellular metabolism of L-d4FC.

human mitochondrial deoxypyrimidine nucleoside kinase, no phosphorylation of L-d4FC occurs *(71)*. Moreover, L-d4FC is not susceptible to deoxycytidine deaminase.

The relative molecular activity of the 5′-TPs of L-d4FC, L-ddFC, and ddC on DNA strand elongation by HIV-1 RT and human DNA polymerases-α, -β, -γ, and -ε has been evaluated *(72)*. The IC_{50} of L-d4FCTP are 0.20, 1.8, and 4.0 μ*M* for HIV RT, polymerase-γ and polymerase-β, respectively, and L-d4FCTP has no or little inhibitory effect on polymerase-ε or polymerase-α. By comparison, the K_i values of 3TCTP have been reported as 1, 0.01, and 1.2 μ*M* for HIV RT, polymerase-γ, and polymerase-β, respectively. L-d4FCTP shows inhibitory effect against mitochondrial DNA polymerase (polymerase-γ). A combination study of L-d4FC showed synergistic activity with AZT or d4T and additive activity with ddI or ddC. A combination with L-d4FC decreased the drug-induced decreases in mitochondrial DNA content but showed additive toxicity toward CEM cells.

In an in vitro study, secretion of HBV DNA from 2.2.15 cells was decreased by 50% at a concentration of 1 n*M* by L-d4FC, which is approx 15 times more potent than 3TC. The level of formation of phosphorylated metabolites in cells is higher for L-d4FC than for 3TC, and L-d4FC metabolites have a much longer retention time in HepG2 cells than 3TC metabolites *(73)*. These results suggest that the dosage required to suppress HBV replication in patients should be lower for L-d4FC than for 3TC.

Short-term administration of L-d4FC transiently inhibited viral replication in vivo in experimentally infected ducklings. At 3 d after inoculation of the virus, the drug was administered intraperitoneally at 25mg/kg/d for 5 d. The inhibition of the peak of viremia reached 97% for L-d4FC. However, after drug withdrawal, a relapse of viral replication occurred. A long-term administration study could not prevent the relapse of viral replication after drug withdrawal, and viral cccDNA was still detected at the end of the treatment, indicating the absence of viral clearance from infected hepatocytes. A 4-wk administration of L-d4FC in acutely infected ducklings not only suppressed viral DNA synthesis but also inhibited viral antigen expression in the liver. No hepatocytes or biliary cells expressing the viral pre-S proteins were detected in the liver of L-d4FC-treated animals.

In a long-term administration study of 3TC, the inhibition of the peak of viremia reached only 80% for 3TC, and viral antigens (pre-S envelope proteins) were expressed up to 90%. However, a significant repulse of viral replication did not occur after 3TC withdrawal. Interestingly, the greater the antiviral effect was during therapy, the higher the rebound of viral replication was after drug withdrawal. This suggested that the suppression of viral replication in acutely infected animals is not associated with a strong specific antiviral response, leading to a delayed peak of viral replication after drug withdrawal.

A new study of L-d4FC was reported in woodchucks chronically infected with WHV *(74)*. L-d4FC was administered at 4 mg/kg to woodchucks for 5 consecutive days as an induction therapy followed by a maintenance therapy three times weekly for 8 more weeks, and the results were compared with those with 3TC at a dose of 10 mg/kg by the same schedule. During the 3TC treatment period, both assays (WHV endogenous polymerase activity and DNA) showed a first phase of viremia drop followed by successive phases of a rise and a drop of viremia. In contrast, in the L-d4FC treatment group a rapid and significant decrease in viremia to the level of detection of the WHV endogenous polymerase activity assay (38.7-fold decrease) or close to the limit of detection of the WHV DNA assay (31-fold decrease) was observed and maintained until the end of therapy. However, after drug withdrawal, viremia levels in all groups of treated woodchucks returned to pretherapy values in 4–6 wk for the L-d4FC group and in 10 wk for the

3TC group. Prolonged L-d4FC treatment for 15 wk was not able to achieve clearance of intrahepatic viral cccDNA. This was associated with the absence of a decline in the number of infected hepatocytes and the WHV surface antigen titer in serum. Residual viral cccDNA is an active template for viral genome expression, allowing the reinitiation of viral replication when therapy is stopped. Analysis of liver histology showed a decrease in the inflammatory activity during chronic hepatitis and a stability of liver fibrosis in woodchucks treated with L-Fd4C; in control animals, hepatitis activity increased, and the level of liver fibrosis decreased. An electron microscopy study showed the absence of ultrastructural changes of hepatic mitochondria, biliary canaliculi, and bile ducts. However, loss of weight was observed in all animals, and skin hyperpigmentation related to an accumulation of melanin was reported during L-d4FC treatment and slowly disappeared after drug withdrawal. Although L-d4FC and 3TC administration inhibited viral replication to different extents and acted on liver disease, 4 of 10 in the L-d4FC group, 1 of 4 in the 3TC group, and 1 of 6 in the control group died of hepatocellular carcinoma during the study period.

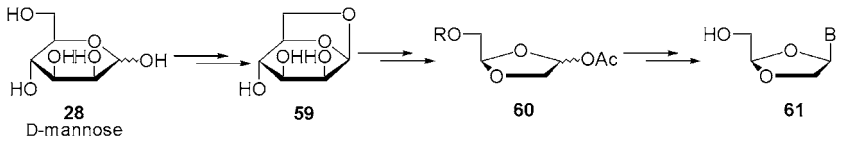
From these continuous studies of L-d4FC, it could be concluded that clearance of viral cccDNA and infected cells from the liver is difficult to achieve with monotherapy. L-d4FC is currently in a late stage of preclinical testing for safety by Achillion Pharmaceuticals.

8. L-I-OddU

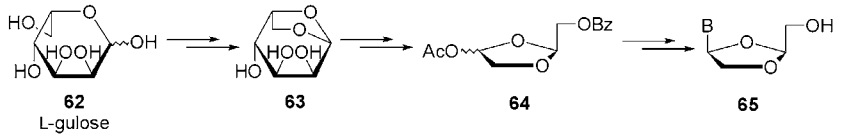
A number of D- and L-5′-halo- and 5′-halovinyl nucleosides and their dioxolane analogs are active against different DNA viruses. Among them, L-I-OddU (**26**, Fig. 4) is the most potent anti-EBV agent so far discovered (EC_{50} 0.03 μ*M*, IC_{50} < 100 μ*M*) and is currently undergoing preclinical studies *(75)*. The antiviral activities of compounds belonging to this series can be related to the size of the halogens (EC_{50} Cl 0.15; Br 0.07 μ*M*]. L-I-OddU is an efficient substrate for EBV TK but not for human cytoplasmic dThy or mt-dPyd kinases, with L-I-OddUMP the major metabolite *(76)*.

The synthesis of D-dioxolane nucleosides has been accomplished analogously to the synthesis of D-oxathiolane nucleosides (Scheme 1) using D-mannose **28** as a chiral starting material (Scheme 12) *(77,78)*. Sugar **28** was converted to the key intermediate **60** via protected 1,6-anhydro-D-mannose **59**. Coupling of intermediate **60** with persilylated purines and pyrimidines followed by deprotection/derivatization reactions afforded D-dioxolane nucleosides **61**.

L-Dioxolane nucleosides have been synthesized in similar fashion using L-gulose **62** as the chiral starting material via its 1,6-anhydro derivative **63** (Scheme 13) *(79,80)*.



Scheme 12. Synthesis of D-dioxolane nucleosides **61**.

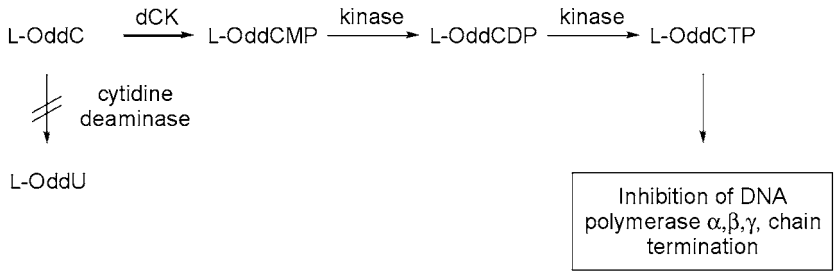


Scheme 13. Synthesis of L-dioxolane nucleosides **65**.

9. TROXACITABINE

The dioxolane analogs of 3TC and FTC, L-OddC **27** (Fig. 4) and its 5-fluoro analog L-OddFC, also display potent antiviral activities but, unlike the oxathiolane nucleosides, also have cellular toxicity *(78,79,81–84)* Thus, L-OddC shows an EC_{50} of 2 n*M* in HIV-1-infected PBM cells and an EC_{50} value of 0.5 n*M* in HBV-infected HepG2 2.2.15 cells but strongly inhibits cellular growth (IC_{50} 0.1 μ*M* in Vero cells and 0.26 μ*M* in CEM cells). The toxicity of L-OddC is caused by the inhibition of DNA polymerases-α, -β, and -γ by its TP. Because this toxicity is selective toward several solid tumor cells, such as hepatocellular and prostate, which are usually unresponsive to nucleoside analogs *(82)*, L-OddC is a potential candidate for the treatment of these tumors. It is the first L-nucleoside showing antitumor activity, and unlike its D-form, it is not metabolized by cytidine deaminase (Scheme 6), which can prolong its effect as a therapeutic agent. Intracellularly, L-OddC is metabolized by dCK to its monophosphate, which is converted to the diphosphate and TP by aspecific cellular kinases (Scheme 14). Interestingly, Cheng and coworkers *(85)* reported that apurinic/apyrimidinic endonuclease, a DNA base excision repair protein, has a higher affinity toward L-nucleosides than the natural substrates, and it is particularly active in removing L-OddC from the 3′-terminus of the DNA chain. L-OddC is currently in phase III clinical trials as an anticancer agent (Troxatyl™, Shire Pharmaceuticals).

The most interesting feature of troxacitabine is its activity in solid tumors, which are usually unresponsive to nucleoside analogs, such as prostatic, renal, hepatic and colon tumors. Besides, its unnatural configuration renders it stable against the metabolic enzyme deoxycytidine deaminase, which may prolong its effect as a drug *(86)*.



Scheme 14. Metabolism of L-OddC.

ACKNOWLEDGMENT

This review chapter was supported by US Public Health Service Research grants AI32351, AI25899, and AI056540 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. Mitsuya, H., Weinhold, K. J., Furman, P. A., et al. 3′-Azido-3′-deoxythimidine (BWA509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro. Proc. Natl. Acad. Sci. USA. 1985;82:7096–7100.
2. Mitsuya, H., Broder, S. Inhibition of the in vitro infectivity and cytopathic effect of human T-lymphotrophic virus type III/limphadenopathy-associated virus (HTLV-III/LAV) by 2′,3′-dideoxynucleosides. Proc. Natl. Acad. Sci. USA. 1986;83: 1911–1915.
3. Lin, T. -S., Schinazi, R. F., Prusoff, W. H. Potent and selective in vitro activity of 3′-deoxythymidin-2′-ene (3′-deoxy-2′,3′-didehydrothymidine) against human immunodeficiency virus. Biochem. Pharm. 1987;36:2713–2718.
4. Schinazi, R. F., Chu, C. K., Peck, A., et al. Activities of the four optical isomers of 2′,3′-dideoxy-3′-thiacytidine (BCH-189) against human immunodeficiency virus type 1 in human lymphocytes. Antimicrob. Agents Chemother. 1992, 36, 672–676.
5. Dobkin J. F. Abacavir enters the clinic. Infect. Med. 1999;16:7–7.
6. Daluge, S. M., Good, S. S., Faletto, M. B., et al. 1592U89, a novel carbocyclic nucleoside analog with potent, selective anti-human immunodeficiency virus activity. Antimicrob. Agents Chemother. 1997;41:1082–1093.
7. Balzarini, J., Aquaro, S., Perno, C. -F., Witvrouw, M., Holy, A., De Clercq, E. Activity of the (R)-enantiomers of 9-(2-phosphonylmethoxypropyl)adenine and 9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine against human immunodeficiency virus in different human cell systems. Biochem. Biophys. Res. Comm. 1996; 219:337–341.
8. Naesens, L., Balzarini, J., De Clercq, E. Therapeutic potential of PMEA as an antiviral drug. Med. Virol. 1994;4:147–159.
9. De Clercq, E. Broad-spectrum anti-DNA virus and anti-retrovirus activity of phosphonylmethoxyalkylpurine and pyrimidines. Biochem. Pharmacol. 1991;42: 963–972.

10. Srinivas, R. V., Robbins, B. L., Connelly, M. C., Gong, Y. -F., Bischofberger, N., Fridland, A. Metabolism and in vitro antiretroviral activities of bis(pivaloyloxymethyl) prodrugs of acyclic nucleoside phosphonates. Antimicrob. Agents Chemother. 1993;37:2247–2250.
11. Galban-Garcia, E., Vega-Sanchez, H., Gra-Oramas, B., et al. Efficacy of ribavirin in patients with chronic hepatitis B. J. Gastroenterol. 2000;35:347–352.
12. Coates, J. A. V., Cammack, N., Jenkinson, H. J., et al. The separated enantiomers of 2′-deoxy-3′-thiacytidine (BCH-189) both inhibit human immunodeficiency virus replication in vitro. Antimicrob. Agents Chemother. 1992;36:202–205.
13. Furman, P. A., Davis, M., Liotta, D. C., et al. The anti-hepatitis B virus activities, cytotoxicities, and anabolic profiles of the (–) and (+) enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine (FTC). Antimicrob. Agents Chemother. 1992;36:2686–2692.
14. Furman, P. A., Wilson , J. E., Reardon , J. E., Painter, G. R. The effect of absolute configuration on the anti-HIV and anti-HBV activity of nucleoside analogs. Antiviral Chem. Chemother. 1995;6:345–355.
15. Wang, P., Hong, J. H., Cooperwood, J. S., Chu, C.K. Recent advances in L-nucleosides: chemistry and biology. Antiviral Res. 1998;40:19–44.
16. Gumina, G., Song, G. -Y., Chu, C. K. L-Nucleosides as chemotherapeutic agents. FEMS Microb. Lett. 2001;202:9–15.
17. Gumina, G., Chong, Y., Choo, H., Song, G. -Y., Chu, C. K. L-Nucleosides: Antiviral activity and molecular mechanism. Curr. Top. Med. Chem. 2002;2:1065–1086.
18. Smejkal, J., Sorm, F. Nucleic acid components + their analogs. 53. Preparation of 1-(2-deoxy-β-L-ribofuranosyl)thymine (L-thymidine). Collec. Czech. Chem. Commun. 1964;29:2809–2813.
19. Coates, J. A. V., Cammack, N., Jenkinson, H. J., et al. The separated enantiomers of 2′-deoxy-3′-thiacytidine (BCH-189) both inhibit human immunodeficiency virus replication in vitro. Antimicrob. Agents Chemother. 1992;36:202–205.
20. Jarvis, B., Faulds, D. Lamivudine. A review of its therapeutic potential in chronic hepatitis B. Drugs 1999;58:101–141.
21. Chu, C. K., Beach, J. W., Jeong, L. S., et al. Enantiomeric synthesis of (+)-BCH-189-[(+)-(2S,5R)-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-cytosine] from D-Mannose and its anti-HIV activity. J. Org. Chem. 1991;56:6503–6505.
22. Jeong, L. S., Alves, A. J., Carrigan, S. W., Kim, H. O., Beach, W., Chu, C. K. An efficient synthesis of enantiomerically pure (+)-(2S,5R)-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-cytosine [(+)-BCH-189] from D-galactose. Tetrahedron Lett. 1992;33:595–598.
23. Beach, J. W., Jeong, L. S., Alves, A. J., et al. Synthesis of enantiomerically pure (2′R,5′S)-(–)-1-[2-(hydroxymethyl)oxathiolan-5-yl]-cytosine as a potent antiviral agent against hepatitis B virus (HBV) and human immunodeficiency virus (HIV). J. Org. Chem. 1992;57:2217–2219.
24. Chang, C. -N., Doong, S. -L., Zhou, J. H., et al. Deoxycytidine deamination-resistant stereoisomer is the active form of (–)-2′,3′-dideoxy-3′-thiacytidine in the inhibition of hepatitis B virus replication. J. Biol. Chem. 1992;267: 13,938–13,942.
25. Severini, A., Liu, X. -Y., Wilson, J. S., Tyrrell, D. L. J. Mechanism of inhibition of duck hepatitis B virus polymerase by (–)-β-L-2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. 1995;39:1430–1435.

26. Shewach, D. S., Liotta, D. C., Schinazi, R. F. Affinity of the antiviral enantiomers of oxathiolane cytosine nucleosides for human 2′-deoxycytidine kinase. Biochem. Pharmacol. 1993;45:1540–1543.
27. Hart, G. J., Orr, D. C., Penn, C. R., et al. Effects of (–)-2′-deoxy-3′-thiacytidine (3TC) 5′-triphosphate on human immunodeficiency virus reverse trancriptase and mammalian DNA polymerase α, polymerase β, and polimerase γ. Antimicrob. Agents Chemother. 1992;36:1688–1694.
28. Lee, K., Chu, C. K. Molecular modeling approach to understanding the mode of action of L-nucleosides as antiviral agents. Antimicrob. Agents Chemother. 2001; 45:138–144.
29. Sarafianos, S. G., Das, K., Clark, A. D., Jr., et al. Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with β-branched amino acids. Proc. Natl. Acad. Sci. USA. 1999;96:10,027–10,032.
30. Tisdale, M., Kemp, S. D., Parry, N. R., Larder, B. A. Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc. Natl. Acad. Sci. USA. 1993;90:5653–5656.
31. Stuyver, L. J., Locarnini, S. A., Lok, A., et al. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. Hepatology 2001;33:751–757.
32. Fu, L., Cheng, Y. -C. Role of additional mutations outside the YMDD motif of hepatitis B virus polymerase in L(–)SddC (3TC) resistance. Biochem. Pharmacol. 1998;55:1567–1572.
33. Fu, L., Liu, S.-H., Cheng, Y.-C. Sensitivity of L-(–)2′,3′-dideoxythiacytidine resistant hepatitis B virus to other antiviral nucleoside analogs. Biochem. Pharmacol. 1999;57:1351–1359.
34. Chong, Y., Stuyver, L., Otto, M. J., Shinazi, R. F., Chu, C. K. Mechanism of antiviral activities of 3′-substituted L-nucleosides against 3TC-resistant HBV polymerase: a molecular modelling approach. Antiviral Chem. Chemother. 2003;14:309–319.
35. Rusconi, S., De Pasquale, M. P., Milazzo, L., et al. In vitro effects of continuous pressure with zidovudine (ZDV) and lamivudine on a ZDV-resistant HIV-1 isolate. AIDS 1997;11:1406–1410.
36. Murray, L., Kelly, G. L., Deutsch, M., Wyble, C. Combivir® tablets (lamivudine/zidovudine tablets). In: Physicians, Desk Reference. 55th ed. 2001, www.pdr.net, pp. 1365–1368.
37. Patick, A. K., Boritzki, T. J., Bloom, L. A. Activities of the human immunodeficiency virus type 1 (HIV-1) protease inhibitor nelfinavir mesylate in combination with reverse transcriptase and protease inhibitors against acute HIV-1 infection in vitro. Antimicrob. Agents Chemother. 1997;41:2159–2164.
38. Landers, M. B., Fraser, V. J. Antiviral chemoprophylaxis after occupational exposure to human immunodeficiency virus: why, when, where, and what. Am. J. Ophthal. 1997;124:234–239.
39. Notermans, D. W., Jurriaans, S., De Wolf, F., et al. Decrease of HIV-1 RNA levels in lymphoid tissue and peripheral blood during treatment with ritonavir, lamivudine and zidovudine. AIDS 1998;12:167–173.
40. Yuen, G. Y., Lou, Y., Thompson, N., et al. Abacavir/lamivudine/zidovudine as a combined formulation tablet: bioequivalence compared with each component administered concurrently and the effect of food on absorption. J. Clin. Pharmacol. 2001;41:277–288.

41. Schinazi, R. F., McMillan, A., Cannon, D., et al. Selective inhibition of human immunodeficiency viruses by racemates and enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. Antimicrob. Agents Chemother. 1992;36:2423–2431.
42. Furman, P. A., Davis, M., Liotta, D. C., et al. The anti-hepatitis B virus activities, cytotoxicities, and anabolic profiles of the (–) and (+) enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. Antimicrob. Agents Chemother. 1992;36:2686–2692.
43. Schinazi, R. F., Boudinot, F. D., Ibrahim, S. S., Manning, C., McClure, H. M., Liotta, D.C. Pharmacokinetics and metabolism of racemic 2′,3′-dideoxy-5-fluoro-3′-thiacytidine in rhesus monkeys. Antimicrob. Agents Chemother. 1992;36: 2432–2438.
44. Bridges, E. G., Dutschman, G. E., Gullen, E. A., Cheng, Y. -C. Favorable interaction of β-L-(–) nucleoside analogs with clinically approved anti-HIV nucleoside analogs for the treatment of human immunodeficiency virus. Biochem. Pharmacol. 1996;51:731–736.
45. Bryant, M., Bridges, E. G., Placidi, L., et al. Antiviral L-nucleosides specific for hepatitis B virus infection. Antimicrob. Agents Chemother. 2001;45:229–235.
46. Bridges, E. G., Juodawikis, A., Faraj, A., et al. Antiviral activity of β-L-thymidine and β-L-2′-deoxycytidine in the woodchuck model of chronic hepatitis B infection. Antiviral Res. 2000;46:91–91.
47. Chong, Y., Chu, C. K. Efficient synthesis of 2-deoxy-L-erithro-pentose (2-deoxy-L-ribose) from L-arabinose. Carbohydr. Res. 2002;337:397–402.
48. Fujimori, S., Iwanami, N., Hashimoto, Y., Shudo, K. A. Convenient and stereoselective synthesis of 2′-deoxy-β-L-ribonucleosides. Nucleosides Nucleotides 1992; 11:341–349.
49. Robins, M., Khwaja, T. A., Robins, R. K. Purine nucleosides. XXIX. The synthesis of 2′-deoxy-L-adenosine and 2′-deoxy-L-guanosine and their α anomers. J. Org. Chem. 1970;35:636–639.
50. Abbruzzese, J. L., Schmidt, S., Raber, M. N., et al. Phase I trial of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) terminated by severe neurologic toxicity. Invest. New Drugs 1989;7:195–201.
51. Chu, C. K., Ma, T. W., Shanmuganathan, K., et al. Use of 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil as a novel antiviral agent for hepatitis B virus and Epstein-Barr virus. Antimicrob. Agents Chemother. 1995;39:979–981.
52. Pai, S. B., Liu, S. H., Zhu, Y. L., Chu, C. K., Cheng, Y. C. Inhibition of hepatitis B virus: a novel L-nucleoside, 2′-fluoro-5-methyl-β-L-arabinofuranosyl uracil. Antimicrob. Agents Chemother. 1996;40:380–386.
53. Yao, G. -Q., Liu, S. H., Chou, E., Kukhanova, M., Chu, C. K., Cheng, Y. C. Inhibition of Epstein-Barr virus replication by a novel L-nucleoside, 2′-fluoro-5-methyl-β-L-arabinofuranosyl uracil. Biochem. Pharm. 1996;51:941–947.
54. Du, J., Choi, Y., Lee, K., Chun, B. K., Hong, J. H., Chu, C. K. A practical synthesis of L-FMAU from L-arabinose. Nucleosides Nucleotides 1999;18:187–195.
55. Ma, T., Pai, S. B., Zhu, Y. L., et al. Structure-activity relationships of 1-(2-deoxy-2-fluoro-β-L-arabinofuranosyl)pyrimidine nucleosides as anti-hepatitis B virus agents. J. Med. Chem. 1996;39:2835–2843.
56. Liu, S. -H., Grove, K. L., Cheng, Y. -C. Unique metabolism of a novel antiviral L-nucleoside analog, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil: a substrate for both thymidine kinase and deoxycytidine kinase. Antimicrob. Agents Chemother. 1998;42;833–839.

57. Kukhanova, M., Lin, Z.-Y., Yas-co, M., Cheng, Y.-C. Unique inhibitory effect of 1-(2′-deoxy-2′-fluoro-β-L-arabinofuranosyl)-5-methyluracil 5′-triphosphate on Epstein-Barr virus and human DNA polymerase. Biochem. Pharmacol. 1998;55: 1181–1187.
58. Chu, C. K., Boudinot, F. D., Peek, S. F., et al. Preclinical investigation of L-FMAU as an anti-hepatitis B virus agent. In: Schinazi, R. F., Sommadossi, J.-P. and Thomas, H. C., eds., Therapies for Viral Hepatitis. Vol. 32. UK: International Medical Press; 1999, pp. 303–312.
59. Aguesse-Germon, S., Liu, S.-H., Chevallier, M., et al. Inhibitory effect of 2′-fluoro-5-methyl-β-L-arabinofuranosyl-uracil on duck hepatitis B virus replication. Antimicrob. Agents Chemother. 1998;42:369–376.
60. Peek, S. F., Cote, P. J., Jacob, J. R., et al. Antiviral activity of clevudine [L-FMAU, (1-(2-fluoro-5-methyl-β,L-arabinofuranosyl)uracil)] against woodchuck hepatitis virus replication and gene expression in chronically infected woodchucks (*Marmota monax*). Hepatology 2001;33:254–266.
61. Chu, C. K., Boudinot, F. D., Peek, S. F., et al. Preclinical investigation of L-FMAU as an anti-hepatitis B virus agent. Antivir. Ther. 1998, 3(suppl. 3), 113–121.
62. Gumina, G., Song, G. -Y., Chu, C. K. Advances in antiviral agents for hepatitis B virus. Antiviral Chem. Chemother. 2001;12:89–112.
63. Zhu, Y., Yamamoto, T., Cullen, J., et al. Kinetics of hepadnavirus loss from the liver during inhibition of viral DNA synthesis. J. Virol. 2001;75:311–322.
64. Lee, K., Choi, Y., Gullen, E., et al. Synthesis and anti-HIV and anti-HBV activities of 2′-fluoro-2′,3′-unsaturated L-nucleosides. J. Med. Chem. 1999;42:1320–1328.
65. Pai, S. B., Liotta, D. C., Chu, C. K., Schinazi, R. F. Inhibition of hepatitis B virus by novel, 2′-fluoro-2′,3′-unsaturated D- and L-nucleosides. Abstracts of Papers, Third International Conference on Therapies for Viral Hepatitis, Maui, HI. 1999, Abstract 125.
66. Pai, S. B., Chu, C. K., Liotta, D. C., Schinazi, R. F. 2′-Fluoro-2′,3′-unsaturated nucleosides as selective antivirals against hepatitis B virus. Antiviral Ther. 2000, 5(suppl. 1), B66–B66.
67. Lee, K., Choi, Y., Gumina, G., Zhou, W., Schinazi, R. F., Chu, C. K. Structure–activity relationships of 2′-fluoro-2′,3′-unsaturated D-nucleosides as anti-HIV-1 agents. J. Med. Chem. 2002;45:1313–1320.
68. Gosselin, G., Boudou, V., Griffon, J.-F., et al. New unnatural L-nucleoside enantiomers: from their stereospecific synthesis to their biological activities. Nucleosides Nucleotides, 1997;16:1389–1398.
69. Lin, T. -S., Luo, M. -Z., Liu, M. -C., et al. Design and synthesis of 2′,3′-dideoxy-2′,3′-didehydro-β-L-cytidine (β-L-d4C) and 2′,3′-dideoxy-2′,3′-didehydro-β-L-5-fluorocytidine (β-L-Fd4C), two exceptionally potent inhibitors of human hepatitis B virus (HBV) and potent inhibitors of human immunodeficiency virus (HIV) in vitro. J. Med. Chem. 1997;39:1757–1759.
70. Le Guerhier, F., Pichoud, C., Guerret, S., et al. Characterization of the antiviral effect of and 2′,3′-dideoxy-2′,3′didehydro-β-L-5-fluorocytidine in the duck hepatitis B virus infection model. Antimicrob. Agents Chemother. 2000;44:111–122.
71. Dutschman, G. E., Bridges, E. G., Liu, S. -H., et al. Metabolism of 2′,3′-dideoxy-2′,3′-didehydro-β-L-(–)-5-fluorocytidine and its activity in combination with clinically approved anti-human immunodeficiency virus β-D-(+) nucleoside analogs in vitro. Antimicrob. Agents Chemother. 1998;42:1799–1804.
72. Kukhanova, M., Li, X., Chen, S. -H., et al. Interaction of β-L-2′,3′-dideoxy-2′,3′-didehydro-5-fluoro-CTP with human immunodeficiency virus-1 reverse transcrip-

tase and human DNA polymerases: Implication for human immunodeficiency virus drug design. Mol. Pharmacol. 1998;53:801–807.
73. Zhu, Y. -L., Dutschman, G. E., Liu, S. -H., Bridges, E. G., Cheng, Y.-C. Anti-hepatitis B virus activity and metabolism of 2′,3′-dideoxy-2′,3′-didehydro-β-L-(–)-5-fluorocytidine. Antimicrob. Agents Chemother. 1998;42:1805–1810.
74. Le Guehier, F., Pichoud, C., Jamard, C., et al. Antiviral activity of β-L-2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine in woodchucks chronically infected with woodchuck hepatitis virus. Antimicrob. Agents Chemother. 2001;45:1065–1077.
75. Lin, J.-S., Kira, T., Gullen, E., et al. Structure–activity relationships of L-dioxolane uracil nucleosides as anti-Epstein Barr virus agents. J. Med. Chem. 1999;42: 2212– 2217.
76. Kira, T., Grill, S. P., Dutschman, G. E., et al. Anti-Epstein-Barr virus (EBV) activity of -L-5-iododioxolane uracil is dependent on EBV thymidine kinase. Antimicrob. Agents Chemother. 2000;44:3278–3284.
77. Kim, H. O., Ahn, S. K., Alves, A. J., et al. Asymmetric synthesis of 1,3-dioxolane pyrimidine nucleosides and their anti-HIV activity. J. Med. Chem. 1992;35: 1987–1995.
78. Kim, H. O., Schinazi, R. F., Nampalli, S., et al. 1,3-Dioxolanylpurine nucleosides (2R, 4R) and (2R, 4S) with selective anti-HIV activity in human lymphocytes. J. Med. Chem. 1993;36:30–37.
79. Kim, H. O., Schinazi, R. F., Shanmuganathan, K., et al. L-β-(2S,4S)- and L-α-(2S,4S)-dioxolanyl nucleosides as potential anti-HIV agents: asymmetric synthesis and structure-activity relationships. J. Med. Chem. 1993;36:519–528.
80. Kim, H. O., Shanmuganathan, S., Alves, A. J., et al. Potent anti-HIV and anti-HBV activities of (–)-L-β-dioxolane-C and (+)-L-β-dioxolane-T and their asymmetric syntheses. Tetrahedron Lett. 1992;33:6899–6902.
81. Grove, K. L., Guo, X., Liu, S. H., Kukhanova, M., Chu, C. K., Cheng, C. -Y. β-L-(–)-Dioxolane cytidine (β-L-(–)-OddC) as a potent compound for the treatment of cancer. Nucleosides Nucleotides 1997;16:1229–1233.
82. Grove, K. L., Guo, X., Liu, S. H., Gao, Z. L., Chu, C. K., Cheng, Y. -C. Anticancer activity of β-L-dioxolane cytidine, a novel nucleoside analog with the unnatural L-configuration. Cancer Res. 1995;55:3008–3011.
83. Grove, K. L., Cheng, Y.-C. Uptake and metabolism of the new anticancer compound β-L-dioxolane cytidine in human prostate carcinoma DU-145 cells. Cancer Res. 1996;56:4187–4191.
84. Kim, H. O., Shanmuganathan, K., Alves, A. J., et al. Potent anti-HIV and anti-HBV activities of (–)-L-β-dioxolane-C and (+)-L-β-dioxolane-T and their asymmetric synthesis. Tetrahedron Lett. 1992;33:6899–6902.
85. Chou, K.-M., Kukhanova, M., Cheng, Y. -C. A novel action of human apurinic/apyrimidinic endonuclease. J. Biol. Chem. 2000;275:31,009–31,015.
86. Moore, M., Belanger, K., Jolivet, J., Baker, S., Wainman, N. NCIC CTG IND 103: a phase I and pharmacokinetic (PK) study of the novel L-nucleoside analog troxacitabine (BCH-4556) given every 21 d. Eur. J. Cancer 1999;35:S284–S284.

9 Troxacitabine (Troxatyl™)

A Deoxycytidine Nucleoside Analog With Potent Antitumor Activity

Henriette Gourdeau, PhD, and Jacques Jolivet, MD

CONTENTS

SUMMARY

Nucleoside analogs are commonly used in the treatment of hematological malignancies and solid tumors. As antimetabolites, these drugs act by disrupting DNA synthesis and inducing apoptosis following their incorporation into DNA. Troxacitabine (Troxatyl™ Shire Biochem, Inc., exclusively licensed to SGX Pharmaceuticals, Inc.) is the first nucleoside analog with anticancer activity that has an unnatural stereochemical configuration. Its broad preclinical antineoplastic spectrum led to its clinical development. Summaries of the preclinical data and of the initial phase I and II clinical trials are presented.

Key Words: Chemotherapy; clinical treatment; hematological malignancies; solid tumors; troxacitabine.

1. INTRODUCTION

The majority of nucleoside analogs known to have antineoplastic or antiviral activities are in the β-D configuration. These drugs were developed based

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

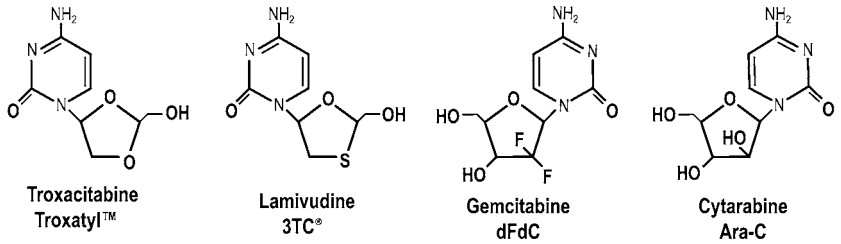


Fig. 1. Chemical structure of clinically relevant cytidine analogs.

on the belief that the corresponding L-enantiomers would be ineffective because metabolic enzymes required for their activation would not recognize the unnatural stereochemistry. This assumption was dismissed with the discovery of lamivudine, an L-nucleoside with potent anti-human immunodeficiency virus and anti-hepatitis B virus activities *(1–4)*. Exchange of the sulfur atom with an oxygen in the dioxolane ring of lamivudine resulted in the formation of troxacitabine [Troxatyl™, BCH-4556, (–)-2′-deoxy-3′-oxacytidine] (Fig. 1). Original screening assays revealed that troxacitabine is cytotoxic and has much lower anti-human immunodeficiency virus activity than lamivudine *(5)*. Initial in vitro assessment by the National Cancer Institute in 1990 indicated moderate antineoplastic activity. Subsequent in vivo work by Cheng and collaborators *(6)* demonstrated the potential of troxacitabine to become an important anticancer agent.

2. STRUCTURE AND MECHANISM OF ACTION

As with other deoxycytidine nucleotide analogs, such as ara-C (1-β-D-arabinofuranosylcytosine, cytarabine) and gemcitabine (2′,2′-difluorodeoxycytidine), deoxycytidine kinase catalyzes the monophosphorylation of troxacitabine, thereby indicating that deoxycytidine kinase may lack chiral specificity *(7)*. In contrast, cytidine deaminase (CDA) is more chirally specific and is incapable of deaminating troxacitabine to an inactive metabolite. This is in contrast to ara-C and gemcitabine, which are both inactivated via deamination. These results suggest that troxacitabine may continue to be effective when deamination is a pathway of tumor resistance to cytidine analogs.

Once monophosphorylated, these antimetabolites are phosphorylated to their di- and triphosphate forms by other cellular kinases *(8–10)*. The triphosphate form of troxacitabine is a good substrate and inhibitor of the replicative and repair deoxyribonucleic acid (DNA) polymerases in vitro *(11)*. Although inhibition of such DNA polymerases could in part explain

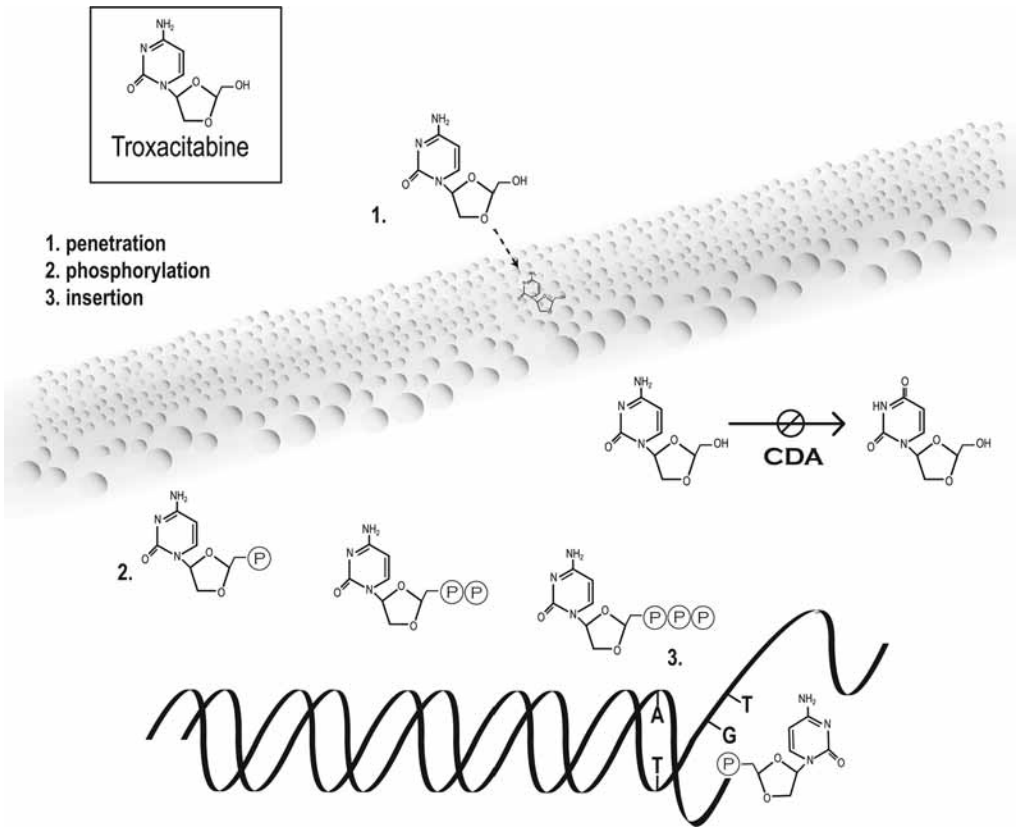
its cytotoxicity, other evidence has indicated that troxacitabine is a complete DNA chain terminator (Fig. 2). This can be rationalized because the dioxolane ring from the nucleoside structure lacks the necessary hydroxyl moiety for chain elongation. In contrast, gemcitabine incorporation allows addition of a single deoxynucleotide *(12,13)*; cytarabine incorporation allows addition of multiple deoxynucleotides before chain termination *(9,14)*.

Excision of incorporated analogs from the 3′ termini of DNA has been demonstrated in vitro and represents a way for cells to recuperate and survive the action of these nucleoside analogs. It was found that a human apurinic/apyrimidic DNA endonuclease (APE1) would be the major enzyme involved in the removal of troxacitabine monophosphate from DNA *(15)*, whereas ara-C and gemcitabine removal would be more dependent on the 3′ to 5′ exonuclease associated with DNA polymerases *(16,17)*.

Other mechanistic features of troxacitabine relate to its cellular metabolism. Although the mono- or triphosphate forms are normally the predominant metabolites for most nucleoside analogs, troxacitabine diphosphate is a major metabolite, and its formation increases linearly with increasing extracellular drug concentration *(18)*. Thus, a large pool of troxacitabine diphosphate is created that serves as a reservoir for the generation of the triphosphate. In addition, although nucleoside diphosphate kinase phosphorylates 2′-deoxyadenosine 5′-diphosphate, 2′-deoxycytidine 5′-diphosphate, and 2′-deoxyguanosine 5′-diphosphate into their corresponding triphosphates and pharmacologically active species *(19)*, data demonstrated that troxacitabine diphosphate was selectively phosphorylated by 3-phosphoglycerate kinase *(20–22)*.

The pharmacokinetic behavior of troxacitabine is also substantially different from that of other deoxynucleoside analogs, which are characterized by rapid disappearance from plasma because of deamination. In contrast to ara-C and gemcitabine, which undergo rapid and monophasic elimination, elimination of troxacitabine triphosphate from cells is slow and biphasic as denoted by intracellular half-lives of 3.5 h and longer than 20 h for the first and second phases, respectively *(23)*. Additional results indicated that mitochondrial DNA synthesis is not inhibited in vitro by troxacitabine at any concentrations that are cytotoxic to tumor cells *(24)*. These results suggest that treatment with troxacitabine would not result in delayed toxicities.

Another characteristic that distinguishes troxacitabine from ara-C and gemcitabine is the uptake of these analogs into cells. Results have demonstrated that the major route of cellular uptake of troxacitabine in leukemia cells was passive diffusion *(25)*, which implies that deficiencies in nucleoside transport are unlikely to impair its activity. On the other hand, ara-C and gemcitabine uptake into cells is highly dependent on nucleoside


Troxacitabine
1.
1. penetration
2. phosphorylation
3. insertion
CDA
2.
3.
A
T
G
T

transporters *(26,27)*, and the level of equilibration-sensitive transporters correlates with the in vitro sensitivity of leukemia blasts from patients to ara-C *(28)*.

3. PRECLINICAL EVALUATION

Troxacitabine has demonstrated broad activity against both solid and hematopoietic malignancies in vitro and in vivo. Antitumor efficacy studies were done in two prostate, four renal, one colon, one head-and-neck, three non-small cell lung, one hepatocellular, two pancreatic, and three leukemia human xenograft tumor models. The activity of troxacitabine against these various tumor types is summarized in Table 1. For comparison purposes, the results are presented using the single or twice daily administration for 5 d because this regimen is one of the optimal dosing schedules. Troxacitabine was administered intraperitoneally with the exception of three studies in which it was administered orally (HepG2) and intravenously (Panc-01 and MiaPaCa). Controls consisted of saline (negative control) and one of the following positive control agents: doxorubicin, cisplatin, 5-fluorouracil (5-FU), cyclophosphamide, mitomycin C, gemcitabine, vinblastine, ara-C, and imatinib mesylate (Gleevec®). Responses in solid tumors were measured by reductions in tumor growth; for the leukemia studies, response rate was evaluated by the length of survival.

Troxacitabine was highly active in both human prostate xenograft tumor models. In the DU-145 xenograft *(7)*, troxacitabine produced tumor regression, with half the tumors regressing completely by d 15. Regrowth occurred by d 25, but ceased by d 47. At d 60, when animals were sacrificed, tumors showed necrotic morphology, with few viable cells found by trypan blue exclusion. In the human hormone-independent prostate adenocarcinoma (PC-3) xenograft, troxacitabine was significantly active at all doses and schedules tested. There was greater antitumor activity when using a 5-d treatment, and the efficacy was the same when the compound was administered once or twice daily. Troxacitabine showed marked activity in all four renal xenograft models and, in all cases, was more effective than the positive control drug *(29)*. Similar results were obtained in the human colon

Fig. 2. (*Opposite page*) Representation of the metabolism and drug target interactions of troxacitabine in proliferating cells. Troxacitabine enters cells via passive diffusion. Once inside the cell, troxacitabine undergoes a series of phosphorylation (P) steps through a first rate-limiting step catalyzed by deoxycytidine kinase (dCK) to form the active triphosphate nucleotide. Troxacitabine triphosphate is incorporated into DNA and since it is a dideoxy analog its incorporation results in chain termination and cell death. Unlike cytidine and other analogs, troxacitabine is resistant to deamination via cytidine deaminase (CDA) and is therefore not converted to a nonactive uridine metabolite.

Table 1
Antitumor Efficacy Studies of Troxacitabine Against Various Human Tumor Xenografts in Nude Mice

	Troxacitabine[a]		*Positive control*	
Tumor type	*Study end point (days)*	*%T/C*[b]	*Drug (regimen)*	*% T/C*[b]
Prostate (PC-3)	60	4	Doxorubicin (10 mg/kg/wk × 3)	34
Prostate (DU-145)	25	7	ara-C (25 mg/kg bid × 5)	100
Renal (CAKI-1)	61	16	Cisplatin (6.7 mg/kg q4d × 3)	108
Renal (A498)	56	27	Doxorubicin (8 mg/kg q4d × 3)	41
Renal (RXF-393)	34	13	Cyclophosphamide (90 mg/kg q4d × 3)	38
Renal (SN-12C)	44	8	Cyclophosphamide (90 mg/kg q4d × 3)	122
Colon (HT-29)	46	5[c]	5-FU (40 mg/kg qd × 5 for 2 wk)	84
Head and neck (KBV)	49	19	Doxorubicin (10 mg/kg/wk × 2)	32
NSC lung (NCI-H322M)	58	62	Mitomycin C (3 mg/kg/wk × 3)	28
NSC lung (NCI-H460)	27	46	Mitomycin C (3 mg/kg q4d × 3)	26
NSC lung (A549)	67	41	Mitomycin C (3 mg/kg q4d × 3)	25
Liver (HepG2)	10	38	ara-C (25 mg/kg bid × 5)	93
Liver (HepG2)	10	26[d]	ara-C (25 mg/kg bid × 5)	93

Pancreas (Panc-01)	29	16[e,f]	Gemcitabine (80 mg/kg q3d × 4)	76
Pancreas (MiaPaCa)	32	77[e,f]	Gemcitabine (80 mg/kg q3d × 4)	87
Leukemia (HL60)	98	422[f,g]	ara-C (25 mg/kg qd × 5)	106[g]
Leukemia (CCRF-CEM)	87	152[f,g]	ara-C (25 mg/kg qd × 5)	146[g]
Leukemia (KBM5)	100	171[f,g]	Imatinib mesylate (50 mg/kg bid × 10)	109[g]

[a]Troxacitabine was administered intraperitoneally twice a day (6-h interval) at a concentration of 25 mg/kg unless specified.

[b]Tumor measurements were taken twice weekly using calipers and were converted to tumor volumes using the following formula: Width (mm^2) × Length (mm) × 0.52. T/C's were generated from the mean treated tumor volume divided by the mean control tumor volume times 100. By National Cancer Institute criteria, %T/C < 42% indicates that the drug is active *(56,57)*.

[c]Two cycles of drug were administered.

[d]The drug was administered orally instead of intraperitoneally.

[e]The drug was administered intravenously instead of intraperitoneally.

[f]Once daily (qd) administration was used instead of twice daily (bid).

[g]For the leukemia studies, the results are expressed as percentage of mean survival time of treated animals over the mean survival time of the control group (treated vs control, T/C%). By National Cancer Institute criteria, %T/C exceeding 125% indicate that the drug has significant antitumor activity *(57)*.

adenocarcinoma (HT-29) xenograft tumor model, well known for its chemorefractory status *(30)*. On the other hand, troxacitabine produced moderate inhibition of tumor growth in the three human lung xenografts.

In the HepG2 human hepatocellular tumor model, troxacitabine produced marked tumor growth inhibition when given either orally or intraperitoneally; no effect was observed with ara-C *(7)*. Indeed, preclinical pharmacokinetic studies in rats indicated that troxacitabine was slowly absorbed, and bioavailability was around 37–41% *(18)*.

Troxacitabine was also found to be active in the HL60 promyelocyte human leukemia xenograft *(31,32)*, resulting in a significant increase in survival time with complete cures at higher doses. In contrast, ara-C was ineffective in this model *(31)*, possibly as a result of the high levels of CDA present in HL60 cells. CDA activity in the HL60 cell line is similar to levels found in solid tumors, which are also refractory to ara-C. This is further indicated by the observation that ara-C was as effective as troxacitabine in the CCRF-CEM T-lymphoblastoid human leukemia xenograft with low levels of CDA *(31)*. When compared with gemcitabine, which is approved for the palliative treatment of pancreatic cancer, troxacitabine was significantly more effective in the Panc-01 model and showed moderate activity in the gemcitabine-refractory MiaPaCa model *(33)*. Gemcitabine, which is also a substrate for CDA, although with less affinity than ara-C *(34)*, was shown to be less effective in several tumors with high CDA levels *(35)*. These findings indicate that troxacitabine is likely to be effective not only against solid tumors with high CDA activity but also in leukemias that have developed resistance to ara-C because of increased CDA levels.

Following the promising antitumor efficacy of troxacitabine in hematological malignancies, we investigated its combination with ara-C or imatinib mesylate *(36–38)*. Combination of ara-C and troxacitabine was better at slowing the progression of leukemia in severe combined immunodeficient mice than either agent used alone without additive toxicities. These effects appear to be related to their interaction at the level of DNA repair rather than to pharmacokinetic interactions *(36)*. Moreover, troxacitabine showed strong synergy with imatinib mesylate, resulting in complete cures at doses at which imatinib mesylate was ineffective when given in monotherapy *(37,38)*.

Multidrug resistance (MDR) is believed to be one of the relevant mechanisms of primary or acquired resistance in human leukemias and solid tumors *(39,40)*. The MDR phenotypes have been associated with the overexpression of various members of the ATP-binding cassette transporters. The first and most common, P-glycoprotein, is the product of the *mdr 1* gene. P-Glycoprotein was demonstrated to act as an efflux pump for anthracyclines as well as for various unrelated cytotoxic drugs.

Because troxacitabine most likely enters cells by passive diffusion, we were interested to evaluate its efficacy against MDR tumors. The in vivo activity of troxacitabine was therefore examined against MDR and multidrug resistance-associate protein (MRP) hematological and solid tumor xenografts *(32)*. The human promyelocytic leukemia (HL60/R10, doxorubicin-resistant) and the human nasopharyngeal epidermoid carcinoma (KBV, vincristine-resistant epidermal carcinoma) cell lines have alterations in the *mdr* 1 gene while the promyelocytic leukemia adriamicin-resistant cell line (HL60/ADR) has an alteration in the *MRP* gene. Tumor regression was observed in the KBV xenograft following a 5-d treatment with 20, 50, and 100 mg/kg of troxacitabine, with percentages of total growth inhibition of 81, 96, and 97, respectively, and some cures at the two highest dosage levels. In the HL60/R10 and HL60/ADR xenografts, the effect of troxacitabine was evaluated on survival time. Troxacitabine treatment (25, 50, and 100 mg/kg/d for 5 d) was initiated 10 d after tumor cell inoculation, once animals had developed disseminated tumors. In both leukemic models, troxacitabine was quite potent, producing treated-vs-control values of 162–315% as well as complete cures at the higher dosage levels.

In mice and rats, troxacitabine was generally well tolerated by intraperitoneal or intravenous administration. In antitumor studies in mice, troxacitabine did not produce significant toxicity at doses up to 50 mg/kg when administered twice daily for 5 d. In rats, troxacitabine was relatively nontoxic when given as a single intravenous injection (no effects up to 2000 mg/kg). However, on multiple dosing in rats, troxacitabine produced spermatocyte depletion and testicular tubular atrophy at doses of 100 mg/kg/d or higher administered over 5 d and decreases in white and red blood cell counts at doses of 25 mg/kg/d or higher over 5 d. Cynomolgus monkeys were much more sensitive than rodents to single- and multiple-dose exposures of troxacitabine. The maximum tolerated dose in the monkey following a single intravenous dose was 1 mg/kg and following 5-d dosing was 0.20 mg/kg/d.

4. CLINICAL TRIALS

4.1. Solid Tumors

Three phase I studies of troxacitabine have been performed in patients with solid tumors. In the first study, troxacitabine was initially given as a 30-min infusion every 3 wk *(41)*. Granulocytopenia was dose limiting, and 10 mg/m^2 was chosen as the recommended dose. A second study administered troxacitabine as a 30-min intravenous infusion daily for 5 d every 3 wk. The recommended dose was 1.2 mg/m^2/d for heavily pretreated patients and 1.5 mg/m^2/d for lightly pretreated patients; neutropenia and skin rash were the dose-limiting toxicities *(42)*. A third study gave the drug

as a 30-min infusion weekly for 3 wk, with prolonged myelosuppression defining the recommended dose of 3.2 mg/m^2 weekly three times every 4 wk *(43)*. The pharmacokinetics of troxacitabine were linear and consistent across the phase I trials, with urinary excretion of unchanged troxacitabine accounting for most of drug elimination *(41,42)*. No metabolite was identified in the plasma or urine of treated patients.

Pilot solid tumor phase II clinical trials were performed with 10 mg/m^2 administered every 3 wk in prostate, colorectal, pancreatic, renal cell *(44)*, and non-small cell lung *(45)* cancers and malignant melanoma. Two partial responses were observed in 33 patients in the renal cancer trial, with 21 patients having stable disease (median duration 4.4 mo), and 10 with progressive disease. Eight patients remained stable for more than 6 mo, of whom 6 remain free of progression. The median survival was 18 mo for patients classified as intermediate risk and 8 mo for the high-risk patients compared to 10 and 4 mo, respectively, in Motzer's retrospective analysis *(46)*. A phase III study would be required to validate these observations.

The pancreatic cancer study enrolled 15 patients, 9 previously treated with either a gemcitabine or 5-FU-containing regimen and 6 chemotherapy naïve. Two patients with no prior chemotherapy met the criteria for attaining a clinical benefit response to troxacitabine, and one of these had a partial response. In addition, three chemotherapy naive patients had decreased serum concentrations of CA 19-9. Troxacitabine was then evaluated as first-line therapy in 54 patients with advanced adenocarcinoma of the pancreas but was administered at 1.5 mg/m^2 daily five times every 4 wk *(47)*. Median time-to-tumor progression was 3.5 mo, median survival was 5.6 mo, and the 1-yr survival rate was 19%. Best responses were stable disease in 24 patients, with 8 patients having stable disease for at least 6 mo (15%). A 50% or greater decrease in CA 19-9 was seen in 7 of 44 assessed patients (16%). Four of 34 (12%) patients who had sufficient symptoms and follow-up data for clinical benefit response evaluation had a positive response. Two additional patients who were not sufficiently symptomatic to meet the criteria for clinical benefit response evaluation increased their weight by more than 7% on the study while receiving 8 and 14 cycles of therapy, respectively. These results appear to be comparable overall to those reported with gemcitabine in recently published randomized trials *(48)*.

Troxacitabine and gemcitabine were demonstrated to be synergistic in vitro in four human pancreatic adenocarcinoma cell lines and to be at least additive in an in vivo model *(49)*. A phase I trial examined the clinical feasibility of a troxacitabine/gemcitabine combination, and preliminary results suggest that both drugs can be safely combined at effective doses *(50)*. Recommended doses for the combination are 8 mg/m^2 troxacitabine on d 1 with 1000 mg/m^2 gemcitabine on d 1 and 8 of a 3-wk cycle. Further

evaluation of troxacitabine in pancreatic cancer in combination with gemcitabine appears warranted.

The most commonly observed toxicities in the phase II studies were hematological (neutropenia, sometimes with fever) and cutaneous (skin rash, dry skin, pruritus, and hand-foot syndrome). Other observed toxicities were nausea, fatigue, anorexia, vomiting, taste disturbance, alopecia, and stomatitis. Most observed toxicities were grade 1 or 2. The incidence of grade 3 skin rash appeared to be decreased by prophylactic steroid therapy.

4.2. Acute Leukemia

A phase I/II study of troxacitabine was performed in patients with refractory acute leukemia using a 30-min infusion daily for 5 d. The dose-limiting toxicities were stomatitis and hand-foot syndrome, and the recommended dose was defined as 8 mg/m^2/d *(51)*. In the subsequent phase II part of this study *(52)*, 42 patients were treated at the recommended dose with 2 complete and 1 partial responses (18%) observed in 16 patients with relapsed acute myeloid leukemia. None of the 6 patients with acute lymphoblastic leukemia responded, but 6 (37%) of 16 patients with chronic myelogenous leukemia in blastic phase were transformed back to chronic phase *(52)*. In a subsequent phase II study, 4 of 31 patients with chronic myelogenous leukemia in blastic phase, including 29 refractory to imatinib mesylate (Gleevec), had a response to troxacitabine *(53)*.

In another phase I/II study, troxacitabine was administered in association with ara-C, idarubicin, or topotecan *(54)*. Four complete responses and an additional four complete responses with incomplete platelet recovery were observed in 31 patients with refractory/relapsed acute myeloid leukemia treated with the ara-C/troxacitabine combination. Respectively, 2/14 and 1/17 complete responses were seen with the idarubicin/troxacitabine and topotecan/troxacitabine combinations *(54)*. These data suggest that further exploration of troxacitabine combinations may be warranted in patients with relapsed acute myeloid leukemia.

A recently published prospective randomized study was conducted in patients aged 50 yr or older with untreated, adverse karyotype, acute myeloid leukemia *(55)*. Patients were adaptively randomized to receive idarubicin and ara-C (IA) vs troxacitabine and ara-C (TA) vs troxacitabine and idarubicin (TI). A Bayesian design was used to adaptively randomize patients to a treatment arm. Thus, although initially there was an equal chance for randomization to IA, TA, or TI, treatment arms with more initial complete responses eventually received a greater proportion of the patients. Thirty-four patients were treated. Randomization to TI stopped after 5 patients and randomization to TA after 11 patients. Complete response rates were 10/18 (55%) with IA, 5/11 (45%) with TA, and 1/5 (20%) with TI. Survival was equivalent with all three regimens. Risk factors were not

equilibrated between the three treatment groups because of the small number of patients randomized. The control group had more patients with more favorable cytogenetics and fewer patients with antecedent hematological disorders compared to the experimental arms, but there were too few patients to evaluate the impact of this imbalance on the observed results. The study was stopped when it was determined that neither troxacitabine combination was likely to be superior to the control arm in elderly patients with previously untreated adverse karyotype acute myeloid leukemia.

5. CONCLUSION

Troxacitabine is a deoxycytidine analog with an unnatural stereochemical configuration and different pharmacological characteristics compared to other clinically relevant deoxycytidine analogs such as ara-C and gemcitabine. Nucleoside transporters do not mediate troxacitabine membrane permeation, and the drug is not inactivated by CDA or excised from DNA by the same enzymes as the naturally occurring nucleosides and deoxycytidine analogs. Troxacitabine has shown significant activity in a number of preclinical tumor models, including models with the MDR phenotype as well as relatively chemoresistant tumor models such as the HT-29 human colon cancer xenograft. This notable preclinical activity led to troxacitabine's clinical development. More than 700 patients have received the drug thus far in phase I and II clinical trials. Encouraging signs of anticancer activity have been seen in patients with acute myeloid leukemia, chronic myelogenous leukemia in blastic phase, and renal cell and pancreatic cancer. Following these trials, SGX Pharmaceuticals has initiated a pivotal phase II/III trial in relapsed refractory AML.

ACKNOWLEDGMENT

The authors would like to thank the scientists who contributed to the preclinical and clinical development of troxacitabine. We would also like to thank Ms. Devon Nykaza for the illustrations.

REFERENCES

1. Regev A, Schiff ER. Drug therapy for hepatitis B. Adv Intern Med 2001;46: 107–135.
2. van Leeuwen R, Katlama C, Kitchen V, et al. Evaluation of safety and efficacy of 3TC (lamivudine) in patients with asymptomatic or mildly symptomatic human immunodeficiency virus infection: a phase I/II study. J Infect Dis 1995;171: 1166–1171.
3. Gulick RM, Mellors JW, Havlir D, et al. Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy. N Engl J Med 1997;11:734–739.

4. Hammer SM, Squires KE, Hughes MD, et al. A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. N Engl J Med 1997;337:725–733.
5. Mansour TS, Jin H, Wang W, et al. Structure–activity relationship among a new class of antiviral and heterosubstituted 2′,3′-dideoxynucleoside analogues. Nucleosides Nucleotides 1995;14:627–635.
6. Chu CK, Cheng YC. Compounds and methods for the treatment of cancer. WO9607413 1996.
7. Grove KL, Guo X, Liu SH, Gao Z, Chu CK, Cheng YC. Anticancer activity of β-L-dioxolane-cytidine, a novel nucleoside analogue with the unnatural L configuration. Cancer Res 1995;55:3008–3011.
8. Chabner BA, Myers CE. Clinical pharmacology of cancer chemotherapy. In: DeVita VT, Hellmnan S, Rosenberg SA, eds. Cancer: Principles and Practice of Oncology. ;3rd ed. Philadelphia: JB Lippincott;1989:362–365.
9. Grant S. Ara-C: cellular and molecular pharmacology. Adv Cancer Res 1998;72:197–233.
10. Plunkett W, Huang P, Xu Y-Z, Heinemann V, Grunewald R, Gandhi V. Gemcitabine: metabolism, mechanisms of action, and self-potentiation. Semin Oncol 1995;22:3–10.
11. Kukhanova M, Liu SH, Mozzherin D, Lin TS, Chu CK, Cheng YC. L-and D-Enantiomers of 2′,3′-dideoxycytidine 5′-triphosphate analogs as substrates for human DNA polymerases. Implications for the mechanism of toxicity. J Biol Chem 1995;270:23,055–23,059.
12. Huang P, Chubb S, Hertel L, Grindey GB, Plunkett W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res 1991;51:6110–6117.
13. Plunkett W, Huang P, Gandhi V. Preclinical characteristics of gemcitabine. Anticancer Drugs 1995;6:7–13.
14. Ohno Y, Spriggs D, Matsukage A, Ohno T, Kufe D. Effects of 1-β-D-arabinofuranosylcytosine incorporation on elongation of specific DNA sequences by DNA polymerase β. Cancer Res 1988;48:1494–1498.
15. Chou KM, Kukhanova M, Cheng YC. A novel action of human apurinic/apyrimidinic endonuclease: excision of L-configuration deoxyribonucleoside analogs from the 3′ termini of DNA. J Biol Chem 2000;275:31,009–31,015.
16. Gandhi V, Legha J, Chen F, Hertel LW, Plunkett W. Excision of 2′,2′-difluorodeoxycytidine (gemcitabine) monophosphate residues from DNA. Cancer Res 1996;56:4453–4459.
17. Pelicano H, Kukhanova M, Cheng YC. Excision of β-D- and β-L-nucleotide analogs from DNA by the human cytosolic 3′-to-5′ exonuclease. Mol Pharmacol 2000;57:1051–1055.
18. Moore LE, Boudinot FD, Chu CK. Preclinical pharmacokinetics of β-L-dioxolane-cytidine, a novel anticancer agent, in rats. Cancer Chemother Pharmacol 1997;39: 532–536.
19. Reichard P. Interactions between deoxyribonucleotide and DNA synthesis. Annu Rev Biochem 1988;57:349–374.
20. Krishnan P, Liou JY, Cheng Y. Phosphorylation of pyrimidine L-deoxynucleoside analog diphosphates. J Biol Chem 2002;277:31,593–31,600.
21. Krishnan P, Fu Q, Lam W, Liou JY, Dutshman G, Cheng YC. Phosphorylation of pyrimidine deoxynucleoside analog diphosphates. J Biol Chem 2002;277: 5453–5459.

22. Krishnan P, Gullen EA, Lam W, Dutschman GE, Grill SP, Cheng YC. Novel role of 3-phosphoglycerate kinase, a glycolytic enzyme, in the activation of L-nucleoside analogs, a new class of anticancer and antiviral agents. J Biol Chem 2003;278: 36,726–36,732.
23. Grove KL, Cheng YC. Uptake and metabolism of the new anticancer compound β-L-(–)-dioxolane-cytidine in human prostate carcinoma DU-145 cells. Cancer Res 1996;56:4187–4191.
24. Lee M, Chu CK, Pai SB, et al. Dioxolane cytosine nucleosides as anti-hepatitis B agents. Bioorg Med Chem Lett 1995;5:2011–2014.
25. Gourdeau H, Clarke ML, Ouellet F, et al. Mechanisms of uptake and resistance to troxacitabine, a novel deoxycytidine nucleoside analogue, in human leukemic and solid tumor cell lines. Cancer Res 2001;61:7217–7224.
26. Ullman B. Dideoxycytidine metabolism in wild type and mutant CEM cells deficient in nucleoside transport or deoxycytidine kinase. Adv Exp Med Biol 1989; 253B:415–420.
27. Mackey JR, Mani RS, Selner M, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 1998;58:4349–4357.
28. Gati WP, Paterson AR, Larratt LM, Turner AR, Belch AR. Sensitivity of acute leukemia cells to cytarabine is a correlate of cellular *es* nucleoside transporter site content measured by flow cytometry with SAENTA-fluorescein. Blood 1997;90: 346–353.
29. Kadhim SA, Bowlin TL, Waud WR, et al. Potent antitumor activity of a novel nucleoside analogue, BCH-4556 (β-L-dioxolane-cytidine), in human renal cell carcinoma xenograft tumor models. Cancer Res 1997;57:4803–4810.
30. Okuno S, Harada M, Yano T, et al. Complete regression of xenografted human carcinomas by camptothecin analogue-carboxymethyl dextran conjugate (T-0128). Cancer Res 2000;60:2988–2995.
31. Gourdeau H, Bibeau L, Ouellet F, Custeau D, Bernier L, Bowlin T. Comparative study of a novel nucleoside analogue (Troxatyl, troxacitabine, BCH-4556) and araC against leukemic human tumor xenografts expressing high or low cytidine deaminase activity. Cancer Chemother Pharmacol 2001;47:236–240.
32. Gourdeau H, Genne P, Kadhim S, et al. Antitumor activity of troxacitabine (Troxatyl) against anthracycline-resistant human xenografts. Cancer Chemother Pharmacol 2002;50:490–496.
33. Weitman S, Marty J, Jolivet J, Locas C, Von Hoff DD. The new dioxolane, (–)-2′-deoxy-3′-oxacytidine (BCH-4556, troxacitabine), has activity against pancreatic human tumor xenografts. Clin Cancer Res 2000;6:1574–1578.
34. Heinemann V, Hertel LW, Grindey GB, Plunkett W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-arabinofuranosylcytosine. Cancer Res 1988;48:4024.
35. Miwa M, Eda H, Ura J, et al. High susceptibility of human cancer xenografts with higher levels of cytidine deaminase to a 2′-deoxycytidine antimetabolite, 2′-deoxy-2′-methylidenecytidine. Clin Cancer Res 1998;4:493–497.
36. Bouffard DY, Jolivet J, Leblond L, et al. Complementary antineoplastic activity of the cytosine nucleoside analogues troxacitabine (Troxatyl) and cytarabine in human leukemia cells. Cancer Chemother Pharmacol 2003;52:497–506.
37. Orsolic N, Giles F-J, Beran M, et al. Troxatyl and STI571 combination therapy for chronic myeloid leukemia: preclinical in vitro and in vivo evaluation.

American Society of Hematology 44th Annual Meeting 2002; Philadelphia, PA; December 6–10, 2002;100(11)A631.
38. Orsolic N, Giles F-J., Gourdeau H, et al. Troxacitabine and imatinib mesylate combination therapy of chronic myeloid leukemia: preclinical evaluation. Br J Haematol 2004;124:727–738.
39. Holzmayer TA, Hilsenbeck S, Von Hoff DD, Roninson IB. Clinical correlates of MDR1(P-glycoprotein) gene expression in ovarian and small-cell lung carcinomas. J Natl Cancer Inst 1992;7:1486–1491.
40. Kuwazuru Y, Yoshimura A, Hanada S, et al. Expression of the multidrug transporter, P-glycoprotein, in acute leukemia cells and correlation to clinical drug resistance. Cancer 1990;66:868–873.
41. Bélanger K, Moore M, Baker SD, et al. Phase I and pharmacokinetic study of novel L-nucleoside analog troxacitabine given as a 30-min infusion every 21 d. J Clin Oncol 2002;20:2567–2574.
42. de Bono JS, Stephenson J Jr, Baker SD, et al. Troxacitabine, an L-stereoisomeric nucleoside analog, on a five-times-daily schedule: a phase I and pharmacokinetic study in patients with advanced solid malignancies. J Clin Oncol 2002;20:96–109.
43. Canova A, Yee L, Baker S. A phase I and pharmacokinetic study of β-L-dioxalocytidine (BCH-4556) administered weekly for three weeks every 28 d. J Clin Oncol 1999;20:2567–2574.
44. Townsley CA, Chi K, Ernst DS, et al. Phase II study of troxacitabine (BCH-4556) in patients with advanced and/or metastatic renal cell carcinoma: a trial of the National Cancer Institute of Canada-Clinical Trials Group. J Clin Oncol 2003;21: 1524–1529.
45. Dent SF, Arnold A, Steward D. Phase II study of troxacitabine (BCH-4556) in patients with advanced non-small cell lung cancer. Proc Am Soc Clin Oncol 2001;20:2786.
46. Motzer RJ, Mazumdar M, Bacik J. Survival and prognostic stratification of 670 patients with advanced renal cell carcinoma. J Clin Oncol 1999;17:2530–2540.
47. Lapointe R, Letourneau R, Steward W, et al. Phase 2 study of troxacitabine in chemotherapy naive patients with advanced cancer of the pancreas. Proc Am Soc Clin Oncol 2002;A565.
48. Kurtz JE, Trillet-Lenoir V, Bugat R, et al. Utilisation compassionnelle de la gemcitabine dans les cancers du pancréas localement avancés ou métastatiques: une étude multicentrique française. Bull Cancer 1999;86:202–206.
49. Wong CKW, Clarke ML, Selner M, et al. Synergistic activity of troxacitabine (troxatyl™) and gemcitabine in pancreatic adenocarcinoma cell lines. AACR 2003;94th Annual Meeting; Washington DC July 10–14, 2003, Abstract # 3724.
50. Von Hoff DD, Jolivet J, Steward W. Phase 1 and pharmacokinetic study of troxacitabine in combination with gemcitabine in advanced solid malignancies. Proc Am Soc Clin Oncol 2003;22;141,A566.
51. Giles FJ, Cortes JE, Baker SD, et al. Troxacitabine, a novel dioxolane nucleoside analog, has activity in patients with advanced leukemia. J Clin Oncol 2001;19: 762–771.
52. Giles FJ, Garcia-Manero G, Cortes J. Troxacitabine in patients with refractory leukemia. J Clin Oncol 2002;20:3356–3361.
53. Giles FJ, Garcia-Manero G, Cortes JE, et al. Phase II study of troxacitabine, a novel dioxolane nucleoside analog, in patients with refractory leukemia. J Clin Oncol 2002;20:656–664.

54. Giles FJ, Faderl S, Thomas DA, et al. Randomized phase I/II study of troxacitabine combined with cytarabine: idarubicin, or topotecan in patients with refractory myeloid leukemias. J Clin Oncol 2003;21:1050–1056.
55. Giles FJ, Kantarjian HM, Cortes JE, et al. Adaptive randomized study of idarubicin and cytarabine vs troxacitabine and cytarabine vs troxacitabine and idarubicin in untreated patients 50 years or older with adverse karyotype acute myeloid leukemia. J Clin Oncol 2003;21:1722–1727.
56. Plowman J, Dykes D, Hollingshead M, Simpson-Herren L, Alley MC. Human tumor xenograft models in NCI drug development. In: Teicher BA, ed. Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval. Totowa, NJ: Humana Press; 1997; 101–125.
57. Johnson JI, Decker S, Zaharevitz D, et al. Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials. Br J Cancer 2001;10:1289, 1290.

10 9-β-D-Arabinofuranosylguanine

Sophie Curbo, PhD
and Anna Karlsson, MD, PhD

CONTENTS

SUMMARY

9-β-D-Arabinofuranosylguanine (ara-G) was synthesized in 1964, but because of its difficult chemical synthesis and poor solubility properties, it was 1995 before a prodrug of ara-G, nelarabine, entered clinical trials. Ara-G is a deoxyguanosine analog that needs to be phosphorylated inside the cell for pharmacological activity. In the first and rate-limiting phosphorylation step, ara-G is a substrate of both the mitochondrial deoxyguanosine kinase and the cytosolic deoxycytidine kinase. Once phosphorylated to its triphosphate derivative, ara-GTP acts as a structural analog of deoxyribonucleotide 5′-triphosphate and is thereby incorporated into DNA. The accumulation of the triphosphate form of ara-G results in inhibition of DNA synthesis and subsequent cell death. Although preclinical studies have shown that ara-G has higher toxicity in T than B lymphoblasts, nelarabine has shown promising effect not only in patients with T-cell malignancies but also in patients with B-cell malignancies. Nelarabine has now earned fast track status from the US Food and Drug Administration for the treatment of T-cell acute lymphoblastic leukemia and lymphoblastic lymphoma in those who

From: *Cancer Drug Discovery and Development:*
Deoxynucleoside Analogs in Cancer Therapy
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

have not responded to or whose disease has progressed during treatment with at least two standard regimens. Nelarabine is an interesting new anticancer agent that might be used as a standard treatment in the future.

Key Words: Anticancer; 9-β-D-arabinofuranosylguanine; deoxyguanosine analog; deoxyguanosine kinase; mitochondrial deoxynucleoside kinases; nelarabine; T-cell malignancies.

1. BACKGROUND

In 1975, it was discovered that patients suffering from purine nucleoside phosphorylase (PNP) deficiency accumulated high levels of deoxyribonucleotide 5′-triphosphate (dGTP) in their T-cells, causing T-cell lymphopenia *(1)*. The greater accumulation of dGTP in T-cells compared to B-cells was later suggested to be caused by a higher phosphorylation rate of deoxyguanosine (dGuo) in T-cells than in B-cells. These findings suggested that dGuo analogs would be selectively toxic to T-cells; thereafter, 9-β-D-arabinofuranosylguanine (ara-G), which was synthesized by Reist and Goodman in 1964, obtained more attention *(2)*. However, because of its difficult chemical synthesis and its poor solubility properties, it took until 1995 for nelarabine, a prodrug of ara-G, to enter clinical trials.

2. MECHANISM OF ACTION

Ara-G is a dGuo analog that needs to be phosphorylated inside the cell for pharmacological activity. In the first and rate-limiting phosphorylation step, ara-G is a substrate of both the mitochondrial deoxyguanosine kinase (dGK) and the cytosolic deoxycytidine kinase (dCK). Although ara-G is a very poor substrate of dCK as compared to dGK *(3)*, the relative importance of dGK and dCK for ara-G phosphorylation in vivo is not known. However, it was shown in vitro that dGK was the predominant enzyme at low ara-G concentrations, whereas dCK was the preferred enzyme at higher ara-G concentrations *(4)*. T lymphoblasts have high expression of dCK and therefore also a high capacity for phosphorylation of dGuo *(5,6)*. The accumulation of dGTP results in inhibition of deoxyribonucleic acid (DNA) synthesis and subsequent cell death *(7,8)*. The dGuo itself is not suitable for selective treatment because it is a good substrate for PNP and is thereby quickly hydrolyzed inside the cell. However, its analog ara-G is relatively resistant to hydrolyses by PNP, and the accumulation of the triphosphate derivative of ara-G (ara-GTP) is independent of the degradation of ara-G, which suggests that ara-G could be selectively toxic to T-cells *(9)*.

Studies have shown that ara-G has higher toxicity to T than to B lymphoblasts *(10,11)*. The prominent efficiency of ara-G in T compared to B lymphoblasts has been attributed both to decreased catabolism of ara-GTP and to higher expression of dCK in T lymphoblasts compared to B lymphoblasts

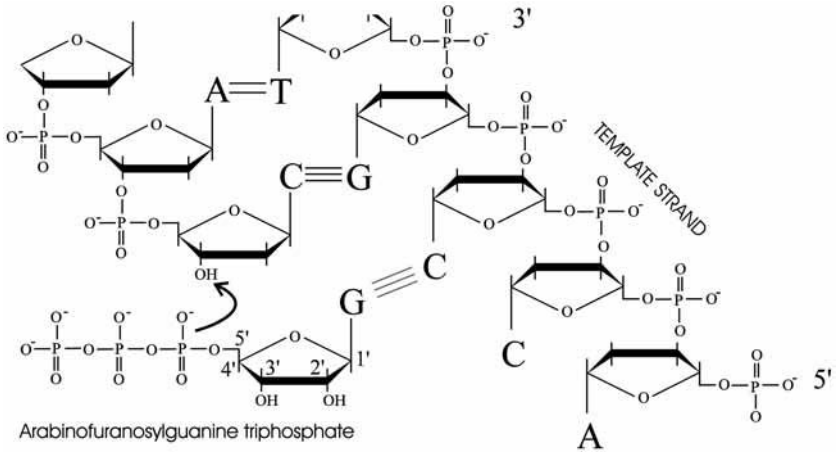


Fig. 1. AraG-triphosphate is a substrate for DNA polymerases, and araG monophosphate is thereby incorporated into the growing DNA chain. It contains no alterations in its 3′-OH group, and subsequently additional nucleotides can be added.

(10,11). Rodriguez and colleagues published a study in which differences of effect of ara-G were analyzed in T lymphoblast (CEM), B lymphoblast (Raji), and myeloid (ML-1) cell lines *(12)*. The pharmacokinetics of ara-GTP accumulation was shown to be favorable, but not the only determinant, for T-lineage-specific cytotoxicity. The incorporation of ara-GMP into replicating DNA of T-cells initiated S-phase-specific cell death and not S-phase arrest, with possible repair of the damage caused by ara-G, as was seen in the other lineages.

It has been shown before that ara-GTP can substitute for deoxyriboadenosine 5′-triphosphate (dATP) in the apoptosome *(13)*, and therefore the signal for apoptosis was suggested to be enhanced by cytosolic ara-GTP by binding to the apoptosome complexes.

Finally, transcriptional and translational upregulation of soluble Fas ligand, which triggers Fas-dependent apoptosis, was only seen in the T lineage and was also suggested to account for the S-phase-independent cell death. However, the clinical effect of the prodrug of ara-G, nelarabine, has demonstrated anticancer effect also in a small number of patients with indolent leukemia, and in vitro studies have also shown sensitivity to ara-G in B-chronic lymphocytic leukemia *(14,15)*. Once phosphorylated to its triphosphate derivative, ara-GTP acts as a structural analog of dGTP and is thereby incorporated into DNA *(16)* (Fig. 1).

The mechanism of action of ara-G is not fully understood. It is hypothesized that a high accumulation of ara-GTP in circulating leukemia cells corresponds to a good prognostic value, and it is likely that ara-GTP is the only

cytotoxic metabolite *(16,17)*. It has been demonstrated that the accumulation of ara-GTP is independent of the cell cycle *(18)*, which is consistent with the constitutive expression of dCK and dGK, the key enzymes of ara-G, throughout the cell cycle. Furthermore, it has been suggested that ara-G exerts its cytotoxic action by inducing apoptosis, and that incorporation of ara-GMP into nuclear DNA is a critical event for triggering apoptosis *(18)*.

Several studies have also demonstrated that ara-G can be incorporated into mitochondrial DNA (mtDNA), but it is presently not known to what extent the mitochondrial incorporation contributes to the cytotoxic action of the analog *(19–21)*. However, it has been shown that the acute cytotoxicity of ara-G is not caused by mtDNA damage, which supports the hypothesis that incorporation of ara-G is necessary for cytotoxicity *(18,21)*. A study by Arpaia et al. suggested a role of mitochondria in the cell-specific toxicity of dGTP with intramitochondrial accumulation of dGTP and inhibition of mtDNA repair *(22)*. It is possible that the mechanism of T-cell-specific toxicity could be similar for ara-G.

3. CLINICAL USE

In 1991, it was reported that ara-G was an effective agent for chemoseparation of malignant T lymphoblasts from human bone marrow *(23)*. Ara-G has poor water solubility, and its clinical use was for a long time hampered because of lack of a more soluble prodrug. In 1995, it was shown that the prodrug 2-amino-6-methoxypurine arabinoside (also called 506U78), which was synthesized enzymatically from diaminopurine arabinoside, was eightfold more soluble than ara-G *(24)*. This prodrug made administration of high drug concentrations possible at physiological pH and in much lower volume than would be required for ara-G *(25)*. 506U78 and ara-G had the same selectivity in vitro to T-cells and did not inhibit B-cell growth in the concentrations tested (IC_{50} > 100 μ*M*). 506U78 was shown not to be a substrate for dCK or PNP but for dGK, which phosphorylated it at a rate of 4% of the rate of ara-G phosphorylation. Furthermore, 506U78 was shown to be converted rapidly to ara-G through demethoxylation by adenosine deaminase *(25)* (Fig. 2).

Based on the accumulated data of 506U78, a phase I trial was initiated in patients with relapsed and refractory hematologic malignancies *(17)*. The overall response rate in this trial was 31% (complete and partial remissions), and of the patients with acute T-cell leukemia, 54% achieved a partial or complete remission after one or two cycles of drug treatment. Including only the evaluable patients, the response rates in patients with T-cell malignancies were even higher (Table 1). In patients who achieved a complete or partial remission, the peak levels of ara-GTP were threefold higher in comparison to patients who failed therapy *(17)*. One patient with B-cell chronic leukemia achieved partial remission from the treatment.

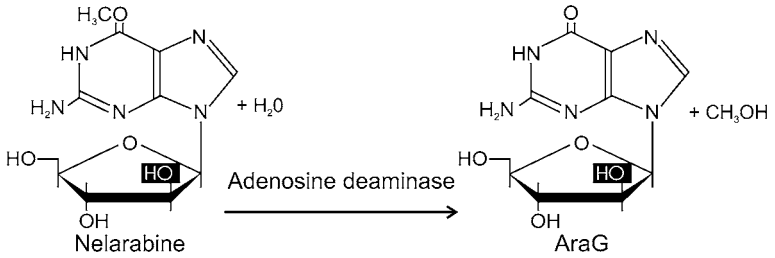


Fig. 2. Adenosine deaminase converts the prodrug nelarabine (506U78) to the biologically active compound araG.

Table 1
Summary of Important Findings in a Phase I Study With GW506U78 *(32)*

Disease	*Evaluable patients*	*Response*
T-ALL	25	CR 44%, PR 32%
T-CLL	9	PR 67%
T-cell lymphoma	12	CR 12%, PR 58%

T-ALL, acute T-cell leukemia; T-CLL, chronic T-cell leukemia; CR, complete remission; PR, partial remission.

The result encouraged further studies with nelarabine, the registered name for 506U78, also in patients with indolent leukemia. To date, one strategy to enhance the effect of nelarabine through increase of cellular ara-GTP has been tested in patients. Clinically achievable levels of F-araATP (fludarabine) can mediate a decrease in deoxynucleotides, especially of the deoxycytidine triphosphate and dGTP pools, which may result in decreased feedback inhibition of dCK and dGK, respectively *(26,27)*. These enzymes are involved in the key activating step of both ara-G and F-ara-A; therefore, a trial was conducted with the combination of nelarabine and fludarabine against indolent leukemia as well as other types of leukemia. The therapy proved to be effective and well tolerated *(28)*. Response was seen in some of the fludarabine-refractory patients, encouraging further evaluation of nelarabine in treatment of patients refractory to fludarabine. Nelarabine has also been evaluated as a single agent and has demonstrated clinical efficacy as such also in the setting of indolent leukemia *(29)*.

4. TOXICITY

Several nucleoside analogs interfere with mtDNA replication, and adverse effects of these compounds are correlated to mitochondrial dysfunction *(30)*. Clinical forms of toxicity often occur in antiviral treatment and span

from mild reversible neuropathy, myopathy, and pancreatitis to severe multiorgan failure *(30,31)*. The dose-limiting toxicity in the clinical trials with nelarabine has been neurotoxicity *(32)*. Although not as pronounced, adverse effects also include myopathy, myelosuppression, and loss of peripheral sensitivity, similar to the symptoms of drugs causing mitochondrial toxicity *(30,33)*.

It has been demonstrated that the acute cytotoxicity of ara-G is not caused by mtDNA damage *(21)*. However, the available data do not exclude that long-term exposure to ara-G might cause mtDNA alterations with subsequent delayed mitochondrial toxicity. 2-Chloro-2′-deoxyadenosine (CdA, cladribine) is also a substrate of dGK and, just like ara-G, has shown side effects similar to mitochondrial toxicity, such as neuropathy and myelosuppression, in the clinical setting *(17,34–36)*. CdA has also been reported to cause an early effect on cellular mitochondrial function, measured as a temporary increase in lactate production *(36)*. In the same study, it was shown that CdA does not cause any decrease in mtDNA content. Taken together, it is thus possible that the subcellular location of the nucleoside analog is of minor importance for mitochondrial toxicity, whereas other determinants (e.g., the affinity for mitochondrial polymerase-γ) are more important.

Recently published results indicated that mutations in the genes coding for the mitochondrial deoxyribonucleoside kinases dGK or thymidine kinase 2 (TK2) are associated with mtDNA depletion *(37,38)*. Because the main supply of deoxyribonucleotides for mitochondrial synthesis comes from the salvage pathway and the rate-limiting step in this pathway is the phosphorylation by dGK or TK2, it is likely that dGK and TK2 are involved in the maintenance of balanced mitochondrial deoxyribonucleotide pools. Although it has been shown that ara-G is predominantly incorporated into mtDNA, the contribution of dGK for the cytotoxicity of ara-G in vivo is not known.

It has been demonstrated that the antiviral compounds 2′,3′-dideoxycytidine, 2′3′-dideoxyinosine, and 2′,3′-didehydro-3′-deoxythymidine induce mitochondrial impairment by reducing the amount of mtDNA. Tissues that are affected by 3′-azido-2′,3′-dideoxythymidine (AZT) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) toxicity consist mainly of nondividing cells. These cells contain undetectable levels of thymidine kinase 1, and it is likely that TK2 is responsible for phosphorylation of the analogs in these tissues.

The importance of nucleoside analog phosphorylation in the mitochondrial matrix for mtDNA damage is, however, still unresolved. The mitochondrial location of TK2 has been suggested to be important for the mitochondrial toxicity of AZT and FIAU *(30)*. It was shown that the human equilibrative nucleoside transporter 1 is expressed also in the mitochondrial

membrane, and that the expression of this transporter in the mitochondrial membrane enhances the toxicity of nucleoside analogs such as FIAU *(39)*. This strengthens the hypothesis that TK2 would be important for mitochondrial toxicity of compounds such as FIAU and AZT. However, 2′,3′-dideoxycytidine, which is initially phosphorylated by dCK outside the mitochondria, does also cause mtDNA damage *(40)*. The identification of a carrier in the mitochondrial membrane that catalyzes the transport of all four deoxy nucleoside analogs and the corresponding deoxyribonucleoside triphosphates in exchange for deoxyribonucleoside diphosphates, adenosine 5′-diphosphate, or adenosine triphosphate provides a possible entering mechanism for phosphorylated analogs into the mitochondrial matrix *(41)*. Although it is suspected that ara-G and CdA might cause mitochondrial toxicity, no purine nucleoside analog, as a substrate of mitochondrial dGK, has yet been shown to induce mitochondrial toxicity.

In addition to ara-G and CdA, another dGuo analog called dFdG (2′,2′-difluorodeoxyguanosine) has been tested as an antileukemic compound. dFdG is also a substrate of dGK, and in vitro studies have shown that it displays potent toxicity to human leukemic cells *(42)*. However, in the preliminary toxicology testing in patients, it caused several deaths because of cardiac toxicity *(43)*. The compound dFdG was subsequently removed from development because of the severe toxicity it caused in patients, and although it has not been proven, the toxicity is assumed to be caused by mitochondrial toxicity.

5. RESISTANCE

So far, nelarabine has only been tested in patients who have been refractory to other treatments. The spectrum of different responses to nelarabine reflects the intrapatient variability and the treatments to which they have been exposed before nelarabine was introduced. No study to reveal the mechanism of resistance to nelarabine has been performed with clinical samples. However, several studies of mechanisms of resistance to ara-G have been performed in cell lines in vitro. These studies have shown partly conflicting results regarding the molecular mechanism of resistance.

Fridland et al. reported ara-G resistance associated with loss of dCK activity *(10)*, whereas ara-G resistance has been reported to occur with retained dCK activity in other cell lines *(16,44,45)*. A study showed that ara-G resistance can occur by two separate molecular mechanisms that can occur sequentially. The first mechanism is associated with a decrease of ara-G incorporation into mtDNA, and the second event is associated with loss of dCK activity *(19)*. In another study, it was also suggested that loss of dCK activity is associated with a higher level of resistance to ara-G *(30,45)*.

It is not yet known how the decreased incorporation of ara-G into mtDNA contributes to the resistance to ara-G, but from a recent study it is known that the acute effect of ara-G does not render mtDNA depletion or altered translation of mtDNA encoded genes *(21)*. Resistance to nucleoside analogs is often caused by several molecular mechanisms, and there is a need for further studies in this area.

6. CONCLUDING REMARKS

It has been shown that nelarabine has activity as a single agent in patients with T-cell malignancies that have relapsed or are refractory to other therapy. Until now, nelarabine has not been approved for the treatment of any disease in any country. However, the ongoing research on nelarabine has earned fast-track status from the US Food and Drug Administration for the treatment of those with T-cell acute lymphoblastic leukemia and lymphoblastic lymphoma who have not responded to or whose disease has progressed during treatment with at least two standard regimens. It is likely that nelarabine will be a useful drug in the treatment of some leukemic diseases in the future, and it is therefore important to learn more about its actions in vivo.

REFERENCES

1. Giblett ER, Ammann AJ, Wara DW, Sandman R, Diamond LK. Nucleoside-phosphorylase deficiency in a child with severely defective T-cell immunity and normal B-cell immunity. Lancet 1975;7914:1010–1013.
2. Reist EJ, Goodman L. Synthesis of 9-β-D-arabinofuranosylguanine. Biochemistry 1964;127:15–18.
3. Johansson M, Karlsson A. Differences in kinetic properties of pure recombinant human and mouse deoxycytidine kinase. Biochem Pharmacol 1995;2:163–168.
4. Rodriguez CO Jr, Mitchell BS, Ayres M, Eriksson S, Gandhi V. Arabinosylguanine is phosphorylated by both cytoplasmic deoxycytidine kinase and mitochondrial deoxyguanosine kinase. Cancer Res 2002;11:3100–3105.
5. Gudas LJ, Ullman B, Cohen A, Martin DW Jr. Deoxyguanosine toxicity in a mouse T lymphoma: relationship to purine nucleoside phosphorylase-associated immune dysfunction. Cell 1978;3:531–538.
6. Carson DA, Kaye J, Seegmiller JE. Lymphospecific toxicity in adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency: possible role of nucleoside kinase(s). Proc Natl Acad Sci USA 1977;12:5677–5681.
7. Mitchell BS, Mejias E, Daddona PE, Kelley WN. Purinogenic immunodeficiency diseases: selective toxicity of deoxyribonucleosides for T cells. Proc Natl Acad Sci USA 1978;10:5011–5014.
8. Scharenberg JG, Spaapen LJ, Rijkers GT, Wadman SK, Staal GE, Zegers BJ. Mechanisms of deoxyguanosine toxicity for human T and B lymphocytes. Adv Exp Med Biol 1986;pt B:191–199.
9. Shewach DS, Mitchell BS. Differential metabolism of 9-β-D-arabinofuranosylguanine in human leukemic cells. Cancer Res 1989;23:6498–6502.

10. Fridland A, Verhoef V. Metabolism and selectivity of arabinonucleoside in human lymphoid cells. Proc Soc Exp Biol Med 1985;4:456–462.
11. Verhoef V, Fridland A. Metabolic basis of arabinonucleoside selectivity for human leukemic T- and B-lymphoblasts. Cancer Res 1985;8:3646–3650.
12. Rodriguez CO Jr, Stellrecht CM, Gandhi V. Mechanisms for T-cell selective cytotoxicity of arabinosylguanine. Blood 2003;5:1842–1848.
13. Genini D, Budihardjo I, Plunkett W, et al. Nucleotide requirements for the in vitro activation of the apoptosis protein-activating factor-1-mediated caspase pathway. J Biol Chem 2000;1:29–34.
14. Bromidge T, Frewin R, Johnson S. In vitro chemo-sensitivity of B-chronic lymphocytic leukaemia to ara-G. Leuk Res 2000;7:623–626.
15. O'Brien SG, Goldman JM. Busulfan alone as cytoreduction before autografting for chronic myelogenous leukemia. Blood 1998;3:1091, 1092.
16. Shewach DS, Daddona PE, Ashcraft E, Mitchell BS. Metabolism and selective cytotoxicity of 9-β-D- arabinofuranosylguanine in human lymphoblasts. Cancer Res 1985;3:1008–1014.
17. Gandhi V, Plunkett W, Rodriguez CO Jr, et al. Compound GW506U78 in refractory hematologic malignancies: relationship between cellular pharmacokinetics and clinical response. J Clin Oncol 1998;11:3607–3615.
18. Rodriguez CO Jr, Gandhi V. Arabinosylguanine-induced apoptosis of T-lymphoblastic cells: incorporation into DNA is a necessary step. Cancer Res 1999;19: 4937–4943.
19. Curbo S, Zhu C, Johansson M, Balzarini J, Karlsson A. Dual mechanisms of 9-β-D-arabinofuranosylguanine resistance in CEM T-lymphoblast leukemia cells. Biochem Biophys Res Commun 2001;1:40–45.
20. Zhu C, Johansson M, Karlsson A. Differential incorporation of 1-β-D-arabinofuranosylcytosine and 9-β-D-arabinofuranosylguanine into nuclear and mitochondrial DNA. FEBS Lett 2000;2–3:129–132.
21. Curbo S, Zhivotovsky B, Johansson M, Karlsson A. Effects of 9-β-D-arabinofuranosylguanine on mitochondria in CEM T-lymphoblast leukemia cells. Biochem Biophys Res Commun 2003;4:942–947.
22. Arpaia E, Benveniste P, Di Cristofano A, et al. Mitochondrial basis for immune deficiency. Evidence from purine nucleoside phosphorylase-deficient mice. J Exp Med 2000;12:2197–2208.
23. Hebert ME, Greenberg ML, Chaffee S, et al. Pharmacologic purging of malignant T cells from human bone marrow using 9-β-D-arabinofuranosylguanine. Transplantation 1991;4:634–640.
24. Krenitsky TA, Koszalka GW, Tuttle JV. Purine nucleoside synthesis, an efficient method employing nucleoside phosphorylases. Biochemistry 1981;12:3615–3621.
25. Lambe CU, Averett DR, Paff MT, Reardon JE, Wilson JG, Krenitsky TA. 2-Amino-6-methoxypurine arabinoside: an agent for T-cell malignancies. Cancer Res 1995;15:3352–3356.
26. Gandhi V, Plunkett W. Modulation of arabinosylnucleoside metabolism by arabinosylnucleotides in human leukemia cells. Cancer Res 1988;2:329–334.
27. Gandhi V, Plunkett W. Interaction of arabinosyl nucleotides in K562 human leukemia cells. Biochem Pharmacol 1989;20:3551–3558.
28. Gandhi V, Plunkett W, Weller S, et al. Evaluation of the combination of nelarabine and fludarabine in leukemias: clinical response, pharmacokinetics, and pharmacodynamics in leukemia cells. J Clin Oncol 2001;8:2142–2152.

29. O'Brien S, Thomas D, Kantarjian HM, et al. Compound 506 has activity in mature lymphoid leukemia. Blood 1998;92:490a–491a.
30. Lewis W, Dalakas MC. Mitochondrial toxicity of antiviral drugs. Nat Med 1995;5:417–422.
31. Feng JY, Johnson AA, Johnson KA, Anderson KS. Insights into the molecular mechanism of mitochondrial toxicity by AIDS drugs. J Biol Chem 2001;26: 23,832–23,837.
32. Kurtzberg J, Ernst TJ, Keating MJ, et al. A phase I study of 2-amino-9-β-D-arabinofuranosyl-6-methoxy-9H-purine (nelarabine) administered on a consecutive five day schedule in children and adults with refractory hematologic malignancies. Blood 1999:629a. Abstract 2794.
33. Brinkman K, ter Hofstede HJ, Burger DM, Smeitink JA, Koopmans PP. Adverse effects of reverse transcriptase inhibitors: mitochondrial toxicity as common pathway. AIDS 1998;14:1735–1744.
34. Aguayo A, Cortes JE, Kantarjian HM, et al. Complete hematologic and cytogenetic response to 2-amino-9-β-D-arabinosyl-6-methoxy-9H-guanine in a patient with chronic myelogenous leukemia in T-cell blastic phase: a case report and review of the literature. Cancer 1999;1:58–64.
35. Galmarini CM, Mackey JR, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. Leukemia 2001;6:875–890.
36. Hentosh P, Tibudan M. 2-Chloro-2′-deoxyadenosine, an antileukemic drug, has an early effect on cellular mitochondrial function. Mol Pharmacol 1997;4:613–619.
37. Mandel H, Szargel R, Labay V, et al. The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. Nat Genet 2001;3:337–341.
38. Saada A, Shaag A, Mandel H, Nevo Y, Eriksson S, Elpeleg O. Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. Nat Genet 2001;3:342–344.
39. Lai Y, Tse CM, Unadkat JD. Mitochondrial expression of the human equilibrative nucleoside transporter 1 (hENT1) results in enhanced mitochondrial toxicity of antiviral drugs. J Biol Chem 2004;6:4490–4497.
40. Chen CH, Cheng YC. The role of cytoplasmic deoxycytidine kinase in the mitochondrial effects of the anti-human immunodeficiency virus compound, 2′,3′-dideoxycytidine. J Biol Chem 1992;5:2856–2859.
41. Dolce V, Fiermonte G, Runswick MJ, Palmieri F, Walker JE. The human mitochondrial deoxynucleotide carrier and its role in the toxicity of nucleoside antivirals. Proc Natl Acad Sci USA 2001;5:2284–2288.
42. Gandhi V, Mineishi S, Huang P, et al. Difluorodeoxyguanosine: cytotoxicity, metabolism, and actions on DNA synthesis in human leukemia cells. Semin Oncol 1995;4(suppl 11):61–67.
43. Andis SL, Bewley JR, Boder GB, et al. Medicinal chemistry of difluoropurines. Semin Oncol 1995;4(suppl 11):54–60.
44. Shewach DS, Mitchell BS. Characterization of arabinosylguanine resistance in a lymphoblastoid cell line. Adv Exp Med Biol 1986;pt B:605–609.
45. Lotfi K, Mansson E, Peterson C, Eriksson S, Albertioni F. Low level of mitochondrial deoxyguanosine kinase is the dominant factor in acquired resistance to 9-β-D-arabinofuranosylguanine cytotoxicity. Biochem Biophys Res Commun 2002; 5:1489–1496.

11 Gemcitabine

Mechanism of Action and Resistance

Andries M. Bergman, MD, PhD
and Godefridus J. Peters, PhD

CONTENTS

SUMMARY

Gemcitabine (2′,2′-difluorodeoxycytidine, Gemzar®) is a deoxycytidine analog with pronounced antitumor activity against a variety of solid tumors, such as non-small cell lung carcinoma and pancreatic and bladder cancer. In this chapter, we summarize the role of the key enzymes in its metabolic activation and deactivation pathways, the role of the various targets, the associated mechanisms of acquired and inherent resistance, and the changes in DNA caused by exposure to this drug. Extensive research has revealed a complex mechanism of action of this relatively new drug. Gemcitabine requires phosphorylation to mono-, di-, and triphosphates (dFdCTP) to be active; this mechanism results in a unique pattern of self-potentiation of the drug, ultimately resulting (when incorporated into the DNA) in a masked chain termination. Similar to the structurally and functionally related deoxycytidine analog 1-β-D-arabinofuranosylcytosine (ara-C), the first, crucial step in phosphorylation is catalyzed by deoxycytidine kinase (dCK). However, unlike ara-C, gemcitabine has multiple intracellular targets; up- or downregulation of these targets may confer resistance to this drug. Resistance is associated

From: *Cancer Drug Discovery and Development:*
Deoxynucleoside Analogs in Cancer Therapy
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

with altered activities of enzymes (e.g., dCK) involved in the metabolism of the drug, of target enzymes (e.g., ribo-nucleotide reductase), and of enzymes involved in programmed cell death. Strong correlations with gemcitabine sensitivity have been observed for dCK activity and dFdCTP pools; microarray analyses have suggested a potentially important role for ribonucleotide reductase.

Key Words: Deoxycytidine kinase; DNA repair; gemcitabine; masked chain termination; ribonucleotide reductase; self-potentiation; thymidine kinase 2.

1. INTRODUCTION

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC, Gemzar®), a deoxy-cytidine (dCyd) analog with two fluorine atoms substituted at the 2′-position of the ribose ring, was synthesized in the 1980s by Eli Lilly (Indianapolis, IN) *(1,2)*. First, it was screened for antiviral activity; however, the drug seemed more promising as a cytostatic than as an antiviral drug. Because the structurally and functionally related dCyd analog 1-β-D-arabinofuranosylcytosine (ara-C) is active in leukemia, gemcitabine was initially screened for activity in hematological malignancies *(3)*. Surprisingly, unlike ara-C, gemcitabine had remarkable activity in solid tumors *(4–7)*. Further research on the mechanism of action of this drug revealed a far more complicated metabolism than that of ara-C, with several self-potentiating mechanisms (Fig. 1). These self-enforcing mechanisms might explain its potency to a variety of malignancies.

In the clinic, gemcitabine is used in combination with other drugs for the treatment of inoperable or metastasized non-small cell lung cancer (NSCLC) and bladder cancer and as a single agent for the treatment of locally advanced or metastasized adenocarcinoma of the pancreas *(8–10)*.

Drug resistance to cytostatic agents is a major problem in the treatment of neoplasms with chemotherapy. Resistance can be either inherent or acquired. Inherent resistance is a quality of several tumor types and is reflected in low response rates in clinical trials *(11)*. Acquired resistance can develop during treatment by selection of cells from a heterogeneous tumor cell population or changes within the cancer cells. Moreover, cells resistant to a closely related drug (e.g., ara-C) might also be resistant to gemcitabine (crossresistant) or be more sensitive (collateral sensitive).

In this chapter, the metabolism of gemcitabine and its mechanism of action are discussed. Theoretically, sensitivity to gemcitabine is determined by the drug's ability to enter the cell; the activity of the catabolizing enzymes, anabolizing enzymes, target enzymes; and cells' ability to repair deoxyribonucleic acid (DNA) damage. A change in activity in the above-mentioned factors might lead to changes in sensitivity of cancer

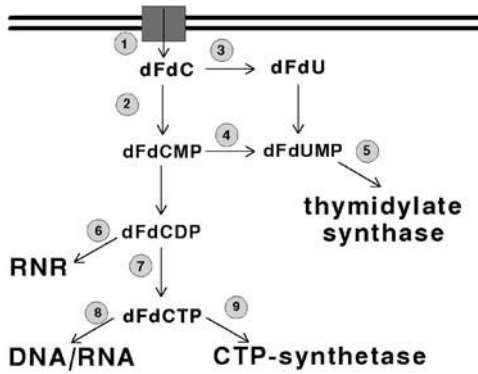


Fig. 1. Metabolism and mechanisms of action of gemcitabine: 1, transport across the cell membrane; 2, phosphorylation of gemcitabine by dCK and TK2; 3, deamination of gemcitabine by dCDA; 4, deamination of gemcitabine by dCMP-deaminase; 5, inhibition of thymidylate synthase by dFdUMP; 6, inhibition of ribonucleotide reductase (RNR) by dFdCDP; 7, accumulation of the triphosphate dFdCTP; 8, incorporation into DNA and RNA; and 9, inhibition of CTP-synthetase by dFdCTP. (Reproduced with permission from ref. *113*.)

cells to gemcitabine. Moreover, altered sensitivity to gemcitabine might be multifactorial. For every step in the metabolism of gemcitabine, the relation to activity of the drug is discussed separately.

2. MECHANISM OF ACTION

2.1. Transport Over the Cell Membrane

2.1.1. Mechanism

Because dCyd and gemcitabine are hydrophilic, they require specialized transport systems for the passage of nucleoside analogs in or out of cells *(12)* (Fig. 2). There are seven distinct carriers for the transport of nucleosides; these are either of the sodium-dependent type (concentrative nucleoside transporter) (CNT) or of the sodium-independent type (equilibrative nucleoside transporter) (ENT) *(13)*. ENTs are classified into two major subtypes: the equilibrative sensitive (*es*) and the equilibrative inhibitor (*ei*) resistant transporters *(13–16)*. Studies in *Xenopus laevis* oocytes overexpressing nucleoside transporters of the CNT and ENT types revealed that gemcitabine is transported by several proteins, except the purine-selective transporters of the CNT type *(17,18)*.

Multidrug resistance proteins (MRPs) pump multiple agents actively out of the cell. Of the MRP family, currently seven members have been identified, all with different drug specificities *(19)*. As far as is known, gemcitabine is only a substrate for the MRP4 and MRP5 efflux pumps *(20)*.

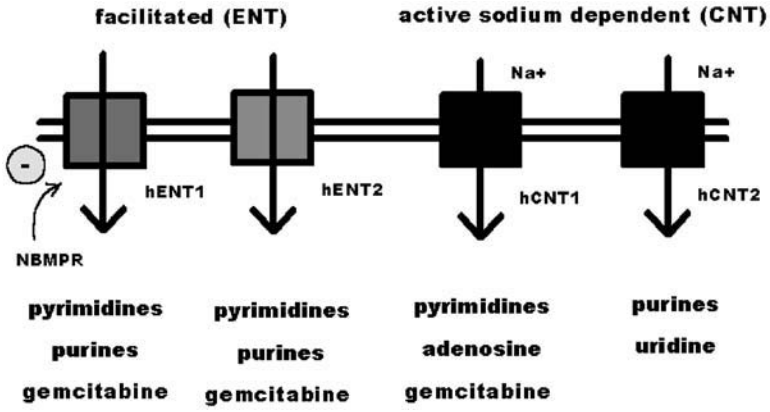


Fig. 2. Classification of human nucleoside transporters (NTs) and substrate specificity of the characterized representatives. ENT, equilibrative NT; CNT, concentrative NT; NBMPR, nitrobenzylmercaptopurine ribonucleoside; *es*, equilibrative and NBMPR inhibition sensitive; *ei*, equilibrative and NBMPR inhibition insensitive. hENT1, hENT2, hCNT1, and hCNT2 are the cloned representatives of the different transporters.

2.1.2. Role in Drug Sensitivity

Several studies in human cancer cell lines reported that inhibition of the facilitated, diffusion-mediated, nucleoside transport resulted in up to a 100-fold decrease in sensitivity to gemcitabine, and that human and murine cells with a nucleoside transport deficiency were highly resistant to gemcitabine *(21–23)*. However, not every cell with an acquired resistance to gemcitabine displays altered nucleoside transport. In those with gemcitabine-resistant variants of head and neck squamous carcinoma and erythroleukemic, no altered nucleoside transport capacity was found *(14,24)*. Moreover, no relation between basal *es* nucleoside transporter and gemcitabine sensitivity could be demonstrated in different human pancreatic cancer cell lines *(25)*. Although gemcitabine is a substrate for MRP4 and MRP5, no in vitro relation between activity of the membrane efflux pump and sensitivity to gemcitabine is reported yet.

2.2. Phosphorylation of Gemcitabine

2.2.1. Mechanism

Once in the cell, ara-C and gemcitabine are phosphorylated to mono-, di-, and triphosphates prior to incorporation into DNA, which is required for their growth-inhibiting activity *(3)*. The first step in phosphorylation is catalyzed by deoxycytidine kinase (dCK), further phosphorylation to di- and triphosphates is catalyzed by less-specific kinases *(3)*. dCK has a K_m

Table 1
Enzymes Involved in Gemcitabine Metabolism, Natural Substrates, and K_m Values of Gemcitabine and ara-C

Enzyme	*Source*	*Substrate*	K_m (μM)	V_m/K_m
dCK	Human leukemic cells	Deoxycytidine	1.5[a]	3.7[a]
	Human leukemic spleen	Deoxyadenosine	120[b]	5[b]
	Human leukemic spleen	Deoxyguanosine	50[b]	5[b]
	Human leukemic cells	Gemcitabine	4.6[a]	14.9[a]
	MOLT-4 human lymphoblasts	Ara-C	14.8[c]	4.2[c]
TK2	Human leukemic spleen	Thymidine	0.3 and 16[d]	1.9[d]
	Human leukemic spleen	Deoxycytidine	36[d]	25[d]
	Human leukemic spleen	Deoxyuridine	6[d]	115[d]
	Human brain cells	Gemcitabine	66[e]	n.d.
	Human brain cells	Ara-C	800[e]	0.8[e]
dCDA	Human leukemic cells	Deoxycytidine	46.3[a]	23.8[a]
	Human leukemic cells	Gemcitabine	95.7[a]	12.5[a]
	Human liver cells	Ara-C	270[f]	15.6[f]

n.d., not determined.
[a]Ref. *26*.
[b]Ref. *114*.
[c]Ref. *36*.
[d]Ref. *115*; biphasic kinetics of TK2 with thymidine as a substrate.
[e]Ref. *27*.
[f]Ref. *116*.
(From ref. *113* with permission.)

value of 4.6 μ*M* for gemcitabine compared to 1.5 μ*M* for dCyd, which makes gemcitabine a good substrate for dCK *(26)*. In addition, it has better efficacy, as shown by the favorable V_m/K_m ratio *(3)* (Table 1). This first step in phosphorylation is the rate-limiting step for further phosphorylation to active metabolites and thus is essential for the activation of gemcitabine. For this reason, dCK plays a pivotal role in gemcitabine activation.

Gemcitabine is also phosphorylated by the mitochondrial enzyme thymidine kinase 2 (TK2), but less efficiently than by dCK *(27)*. The substrate specificity of gemcitabine for TK2 is only 5–10% of the specificity of dCyd. In contrast, the cytosolic cell cycle-dependent enzyme thymidine kinase 1 is not able to phosphorylate dCyd and gemcitabine *(27)*. In reverse, phosphorylating activity is counteracted by 5′-nucleotidases (5′-NTs), which degrades the nucleotide to a lower number of phosphate groups *(24)*.

Gemcitabine is inactivated by deamination, catalyzed by deoxycytidine deaminase (dCDA) to 2′,2′-difluorodeoxyuridine (dFdU) (K_m for dCyd and gemcitabine of 46.3 and 95.7 μ*M*, respectively) *(26)* (Table 1). In addition, the

monophosphate dFdCMP can be deaminated by the action of deoxycytidylate deaminase (dCMP-deaminase), to 2′,2′-difluorodeoxyuridine monophosphate (dFdUMP), which is further degraded to dFdU and transported out of the cell *(28)*. However, dCMP-deaminase is inhibited by dFdCTP, which is one of gemcitabine's mechanisms of self-potentiation *(29)*.

2.2.2. Role in Drug Sensitivity

In a panel of solid tumor cell lines, intrinsic resistance to gemcitabine correlated with dCK activity in the cell *(30)*. Also, in various murine tumors and human tumor xenografts, a clear correlation between dCK levels and gemcitabine sensitivity was found *(31)*.

Human ovarian cancer cells with an acquired highly gemcitabine-resistant phenotype were dCK deficient *(32)*. When these cells were transfected with the dCK gene, various clones of transfected cells were formed with different levels of dCK activity, which strongly correlated with gemcitabine sensitivity *(33)*. Altered dCK activity is the most frequently described mechanism of acquired resistance to gemcitabine in vitro (Table 2). Only a few authors described a discrepancy between dCK expression or activity and acquired resistance to gemcitabine *(30,34)*.

Cells made resistant to other nucleoside analogs can display crossresistance to gemcitabine *(3,24,35)*. Murine and rat leukemia cells with an acquired resistance to ara-C were shown to be crossresistant to gemcitabine, which was related to altered dCK activity *(35)*.

Not only the degree of expression of the dCK enzyme, but also its regulation determines its phosphorylating activity. In a study with purified dCK enzyme from leukemia cells, the best phosphorylating efficiency was achieved with uridine triphosphate (UTP) as the phosphate donor *(36)*. The decreased UTP pools found in gemcitabine-resistant rat and murine human cancer cells may contribute to their gemcitabine resistance *(30,32,35)*. Because the deoxynucleotide dCTP is the most prominent feedback inhibitor of dCK, increased dCTP pools will decrease phosphorylation of gemcitabine and reduce its activity, as was found in gemcitabine-resistant human oropharyngeal epidermoid carcinoma cells *(37)*.

Although gemcitabine is a poor substrate, the mitochondrial enzyme TK2 could play an important role in sensitivity to gemcitabine. Because dCK phosphorylates dCyd and gemcitabine efficiently and TK2 phosphorylates dCyd far more efficiently than gemcitabine, some authors suggested that an increase in a dCK/TK2 activity ratio may lead to increased sensitivity to gemcitabine *(38,39)*.

5′-NT opposes the action of nucleoside kinases by catalyzing the conversion of nucleotides back to nucleosides. The kinetic properties of the human cytosolic 5′-NT II (cN-II) make it unlikely to contribute to pyrimidine dephosphorylation *(40)*. However, gemcitabine resistance of human

Table 2
Summary of the Studies on the Relation Between Deoxycytidine Kinase (dCK) Activity or mRNA Expression and Sensitivity to Gemcitabine in Cell Lines and Human Xenografts

Tumor	*Resistance to*	*Technique*	*dCK*	*Related*	*Reference*
Human ovarian cancer cells	Gemcitabine	Continuous exposure	Activity	+	*32*
Human ovarian cancer cells		transfection	Activity	+	*96*
Human erythroleukemic cells	Gemcitabine	Continuous exposure	Activity	+	*24*
Human oropharyngeal cells	Gemcitabine	Continuous exposure	Activity	+	*37*
Murine leukemia cells	Gemcitabine	Continuous exposure	Activity	+	*117*
Human folicular lymphoma cells	Gemcitabine	Continuous exposure	mRNA	+	*118*
Human NSCLC cells	Gemcitabine	Continuous exposure	mRNA	-	*34*
Human NSCLC cells	Gemcitabine	Continuous exposure	mRNA	+	*119*
Human NSCLC cells	Gemcitabine	Continuous exposure	Activity	+	*120*
Human lung cancer cells		transfection	Activity	-	*30*
Murine colon cancer	Gemcitabine	Continuous exposure	mRNA	+	*63*
Human colon xenografts		transfection	Activity	+	*121*
Intrinsic resistance					
Various solid tumor cells			Activity	+	*33*
Human NSCLC cells			Activity	+	*69*
Various xenografts			Activity	+	*33*
Human pancreatic cancer cells			mRNA	+	*122*
Various human xenografts			Activity	+	*31*
Various human xenografts			Activity	+	*123*
Various solid tumor cells			Activity	+	*124*

(Continued)

Table 2 *(Continued)*

Tumor	*Resistance to*	*Technique*	*dCK*	*Related*	*Reference*
Crossresistance					
Chinese hamster ovary	Ara-C	Continuous exposure	Activity	+	*3*
Murine and rat leukemia cells	Ara-C	Continuous exposure	Activity	+	*35*
Human erythroleukamic cells	Ara-C	Continuous exposure	Activity	+	*24*
Human promyelocytic cells	Cladribine	Continuous exposure	Activity	-	*125*
Human erythroleukemic cells	Cladribine	Continuous exposure	Activity	+	*24*
Human erythroleukemic cells	Fludarabine	Continuous exposure	Activity	+	*24*
Human NSCLC cells		P-gp / MRP transfection[a]	Activity	+	*45*
Human NSCLC cells	Daunorubicine	Continuous exposure[a]	Activity	+	*38*
Human NSCLC cells	VM-26	Continuous exposure	Activity	+	*38*

ara-C, 1-β-D-arabinofuranosylcytosine; cladribine, 2-chlorodeoxyadenosine; fludarabine, 2-fluoroadenine-β-D-arabinoside; NSCLC, non-small cell lung carcinoma. Gemcitabine, ara-C, cladribine, and fludarabine all require phosphorylation by dCK to become active.

[a]Collateral sensitivity.

erythroleukemic cells was associated with increased 5′-NT activity *(24)*. Moreover, transfection of the 5′-NT I gene resulted in cells less sensitive to gemcitabine *(41)*. However, studies of the relation between altered 5′-NT activity and nucleoside analog sensitivity were unequivocal.

dCDA effectively inactivates gemcitabine to dFdU (K_m for dCyd and gemcitabine of 46.3 and 95.7 μ*M*, respectively) *(26)*. Murine and human cells overexpressing dCDA by transfection were resistant to ara-C and gemcitabine *(42–44)*, which was completely reversed by the addition of a specific dCDA inhibitor *(42)*. Collateral sensitivity to gemcitabine in anthracyclin-resistant variants of human lung cancer cells was associated with decreased dCDA activity *(38,45)*. However, the role of dCDA in gemcitabine sensitivity is not clear because in murine and human cells with an acquired resistance or crossresistance to gemcitabine, dCDA activity was unchanged compared to that of parental cells or even decreased *(32,35)*. In a panel of five parental solid tumor cell lines and several head-and-neck, ovarian, and colon xenografts, no relation between dCDA activity and inherent sensitivity to gemcitabine was found *(33)*. In addition, the ratio dCK/dCDA in a panel of 28 xenografts was not related to sensitivity *(46)*.

Another pathway of inactivation is deamination of dFdCMP to dFdUMP by the action of dCMP-deaminase and subsequently to dFdU *(28)*. Because gemcitabine and dFdU are not substrates for pyrimidine nucleoside phosphorylases, dFdU is not further degraded and excreted out of the cell *(28)*.

In one study, an inhibitor of dCMP-deaminase affected the degradation of dFdCTP in human leukemia cells, indicating a role of this enzyme in accumulation of the active metabolite *(28)*.

2.3. Accumulation of Triphosphates and Incorporation Into DNA and Ribonucleic Acid

2.3.1. Mechanism

The triphosphate dFdCTP is incorporated into DNA, which is considered the most important mechanism of action of gemcitabine *(3)*. After incorporation of dFdCTP into DNA, one more nucleotide is incorporated before polymerization stops *(47)*. This so-called masked chain termination after incorporation of dFdCTP prevents exonucleases from excising dFdCMP from DNA *(47)*. Further mechanistic studies of chain termination revealed that incorporated gemcitabine into DNA is recognized by a DNA-dependent protein kinase (DNA-PK) and p53 protein complex *(48)*. After gemcitabine treatment, the quantity of this complex will increase, and p53 will increasingly be phosphorylated at Ser15. Although the wild-type p53 present in the protein complex exhibits 3′-5′ exonuclease activity, it is incapable of excising the incorporated gemcitabine from DNA, resulting in stalling of this complex at the site of drug incorporation *(48)*.

Moreover, dFdCTP is also incorporated into ribonucleic acid (RNA), after which RNA synthesis is completely blocked in leukemia cells and severely hampered in solid tumor cells *(49)*. However, the contribution of incorporation into RNA to the growth-inhibiting properties of gemcitabine is still unclear.

2.3.2. Role in Drug Sensitivity

Accumulation of dFdCTP in solid tumor cell lines is dose and exposure time dependent *(50)*. In a panel of 21 cell lines, dFdCTP concentrations related to sensitivity to gemcitabine in vitro, underlining the important role of dFdCTP *(51)*. In AG6000 cells with an acquired resistance to gemcitabine as a result of dCK deficiency, no dFdCTP was accumulated *(32)*. Also, in xenografts of this cell line, no dFdCTP pools could be measured in the resistant tumors after an intraperitoneal injection of 120 mg/kg gemcitabine *(32)*. These results are in agreement with a five-fold decreased dFdCTP pool in gemcitabine-resistant erythroleukemic K562 cells compared to parental cells *(24)*. In human small cell lung cancer cell lines with collateral sensitivity to gemcitabine, increased sensitivity was associated with increased concentrations of accumulated dFdCTP *(38)*.

Another important factor in sensitivity to gemcitabine is the retention of dFdCTP, even when initial accumulation may be limited. In both in vitro and in vivo models, long retention of dFdCTP was associated with increased sensitivity *(33,50)* In addition, in human NSCLC cell lines collaterally sensitive to gemcitabine, no difference in dFdCTP pools was found; however, dFdCTP was retained longer *(44)*. In contrast to elimination of ara-C triphosphate, which is relatively rapid and monophasic, that of dFdCTP is biphasic and much slower *(52)*. This is because of the self-potentiation of gemcitabine, specifically inhibition of dCMP-deaminase, which will prevent degradation, and to the inhibition of cytidine triphosphate (CTP) synthetase and ribonucleotide reductase (RNR). Inhibition of these last two enzymes will decrease the dCTP concentration, which will activate dCK and synthesis of dFdCTP. Overall, these effects will lead not only to increased dFdCTP accumulation, but also to longer retention *(52)*.

dFdCTP is believed to be one of the most important metabolites of gemcitabine, which is reflected in a strong correlation between dFdCTP pools and sensitivity to gemcitabine. However, other factors may play a role because some studies describe a discrepancy between dFdCTP pools and sensitivity to gemcitabine *(50,53)*. Measurement of dFdCTP in target and surrogate tissue such as lymphocytes may be used as a marker for efficacy.

2.4. Intracellular Targets

2.4.1. Mechanism

Unlike ara-C, gemcitabine has several self-potentiating mechanisms of action. Several metabolites of gemcitabine inhibit enzymes involved in

DNA synthesis and repair and salvage of (deoxy)ribonucleotides. The diphosphate dFdCDP is an inhibitor of RNR, which plays a vital role in the provision of DNA building blocks during cell division and DNA repair *(54)*. The enzyme catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which are subsequently phosphorylated to triphosphates prior to incorporation into DNA. Deoxycytidine triphosphate (dCTP) competes with dFdCTP for incorporation into DNA and is a potent inhibitor of dCK activity *(54,55)*. Thus, depletion of dCTP pools by RNR inhibition enhances both dFdC phosphorylation and incorporation into DNA *(37)*.

Although the deaminated products dFdU and dFdUMP were considered inactive metabolites for a long time, dFdU can inhibit growth in human ovarian carcinoma cells; however, dFdU was over 1000-fold less toxic than gemcitabine *(32)*. Because dFdU is present at relatively high concentrations in the plasma of patients (>100 μ*M* for 24 h) *(56–58)*, it may exert some cytotoxicity.

Thymidylate synthase (TS) catalyzes the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate *(59)*. Because dFdUMP resembles dUMP, it might contribute to the growth-inhibiting activity by inhibiting TS *(60)*. In human ovarian cancer cells and murine colon carcinoma cells, TS activity was inhibited 90% after 24-h exposure to gemcitabine *(60)*. However, the value of this putative activity is not known yet. The triphosphate of gemcitabine, dFdCTP, is an inhibitor of CTP synthetase, which catalyzes the conversion of UTP to CTP *(51,61)*. Because CTP competes with dFdCTP for incorporation into RNA, inhibition of CTP synthetase might also contribute to cell growth inhibition.

2.4.2. Role in Drug Sensitivity

Inhibition of RNR by dFdCDP is concentration, time, and cell line dependent and results in a decrease in deoxyribonucleotide pools *(54)*. Because dCTP is a feedback inhibitor of dCK and competes with dFdCTP for incorporation into DNA, depletion of dCTP pools will lead to increased phosphorylation of gemcitabine and incorporation into DNA *(54)*. Therefore, this self-potentiating mechanism of gemcitabine is considered an important one, and some authors believe it is a more important determinant for gemcitabine sensitivity than dCK activity.

Human erythroleukemic cells with an acquired gemcitabine resistance had increased RNR activity; however, dCK activity was decreased *(24)* (Table 3). As a result of an increased RNR activity in human oropharyngeal epidermoid carcinoma cells made resistant to gemcitabine, increased deoxyadenosine 5′-triphosphate and dCTP pools were found *(37)*. No difference was found in dCK expression, but enzyme activity was decreased in the resistant cells. After removal of the endogenous deoxyribonucleotide

Table 3
Summary of the Studies on RNR Activity or mRNA Expression of the Subunits M1 and M2 and Acquired Resistance to Gemcitabine of Cell Lines and In Vivo Tumors

Tumor	*Technique*	*RNR activity*	*RNRM1 mRNA*	*RNRM2 mRNA*	*Reference*
In vitro					
Murine leukemia cells	Continuous exposure			Decreased	*117*
Human pancreas cancer cells	Continuous exposure			Increased	*126*
Human oropharyngeal cancer cells	Continuous exposure	Increased			*127*
Human oropharyngeal cancer cells	Continuous exposure			Increased	*128*
Human oropharyngeal cells	Continuous exposure	Increased			*37*
Human erythroleukemic cells	Continuous exposure	Increased			*24*
Human NSCLC cells	Continuous exposure	No change	Increased		*34*
In vivo					
Murine colon carcinoma	Repeated treatment		Increased		*63*

triphosphate pools, no difference in dCK activity in extracts from parental and variant cells was found, suggesting that increased RNR activity was the only mechanism of gemcitabine resistance.

By using RNA interference mediated by small interfering RNA of the M2 subunit of RNR, evidence was provided that RNR overexpression is associated with gemcitabine chemoresistance in pancreatic adenocarcinoma cells *(62)*. Synergism between RNR subunit M2 small interfering RNA and gemcitabine resulted in markedly suppressed tumor growth, increased tumor apoptosis, and inhibition of metastasis *(62)*. In gemcitabine-resistant human NSCLC cells, microarray expression profiling showed marked increase in the expression of the M1 subunit of RNR that was related to an increase in M1 messenger RNA (mRNA) and protein expression but not with an increased RNR activity *(34)*. The authors suggested that the increased M1 expression, which is the binding site for dFdCDP, might act as a "molecular sink," by which the drug binds to the subunit, which is irreversibly inactivated *(34)*. In a mouse colon cancer with an in vivo acquired resistance to gemcitabine, an increase in RNR M1 expression was found in a microarray expression profiling assay *(63)*. These results demonstrate the importance of RNR inhibition as a target for gemcitabine. Also, clinical evidence for the importance of RNR inhibition is provided. Pretreatment RNR mRNA levels in tumor specimens predicted the efficacy of gemcitabine-containing chemotherapy regimens in patients with NSCLC *(64)*.

Not all reports on the importance of RNR inhibition in the cytotoxic properties of gemcitabine are consistent. Murine leukemia cells made resistant to the RNR inhibitor hydroxyurea showed increased RNR activity but were not crossresistant to gemcitabine *(65)*. Moreover, human leukemia cells resistant to hydroxyurea as a result of increased RNR activity were even more sensitive to gemcitabine *(66)*.

CTP synthetase provides the cell with CTP, which might compete with dFdCTP for incorporation into RNA; however, CTP synthetase activity is also inhibited by the triphosphate. Thus, increased CTP synthetase activity may maintain CTP pools during gemcitabine exposure, contributing to gemcitabine resistance. Only indirect evidence is provided that this mechanism plays a role in gemcitabine sensitivity. Several studies showed a marked change in normal ribonucleotide pools after exposing human colon and ovarian carcinoma cell lines to gemcitabine *(50,67)*. However, in a study of shifts in NTP pools during gemcitabine exposure in various cell lines, both increased and decreased CTP pools were found *(51)*. The authors attributed the decreased CTP pools to the inhibition of CTP synthetase by dFdCTP, and the increased CTP pools to a rise in ATP pools because ATP is a phosphate donor for the synthesis of all NTPs. Also, in cells with an acquired gemcitabine resistance, increased CTP pools are found. In highly gemcitabine-resistant ovarian carcinoma cells and in

murine colon cancer cells with an in vivo induced gemcitabine resistance, increased CTP pools were found compared to the parent *(32,53)*. No evidence is provided that competition between CTP and dFdCTP for incorporation into RNA affects gemcitabine sensitivity.

2.5. Incorporation Into DNA and RNA

2.5.1. Mechanism

After incorporation of dFdCTP catalyzed by DNA polymerases-α and -ε, one more deoxynucleotide is incorporated before DNA polymerization stops, resulting in single-strand DNA damage *(47,68)*. The rate of dFdCTP incorporation depends on activity of the polymerases, competition with dCTP, and the capacity to excise gemcitabine from DNA. Whether the cell dies as a result of gemcitabine incorporation depends on efficacy of DNA repair mechanisms and apoptotic pathways. Gemcitabine is also incorporated into RNA, which is in some cell lines at similar levels as in DNA *(40,69,70)*.

2.5.2. Role in Drug Sensitivity

Incorporation into DNA is considered an important mechanism of gemcitabine-induced cell death. In human glioblastoma cells, gemcitabine was far more cytotoxic than RNR and DNA polymerase-inhibiting drugs, but less effective in inhibiting DNA synthesis, suggesting that incorporation itself of dFdCTP into DNA is a more lethal event *(71)*. Also, in another study dFdCTP did not show potent inhibition against the DNA polymerases-α, -β, and -γ *(72)*. After exposure to gemcitabine at equitoxic and equimolar concentrations, fewer DNA strand breaks were found in gemcitabine-resistant human ovarian carcinoma cells than in the parental cells, suggesting decreased incorporation of gemcitabine into DNA or an increased DNA repair mechanism as a mechanism of gemcitabine resistance *(73)*.

Of the four mammalian DNA repair pathways, a relation between some pathways, or enzymes involved in these pathways, and sensitivity to gemcitabine has been suggested. Although initial reports described an inability of the exonuclease activity of polymerase ε to excise gemcitabine from DNA *(47)*, gemcitabine incorporated into DNA of *Escherichia coli* bacteria was excised by 3′-5′ exonuclease activity. These results suggest that 3′-5′ exonuclease activity might be a mechanism of resistance to gemcitabine *(74,75)*.

Mismatch repair (MMR) is a postreplicative DNA repair process that corrects single-base mismatches in DNA by removing such errors from the newly synthesized strand *(76)*. In human colon cancer and endometrial adenocarcinoma cells with deficient MMR, resistance to gemcitabine was found compared to the MMR-sufficient parental cells, suggesting an important role of MMR in gemcitabine sensitivity *(77)*.

Incorporation of gemcitabine into RNA was time and concentration dependent, and RNA synthesis was inhibited completely in human leukemia

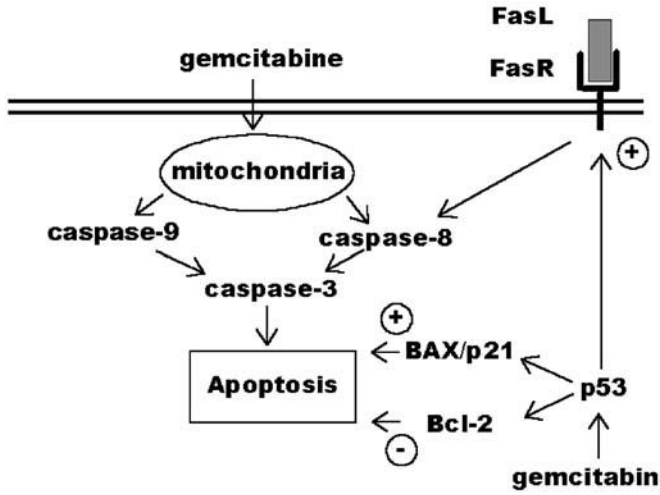


Fig. 3. Schematic representation of the p53-dependent and -independent, gemcitabine-induced apoptotic pathways in NSCLC. In some solid tumor cells lines (e.g., NSCLC), gemcitabine induces apoptosis via a mitochondria-dependent, caspase 8-mediated pathway. This is in contrast to leukemic cells, for example, in which gemcitabine and other cytostatic agents can induce caspase 8 via a Fas receptor-dependent pathway or via a mitochondria-dependent caspase 9 pathway.

cells *(49)*. Although, relations have been found between gemcitabine sensitivity and incorporation into RNA, no evidence has been provided that incorporation into RNA plays an important role in gemcitabine toxicity.

2.6. Induction of Apoptosis

2.6.1. Mechanism

In drug-sensitive cells, cytostatic agents can induce apoptosis irrespective of their intracellular target *(78)*. Also, in gemcitabine-induced cell death, apoptosis seemed to play a significant role *(79)*. Gemcitabine mediates apoptosis by several distinct pathways, both P53 dependent and independent (Fig. 3).

Activation of protein kinase C plays an important role in second messenger pathways in cells with a functional tumor suppressor P53 gene and in the induction of the proapoptotic Bax gene *(80–82)*. Gemcitabine exerts its cytotoxic activity mainly by incorporation into DNA; disruption of further DNA synthesis seems to play a less-important role *(71,72)*. After gemcitabine exposure, a complex of the DNA-PK and p53 is able to interact with the gemcitabine-containing DNA *(48)*. This complex binds to gemcitabine-containing DNA in preference to normal DNA and plays a role in signaling to apoptotic pathways.

Gemcitabine also induces apoptosis via other pathways than P53 (Fig. 3). Apoptosis in gemcitabine-exposed NSCLC cells was positively correlated with induction of the transmembrane receptor Fas, which plays a role in tumor–host interactions *(83)*. Further downstream, events of induction of receptor-mediated apoptosis such as FAS include activation of the caspase 8-initiated cascade *(84,85)*. The observation that in NSCLC cells a cytostatic agent and a caspase 8 peptide inhibitor resulted in a decreased percentage of apoptotic cells is also suggestive for an important role of caspase 8 activity in gemcitabine resistance.

Another possible pathway to apoptosis might be via the mitochondria. Gemcitabine treatment caused apoptosis in multiple myeloma cells as measured by an increase in DNA cleavage, a decrease in the mitochondrial membrane potential as a measure of damage, and activation of caspase activity *(86)*. At 8 h after gemcitabine treatment, cleavage of the caspase substrate poly(ADP-ribose) polymerase and caspase 3 activation were documented. Caspases 8 and 9 were activated by gemcitabine treatment in this cell line, suggesting several mechanisms of action, including death receptor pathway and mitochondrial damage. These results suggest an important role for a caspase 8 mitochondria-dependent cascade in gemcitabine-induced apoptosis in solid tumor cells *(85)*.

The nuclear factor κB (NF-κB) was increased in gemcitabine-resistant pancreatic cancer cells and associated with decreased apoptosis. Moreover, NF-κB was induced in sensitive cells during gemcitabine exposure *(87)*. An inhibitor of NF-κB restored sensitivity to gemcitabine in these cells. These results indicate an important role of this transcription factor in gemcitabine sensitivity.

In one study, the authors suggested that gemcitabine-induced apoptosis is mediated by the MKK3/6-p38 mitogen-activated protein kinase (MAPK)-caspase signaling pathway *(88)*. In human pancreatic cancer cell lines, gemcitabine induced apoptosis by specific activation of p38 MAPK, a member of the MAPK superfamily *(88)*. A selective p38 MAPK inhibitor significantly inhibited gemcitabine-induced apoptosis, suggesting that phosphorylation of p38 MAPK might play a key role in gemcitabine-induced apoptosis in pancreatic cancer cells. Furthermore, gemcitabine-induced cleavage of the caspase substrate poly(ADP-ribose) polymerase was inhibited by pretreatment with a p38 MAPK inhibitor, suggesting that activation of p38 MAPK by gemcitabine induces apoptosis through caspase signaling *(88)*.

2.6.2. Role in Drug Sensitivity

The p53 status of cancer cells influences their sensitivity to gemcitabine cytotoxicity. Evidence is provided that loss of p53 function leads to loss of cell cycle control and alterations in the apoptotic cascade, conferring resistance to gemcitabine in cancer cell lines displaying a mutant p53 *(89)*. Reintroducing wild-type p53 in several pancreatic cancer cell lines resulted

in a decreased percentage of cells in S phase and increased sensitivity to gemcitabine *(90)*.

In pancreatic cancer cells, which have generally an inherent resistance to 5-fluorouracil and gemcitabine, a Bax/Bcl-2 ratio was predictive for chemotherapy sensitivity, whereas Bcl-X(L) levels following repeated exposure to 5-fluorouracil or gemcitabine were associated with resistance to these drugs *(91)*. These findings suggest that the activation of antiapoptotic genes after repeated drug exposure contributes to chemoresistance of pancreatic cancer cells. Transfection of human breast cancer cells with complementary DNA of the Bcl-2 gene resulted in reduced sensitivity to gemcitabine, and the use of antisense mRNA of Bcl-X(L) in pancreatic cancer cells increased sensitivity to gemcitabine *(92,93)*. Transfection of pancreatic cancer cells with the Bax-α gene resulted in increased expression of Bax, activation of the caspase 8 and caspase 3 pathways, and increased sensitivity to gemcitabine *(94)*. Apparently, sensitivity to gemcitabine is closely correlated to activity of suicide genes whether or not P53 mediated.

3. GENOMIC ALTERATIONS AND SENSITIVITY

Exposure to antimetabolite drugs results in genomic instability, which can eventually lead to gene deletions and gene mutations. These genomic alterations might result in resistance to antimetabolite drugs. Also, gemcitabine introduces genomic instability in exposed cells. Gemcitabine exposure induced chromatid exchange and micronuclei in mortal and transformed fibroblasts *(95)*. If these genomic alterations occur in genes involved in gemcitabine metabolism or target enzymes, this might result in altered sensitivity to this drug. In ovarian cancer cells with an acquired resistance to gemcitabine, an absent dCK activity was found that was associated with an altered splice variant by deletion of exon 3 *(96)*. Also, altered activity of oncogenes like *myc* and *ras*, which are frequently amplified in tumor cell lines *(97,98)*, is associated with development of resistance to antimetabolite drugs by amplification of the corresponding target genes *(99)*. However, also in tumors with an inherent resistance to gemcitabine, low sensitivity is associated with genomic alterations. In human lung adenocarcinoma cells, gemcitabine sensitivity was increased by an inhibitor of posttranslational modification of *ras* malignant transformation and human pancreatic adenocarcinoma cells, with an inherent resistance to gemcitabine, carry constitutively active Ki-*ras* and overexpress multiple receptor tyrosine kinases, eventually initiating a survival pathway *(100,101)*.

4. DRUG COMBINATIONS

Gemcitabine is the only dCyd analog with activity against solid tumors. This unique quality makes introduction of gemcitabine in drug combination

regimens interesting. Moreover, gemcitabine was relatively ineffective in colon and other gastrointestinal cancers *(11)*. This type of resistance can be overcome by using combinations of chemotherapeutic agents with different mechanisms of action, such as cisplatin. Combinations of gemcitabine with many drugs with a different mechanism of action have been tested in preclinical models.

The combination of gemcitabine and cisplatin is the most successful and has been extensively studied. These two drugs differ completely in both mechanisms of action and side effects. Gemcitabine is primarily myelotoxic; cisplatin induces mainly nonhematological side effects, such as nephrotoxicity, neurotoxicity, and severe nausea and vomiting *(102,103)*. In in vitro studies, a synergistic interaction was found between gemcitabine and cisplatin that was mainly caused by both an increased platina adduct formation with DNA by cisplatin and decreased repair of these lesions *(104,105)*. Decreased kidney function, severe ototoxicity, or neuropathy can be a reason not to treat a patient with cisplatin. For these reasons, the combination of gemcitabine and carboplatin was investigated, which was found synergistic in NSCLC cell lines *(106)*.

Etoposide is also a compound with a completely different mechanism of action than gemcitabine. Etoposide is a topoisomerase II inhibitor, resulting in double DNA strand breaks in the dividing cell *(107)*. In human ovarian cancer cells, gemcitabine enhanced the formation of double DNA strand breaks by etoposide, which might explain the synergistic interaction in some of the cell lines *(108)*.

The taxanes paclitaxel and docetaxel are used as a second-line treatment for NSCLC and for that reason are interesting to combine with gemcitabine. In human NSCLC cell lines, additive toxicity was found in the combination of gemcitabine and paclitaxel *(109)*. The interaction was sequence dependent, and paclitaxel increased dFdCTP accumulation, gemcitabine incorporation into RNA, and the apoptotic index. Also, in human bladder cancer cells, the combination of gemcitabine and paclitaxel was highly active *(110)*. The combination of gemcitabine and the other taxane docetaxel was highly synergistic in a panel of human NSCLC and human gastric cancer cell lines *(111,112)*. In both studies synergistic cytotoxicity of the drug combination was schedule dependent.

Gemcitabine has been combined with many more drugs in preclinical models and in the clinic. Moreover, gemcitabine has shown excellent preclinical activity as a radiosensitizer.

5. FUTURE PROSPECTS

Although gemcitabine has established its place in the treatment of several solid tumors, response rates are still low, especially in relapsed tumors

after previous treatment. Because gemcitabine is only moderately active as monotherapy, emphasis should be on gemcitabine-based combination chemotherapy. To design intelligent new chemotherapy regimens, further research on the mechanisms of action of gemcitabine is warranted. Gemcitabine has a complicated mechanism of action, with many enzymes involved in the metabolism of the drug or as targets for the different metabolites of gemcitabine. It seems that sensitivity of a cell to gemcitabine cannot be attributed to activity of a single enzyme, but is multifactorial.

However, some parameters seem to predict sensitivity to gemcitabine. Knowledge of these parameters is valuable for predicting which patient is going to respond to gemcitabine-based therapy and who is not. With that knowledge, it might be possible to design a combination therapy based on qualities of the patients' tumor cells. Possible parameters for predicting gemcitabine response might be nucleoside transport, dCK activity, RNR, and accumulation and retention of dFdCTP.

Another promising development is gene therapy. Cells transfected with the dCK gene were increasingly more sensitive to dCyd analogs. It might be possible to sensitize resistant and moderately sensitive tumors by targeted gene delivery, resulting in specific expression of the gene in certain tumors. Although there is no clear relationship between dCDA activity and gemcitabine sensitivity, cells transfected with the dCDA gene were resistant to gemcitabine. Transfection of the dCDA gene may play an important role in protecting bone marrow from gemcitabine toxicity.

REFERENCES

1. Grindey GB, Boder GB, Hertel LW, et al. Antitumor activity of 2′,2′-difluorodeoxycytidine (LY188011). Proc Am Assoc Cancer Res 1986;27. Abstract 1175.
2. Hertel LW, Kroin JS, Misner JW, Tustin JM. Synthesis of 2′-deoxy-2′,2′-difluoro-D-ribose and 2′-deoxy-2′,2′-difluoro-D-ribofuranosyl nucleosides. J Org Chem 1988;53:2406–2409.
3. Heinemann V, Hertel LW, Grindey GB, Plunkett W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. Cancer Res 1988;48:4024–4031.
4. Abratt RP, Rezwoda W, Falkson G, Goedhals L, Hacking D. Efficacy and safety profile of gemcitabine in non-small cell lung cancer. Phase II study. J Clin Oncol 1994;12:1535–1540.
5. Van Moorsel CJA, Peters GJ, Pinedo HM. Gemcitabine: future prospects of single-agent combination studies. Oncologist 1997;2:127–134.
6. Kaye SB. Gemcitabine: current status of phase I and II trials. J Clin Oncol 1994;12:1527–1531.
7. Heinemann V. Gemcitabine: progress in the treatment of pancreatic cancer. Oncology 2001;60:8–18.
8. Ramalingam S, Belani CP. State of the art chemotherapy for advanced non-small cell lung cancer. Semin Oncol 2004;31(1 suppl 1):68–74.

9. Hussain SA, James ND. The systemic treatment of advanced and metastatic bladder cancer. Lancet Oncol 2003;8:489–497.
10. Heinemann V. Gemcitabine in the treatment of advanced pancreatic cancer: a comparative analysis of randomized trials. Semin Oncol 2002;29(6 suppl 20):9–16.
11. Carmichael J. The role of gemcitabine in the treatment of other tumors. Br J Cancer 1998;78 (suppl. 3):21–25.
12. Cass CE. Nucleoside transport. In: Georgopapadakou NH, ed. Drug transport in antimicrobial and anticancer chemotherapy. New York: Marcel Dekker: 1995:403.
13. Griffith DA, Jarvis SM. Nucleoside and nucleobase transport systems of mammalian cells. Biochim Biophys Acta 1996;1286:153–181.
14. Hammond JR, Lee S, Ferguson J. [^{3}H]Gemcitabine uptake by nucleoside transporters in a human head and neck squamous carcinoma cell line. Pharm Exp Ther 1999;288:1185–1191.
15. Fang X, Parkinson FE, Mowles DA, Young JD, Cass CE. Functional characterization of a recombinant sodium-dependent nucleoside transporter with selectivity for pyrimidine nucleosides (cNT1rat) by transient expression in cultured mammalian cells. Biochem J 1996;317:457–465.
16. Burke T, Lee S, Ferguson PJ, Hammond JR. Interaction of 2′,2′-difluorodeoxycytidine (gemcitabine) and formycin B with Na^+-dependent and -independent nucleoside transporters of Ehrlich ascites tumor cells. J Pharm Exp Ther 1998;286: 1333–1340.
17. Mackey JR, Yao SY, Smith KM, et al. Gemcitabine transport in *Xenopus* oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. J Natl Cancer Inst 1999;91:1876–1881.
18. Ritzel MW, Ng AM, Yao SY, et al. Molecular identification and characterization of novel human and mouse concentrative Na^+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). J Biol Chem 2001;276:2914–2927.
19. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst 2000;92:1295–1302.
20. Reid G, Wielinga P, Zelcer N, et al. Characterization of transport of nucleoside analog drugs by the human multidrug resistance proteins MRP4 and MRP5. Mol Pharmacol 2003;63:1094–1103.
21. Jansen WJ, Pinedo HM, Van der Wilt CL, Feller N, Bamberger U, Boven E. The influence of BIBW22BS, a dipyridamole derivative, on the antiproliferative effects of 5-fluorouracil, methotrexate and gemcitabine in vitro and in human tumour xenografts. Eur J Cancer 1995;31:2313–2319.
22. Mackey JR, Mani RS, Selner M, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestations of toxicity in cancer cell lines. Cancer Res 1998;58:4349–4357.
23. Gourdeau H, Clarke ML, Ouellet F, et al. Mechanisms of uptake and resistance to troxacitabine, a novel deoxycytidine nucleoside analogue, in human leukemic and solid tumor cell lines. Cancer Res 2001;61:7217–7224.
24. Dumontet C, Fabianowska-Majewska K, Matincic D, et al. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol 1999;106:78–85.
25. Rauchwerger DR, Firby PS, Hedley DW, Moore MJ. Equilibrative-sensitive nucleoside transporter and its role in gemcitabine sensitivity. Cancer Res 2000;60: 6075–6079.

26. Bouffard DY, Laliberte J, Momparler RL. Kinetic studies on 2′,2′-difluorodeoxycytidine (gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem Pharmacol 1993;45:1857–1861.
27. Wang L, Munch-Petersen B, Herrstrom Sjoberg A, et al. Human thymidine kinase 2: molecular cloning and characterisation of the enzyme activity with antiviral and cytostatic nucleoside substrates. FEBS Lett 1999;443:170–174.
28. Heinemann V, Xu Y-Z, Chubb S, et al. Cellular elimination of 2′,2′-difluorodeoxycytidine 5′-triphosphate: a mechanism of self potentiation. Cancer Res 1992;52:533–539.
29. Xu YZ, Plunkett W. Modulation of deoxycytidylate deaminase in intact human leukemia cells. Action of 2′,2′-difluorodeoxycytidine. Biochem Pharmacol 1992;44:1819–1827.
30. Beausejour CM, Cagnon J, Primeau M, Momparler RL. Cytotoxic activity of 2′,2′-difluorodeoxycytidine, 5-aza-2′-deoxycytidine and cytosine arabinoside in cells transduced with deoxycytidine kinase gene. Biochem Biophys Res Commun 2002;293:1478–1484.
31. Kroep JR, Loves WJ, van der Wilt CL, et al. Pretreatment deoxycytidine kinase levels predict in vivo gemcitabine sensitivity. Mol Cancer Ther 2002;6: 371–376.
32. Ruiz van Haperen VWT, Veerman G, Eriksson S, et al. Development and molecular characterization of a 2′,2′-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. Cancer Res 1994;54:4138–4143.
33. Ruiz van Haperen VWT, Veerman G, Vermorken JB, Pinedo HM, Peters GJ. Regulation of phosphorylation of deoxycytidine and 2′,2′-difluorodeoxycytidine (gemcitabine); effects of cytidine 5′-triphosphate and uridine 5′-triphosphate in relation to chemosensitivity for 2′,2′-difluorodeoxycytidine. Biochem Pharm 1996;51: 911–918.
34. Davidson JD, Ma L, Flagella M, Geeganage S, Gelbert LM, Slapak CA. An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. Cancer Res 2004;64:3761–3766.
35. Bergman AM, Pinedo HM, Jongsma APM, et al. Decreased resistance to gemcitabine of cytosine arabinoside resistant myeloblastic murine and rat leukemia cell lines: role of altered activity and substrate specificity of deoxycytidine kinase. Biochem Pharmacol 1999;57:397–406.
36. Shewach DS, Reynolds KK, Hertel LW. Nucleotide specificity of human deoxycytidine kinase. Mol Pharmacol 1992;42:518–524.
37. Goan YG, Zhou B, Hu E, Mi S, Yen Y. Overexpression of ribonucleotide reductase as a mechanism of resistance to 2′,2′-difluorodeoxycytidine in the human KB cancer cell line. Cancer Res 1999;59:4204–4207.
38. Bergman AM, Munch-Petersen B, Jensen P-B, et al. Collateral sensitivity to gemcitabine (2′,2′-difluorodeoxycytidine) and cytosine arabinoside of daunorubicine and VM-26 resistant variants of human small cell lung cancer cells. Biochem Pharmacol 2001;61:1401–1408.
39. Nielsen SE, Munch-Petersen B, Mejer J. Increased ratio between deoxycytidine kinase and thymidine kinase 2 in CLL lymphocytes compared to normal lymphocytes. Leuk Res 1995;19:443–447.
40. Oka J, Matsumoto A, Hosokawa Y, Inoue S. Molecular cloning of human cytosolic purine 5′-nucleotidase. Biochem Biophys Res Commun 1994;205:917–922.

41. Hunsucker SA, Spychala J, Mitchell BS. Human cytosolic 5′-nucleotidase Int J Biol Chem 2001;276:10,498–10,504.
42. Neff T, Blau CA. Forced expression of cytidine deaminase confers resistance to cytosine arabinoside and gemcitabine. Exp Haematol 1996;24:1340–1346.
43 Eliopoulos N, Cournoyer D, Momparler RL. Drug resistance to 5-aza-2′-deoxycytidine, 2′,2′-difluorodeoxycytidine, and cytosine arabinoside conferred by retroviral-mediated transfer of human cytidine deaminase cDNA into murine cells. Cancer Chemother Pharmacol 1998;42:373–378.
44. Eda H, Ura M, Ouchi KF, Tanaka Y, Miwa M, Ishitsuka H. The antiproliferative activity of DMDC is modulated by inhibition of cytidine deaminase. Cancer Res 1998;58:1165–1169.
45. Bergman AM, Pinedo HM, Talianidis I, et al. Increased sensitivity to gemcitabine of P-glycoprotein and multidrug resistance-associated protein-overexpressing human cancer cell lines. Br J Cancer 2003;88:1963–1970.
46. Ruiz van Haperen VWT, Veerman G, Braakhuis BJM, et al. Deoxycytidine kinase and deoxycytidine deaminase activities, in human tumour xenografts. Eur J Cancer 1993;29:132–137.
47. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res 1991;51:6110–6117.
48. Achanta G, Pelicano H, Feng L, Plunkett W, Huang P. Interaction of p53 and DNA-PK in response to nucleoside analogues: potential role as a sensor complex for DNA damage. Cancer Res 2001;61:8723–8729.
49. Ruiz van Haperen VWT, Veerman G, Vermorken JB, Peters GJ. 2′,2′-Difluorodeoxycytidine (gemcitabine) incorporation into RNA and DNA from tumour cell lines. Biochem Pharmacol 1993;46:762–766.
50. Ruiz van Haperen VWT, Veerman G, Boven E, Noordhuis P, Vermorken JB, Peters GJ. Schedule dependence of sensitivity to 2′,2′-difluorodeoxycytidine (gemcitabine) in relation to accumulation and retention of its triphosphate in solid tumour cell lines and solid tumours. Biochem Pharmacol 1994;48: 1327–1339.
51. Van Moorsel CJA, Bergman AM, Veerman G, et al. Differential effects of gemcitabine on ribonucleotide pools of 21 solid tumour and leukaemia cell lines. Biochim Biophys Acta 2000;1474:5–12.
52. Plunkett W, Huang P, Gandhi V. Gemcitabine: actions and interactions. Nucleosides Nucleotides 1997;16:1261–1270.
53. Ruiz van Haperen VWT, Veerman G, Van Moorsel CJA, Peters GJ. Induction of in vivo resistance against gemcitabine (dFdC, 2′,2′-difluorodeoxycytidine). Adv Exp Med Biol 1998;431:637–640.
54. Heinemann V, Xu Y-Z, Chubb S, et al. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2′,2′-difluorodeoxycytidine. Mol Pharmacol 1990;38:567–572.
55. Datta NS, Shewach DS, Hurley MC, Mitchell BS, Fox IH. Human T-lymphoblast deoxycytidine kinase: purification and properties. Biochemistry 1989;28:114–123.
56. Abbruzzese JL, Grunewald R, Weeks EA, et al. A phase I clinical, plasma, and cellular pharmacology study of gemcitabine. J Clin Oncol 1991;9:491–498.
57. Edzes HT, Peters GJ, Noordhuis P, Vermorken JB. Determination of the antimetabolite gemcitabine (2′,2′-difluoro-2′-deoxycytidine) and 2′,2′-difluoro-2′-deoxyuridine by 19F nuclear magnetic resonance spectroscopy. Anal Biochem 1993;214:25–30.

58. Kroep JR, Giaccone G, Voorn DA, et al. Gemcitabine and paclitaxel: pharmacokinetic and pharmacodynamic interactions in patients with non-small-cell lung cancer. J Clin Oncol 1999;17:2190–2197.
59. Peters GJ, Van der Wilt CL, Van Triest B, et al. Thymidylate synthase and drug resistance. Eur J Cancer 1995;31:1299–1305.
60. Ruiz van Haperen VWT, Veerman G, Smid K, Pinedo HM, Peters GJ. Gemcitabine inhibits thymidylate synthase (TS) activity in solid tumour cell lines. Proc Am Assoc Cancer Res 1995;36:354. Abstract 2107.
61. Heinemann V, Schulz L, Issels RD, Plunkett W. Gemcitabine: a modulator of intracellular nucleotide and deoxynucleotide metabolism. Semin Oncol 1995; 22:11–18.
62. Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine. Oncogene 2004;23:1539–1548.
63. Bergman AM, Eijk PP, Ruiz van Haperen VWT, et al. In vivo induction of resistance to gemcitabine results in increased expression of ribonucleotide reductase subunit M1 as the major determinant. Cancer Res 2005;65:9510–9516.
64. Rosell R, Danenberg KD, Alberola V, et al. Ribonucleotide reductase messenger RNA expression and survival in gemcitabine/cisplatin treated advanced non-small cell lung cancer patients. Clin Cancer Res 2004;10:1318–1325.
65. Cory AH, Hertel LW, Kroin JS, Cory JG. Effects of 2′,2′-difluorodeoxycytidine (gemcitabine) on wild type and variant mouse leukemia L1210 cells. Oncol Res 1993;5:59–63.
66. Wong SJ, Myette MS, Wereley JP, Chitambar CR. Increased sensitivity of hydroxyurea-resistant leukemic cells to gemcitabine. Clin Cancer Res 1999;5: 439–443.
67. Smitskamp-Wilms E, Pinedo HM, Veerman G, Ruiz van Haperen VWT, Peters GJ. Postconfluent multilayered cell line cultures for selective screening of gemcitabine. Eur J Cancer 1998;34:921–926.
68. Ross DD, Cuddy DP. Molecular effects of 2′,2′-difluorodeoxycytidine (gemcitabine) on DNA replication in intact HL-60 cells. Biochem Pharmacol 1994;48: 1619–1630.
69. Kroep JR, Giaccone G, Tolis C, et al. Sequence dependent effect of paclitaxel on gemcitabine metabolism in relation to cell cycle and cytotoxicity in non-small-cell lung cancer cell lines. Br J Cancer 2000;83:1069–1076.
70. Van Moorsel CJA, Pinedo HM, Veerman G, et al. Mechanisms of synergism between cisplatin and gemcitabine in ovarian and non-small cell lung cancer cell lines. Br J Cancer 1999;80:981–990.
71. Ostruszka LJ, Shewach DS. The role of DNA synthesis inhibition in the cytotoxicity of 2′,2′-difluoro-2′-deoxycytidine. Cancer Chemother Pharmacol 2003;52: 325–332.
72. Miura S, Izuta S. DNA polymerases as targets of anticancer nucleosides. Curr Drug Targets 2004;5:191–195.
73. Bergman AM, Ciaccone G, Van Moorsel CJA, et al. Cross-resistance in the 2′,2′-difluorodeoxycytidine (gemcitabine) resistant human ovarian cancer cell line AG6000 to standard and investigational drugs. Eur J Cancer 2000;36:1974–1983.
74. Gandhi V, Legha J, Chen F, Hertel LW, Plunkett W. Excision of 2′,2′-difluorodeoxycytidine (gemcitabine) monophosphate residues from DNA. Cancer Res 1996;56:4453–4459.

75. Feng L, Achanta G, Pelicano H, Zhang W, Plunkett W, Huang P. Role of p53 in cellular response to anticancer nucleoside analog induced DNA damage. Int J Mol Med 2000;5:597–604.
76. Parsons R, Li GM, Longley MJ, et al. Hypermutability and mismatch repair deficiency in RER^+ tumor cells. Cell 1993;75:1227–1236.
77. Lin X, Howell SB. Effect of loss of DNA mismatch repair on development of topotecan-, gemcitabine-, and paclitaxel-resistant variants after exposure to cisplatin. Mol Pharm 1999;56:390–395.
78. Fisher DE. Apoptosis in cancer therapy: crossing the threshold. Cell 1994;78: 539–542.
79. Huang P, Plunkett W. Induction of apoptosis by gemcitabine. Semin Oncol 1995;22:19–25.
80. Kucera GL, Capizzi RL. 1-β-D-Arabinofuranosylcytosine-diphosphate-choline is formed by the reversal of choline phosphotransferase and not via cytidyltransferase. Cancer Res 1992;52:3886.
81. Tolis C, Peters GJ, Ferreira CG, Pinedo HM, Giaccone G. Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. Eur J Cancer 1999;35:796–807.
82. Chen M, Hough AM, Lawrence TS. The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization. Cancer Chemother Pharmacol 45:369–374.
83. Pace E, Melis M, Siena L, et al. Effects of gemcitabine on cell proliferation and apoptosis in non small cell lung cancer (NSCLC) cell lines. Cancer Chemother Pharmacol 2000;46:467–476.
84. Sun XM, MacFarlane M, Zhuang J, Wolf BB, Bgeen DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J Biol Chem 1999;274:5053–5060.
85. Ferreira CG, Span SW, Peters GJ, Kruyt FAE, Giaccone G. Chemotherapy triggers apoptosis in a caspase-8 dependent and mitochondria controlled manner in the non-small cell lung cancer cell line NCI-H460. Cancer Res 2000;60: 7133–7141.
86. Nabhan C, Gajria D, Krett NL, Gandhi V, Ghias K, Rosen ST. Caspase activation is required for gemcitabine activity in multiple myeloma cell lines. Mol Cancer Ther 2002;13:1221–1227.
87. Arlt A, Gehrz A, Muerkoster S, et al. Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. Oncogene 2003;22:3243–3251.
88. Habiro A, Tanno S, Koizumi K, et al. Involvement of p38 mitogen-activated protein kinase in gemcitabine-induced apoptosis in human pancreatic cancer cells. Biochem Biophys Res Commun 2004;316:71–77.
89. Galmarini CM, Clarke ML, Falette N, Puisieux A, Mackey JR, Dumontet C. Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. Int J Cancer 2002;97:439–445.
90. Cascallo M, Calbo J, Gelpi JL, Mazo A. Modulation of drug cytotoxicity by reintroduction of wild-type p53 gene (Ad5CMV-p53) in human pancreatic cancer. Cancer Gene Ther 2000;7:545–556.
91. Shi X, Liu S, Kleeff J, Friess H, Buchler MW. Acquired resistance of pancreatic cancer cells towards 5-fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. Oncology 2002;62:354–362.

92. Orlandi L, Bearzatto A, Abolafio G, De Marco C, Daidone MG, Zaffaroni N. Involvement of bcl-2 and p21waf1 proteins in response of human breast cancer cell clones to Tomudex. Br J Cancer 1999;81:252–260.
93. Xu ZW, Friess H, Solioz M, et al. Bcl-x(L) antisense oligonucleotides induce apoptosis and increase sensitivity of pancreatic cancer cells to gemcitabine. Int J Cancer 2001;94:268–274.
94. Xu ZW, Friess H, Buchler MW, Solioz M. Overexpression of Bax sensitizes human pancreatic cancer cells to apoptosis induced by chemotherapeutic agents. Cancer Chemother Pharmacol 2002;49:504–510.
95. Auer H, Oehler R, Lindner R, et al. Characterization of genotoxic properties of 2′,2′-difluorodeoxycytidine. Mutat Res 1997;393:165–173.
96. Al-Madhoun AS, Van der Wilt CL, Loves WJP, et al. Detection of an alternatively spliced form of deoxycytidine kinase mRNA in the 2′-2′-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cancer cell line AG6000. Biochem Pharmacol 2004;68:601–609.
97. Schwab M, Amler LC. Amplification of cellular oncogenes. A predictor of clinical outcome of cancer. Genes Chromosomes Cancer 1990;1:181–193.
98. Brennan J, O'Connor T, Makuch RW, et al. Myc family DNA amplification in 107 tumours and tumour cell lines from patients with small cell lung cancer treated with different combination chemotherapy regimens. Cancer Res 1991;51: 1708–1712.
99. Stark GR, Wahl GM. Gene amplification. Annu Rev Biochem 1984;53:447–491.
100. Sun J, Blaskovich MA, Knowles D, et al. Antitumor efficacy of a novel class of non-thiol-containing peptidomimetic inhibitors of farnesyltransferase and geranylgeranyltransferase I: combination therapy with the cytotoxic agents cisplatin, taxol and gemcitabine. Cancer Res 1999;59:4919–4926.
101. Ng SW, Tsao MS, Chow S, Hedley DW. Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. Cancer Res 2000;60:5451–5455.
102. Abratt RP, Bezwoda WR, Falkson G, Goedhals L, Hacking D, Rugg TA. Efficacy and safety profile of gemcitabine in non-small-cell lung cancer: a phase II study. J Clin Oncol 1994;12:1535–1540.
103. Von Hoff DD, Schilsky R, Reichert CM, et al. Toxic effects of cis-dichlorodiammineplatinum(II) in man. Cancer Treat Rep 1979;63:1527–1531.
104. Bergman AM, Ruiz van Haperen VW, Veerman G, Kuiper CM, Peters GJ. Synergistic interaction between cisplatin and gemcitabine in vitro. Clin Cancer Res 1996;2:521–530.
105. van Moorsel CJ, Pinedo HM, Smid K, et al. Schedule-dependent pharmacodynamic effects of gemcitabine and cisplatin in mice bearing Lewis lung murine non-small cell lung tumours. Eur J Cancer 2000;36:2420–2429.
106. Edelman MJ, Quam H, Mullins B. Interactions of gemcitabine, carboplatin and paclitaxel in molecularly defined non-small-cell lung cancer cell lines. Cancer Chemother Pharmacol 2001;48:141–144.
107. Chow KC, Macdonald TL, Ross WE. DNA binding by epipodophyllotoxins and N-acyl anthracyclines: implications for mechanism of topoisomerase II inhibition. Mol Pharmacol 1988;34:467–473.
108. van Moorsel CJ, Pinedo HM, Veerman G, et al. Combination chemotherapy studies with gemcitabine and etoposide in non-small cell lung and ovarian cancer cell lines. Biochem Pharmacol 1999;57:407–415.

109. Kroep JR, Giaccone G, Tolis C, et al. Sequence dependent effect of paclitaxel on gemcitabine metabolism in relation to cell cycle and cytotoxicity in non-small-cell lung cancer cell lines. Br J Cancer 2000;83:1069–1076.
110. Perabo FG, Lindner H, Schmidt D, et al. Preclinical evaluation of gemcitabine/paclitaxel-interactions in human bladder cancer lines. Anticancer Res 2003;23:4805–4814.
111. Zoli W, Ricotti L, Dal Susino M, et al. Docetaxel and gemcitabine activity in NSCLC cell lines and in primary cultures from human lung cancer. Br J Cancer 1999;81:609–615.
112. Ricotti L, Tesei A, De Paola F, et al. In vitro schedule-dependent interaction between docetaxel and gemcitabine in human gastric cancer cell lines. Clin Cancer Res 2003;9:900–905.
113. Bergman AM, Pinedo HM, Peters GJ. Determinants of resistance to 2′,2′-difluorodeoxycytidine (gemcitabine). Drug Resist Updat 2002;5:19–33.
114. Bohman C, Eriksson S. Deoxycytidine kinase from human leukemic spleen: preparation and characteristics of homogenous enzyme. Biochemistry 1988;27:4258–4265.
115. Munch-Petersen B, Cloos L, Tyrsted G, Eriksson S. Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. J Biol Chem 1991;266:9032–9038.
116. Fanuchi MP, Watanabe KA, Fox JJ, Chou T-C. Kinetics and substrate specificity of human and canine cytidine deaminase. Biochem Pharmacol 1986;35:1199–1201.
117. Jordheim LP, Cros E, Gouy MH, et al. Characteristics of a gemcitabine resistant murine leukemic cell line: reversion of in vitro resistance by a mononucleotide prodrug. Clin Cancer Res 2004;10:5614–5621.
118. Calmarini CM, Clarke ML, Jordheim L, et al. Resistance to gemcitabine in a human follicular lymphoma cell line is to partial deletion of the deoxycytidine kinase gene. BMC Pharmacol 2004;4:8.
119. Dong M, Feng FY, Lin C, et al. Mechanisms of the drug resistance of a 2′, 2′-difluoro-deoxycytidine (gemcitabine)-resistant variant of a human adenocarcinoma cell line. Zhonhua Yi Xue Za Zhi 2004;84:323–328.
120. Van Bree C, Castro Kreder N, Loves WJ, Franken NA, Peters GJ, Haveman J. Sensitivity to ionizing radiation and chemotherapeutic agents in gemcitabine-resistant human tumor cell lines. Int J Radiat Oncol Biol Phys 2002;54:237–244.
121. Blackstock AW, Lightfoot H, Case LD, et al. Tumor uptake and elimination of 2′,2′-difluoro-2′-deoxycytidine (gemcitabine) after deoxycytidine gene transfer: correlation with in vivo tumor response. Clin Cancer Res 2001;7:3263–3268.
122. Giovannetti E, Mey V, Danesi R, Mosca I, Del Tacca M. Synergistic cytotoxicity and pharmacogenetics of gemcitabine and pemetrexed combination in pancreatic cancer cell lines. Clin Cancer Res 2004;10:2936–2943.
123. Sigmond J, Kroep JR, Loves W, Codacci-Pisanelli G, Peters GJ. Quantitative real time PCR of deoxycytidine kinase mRNA by Light Cycler PCR in relation to enzyme activity and gemcitabine sensitivity. Cancer Lett 2004;213:173–179.
124. Gregoire V, Rosier JF, De Bast M, et al. Role of deoxycytidine kinase (dCK) activity in gemcitabine's radioenhancement in mice and human cell lines in vitro. Radiother Oncol 2002;63:329–338.
125. Schirmer M, Stegmann AP, Geisen F, Konwalinka G. Lack of cross-resistance with gemcitabine and cytarabine in cladribine-resistant HL60 cells with elevated 5′-nucleotidase activity. Exp Hematol 1998;26:1223–1228.

126. Duxbury MS, Ito H, Benoit E, Zinner MJ, Ashley SW, Whang EE. Retrovirally mediated RNA interference targeting the M2 subunit of ribonucleotide reductase: a novel therapeutic strategy in pancreatic cancer. Surgery 2004;136:261–269.
127. Liu X, Zhou B, Xue L, et al. Nuclear factor Y regulation and promoter transactivation of human ribonucleotide reductase subunit M2 gene in a gemcitabine resistant KB clone. Biochem Pharmacol 2004;67:1499–1511.
128. Zhou B, Mo X, Liu X, Qiu W, Yen Y. Human ribonucleotide reductase M2 subunit gene amplification and transcriptional regulation in a homogenous staining chromosome region responsible for the mechanism of drug resistance. Cytogenet Cell Genet 2001;95:34–42.

12 Clinical Activity of Gemcitabine as a Single Agent and in Combination

Judith R. Kroep, MD, PhD, Godefridus J. Peters, PhD, and Robert A. Nagourney, MD

CONTENTS

SUMMARY

Gemcitabine (2′,2′-difluoro-deoxycytidine, dFdC, Gemzar®) is active in various solid tumors and hematological malignancies. It is attractive for combination chemotherapy based on its multiple mechanisms of action and relatively mild toxicity profile. This chapter summarizes the current preclinical and clinical knowledge of gemcitabine as a single agent and in combinations in non-small cell lung cancer, and pancreatic, bladder, breast, ovarian, gastric, and esophageal cancer.

Key Words: Breast cancer; cisplatin; gemcitabine; gemcitabine combinations; non-small cell lung cancer; ovarian cancer; pancreatic cancer; radiotherapy; taxanes.

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

Gemcitabine (2′,2′-difluoro-deoxycytidine, dFdC, Gemzar®), a pyrimidine derivative characterized by its geminyl difluoro substitution on the ribose ring, entered cancer therapeutics in the 1990s as the first nucleoside analog with broad solid tumor activity since the introduction of 5-fluorouracil (5-FU) by Heidelberger in the late 1950s. With proven activity in a wide array of solid tumors (Table 1) and hematologic malignancies *(1)*, gemcitabine's role both alone and in combination with other anticancer drugs and irradiation continues to be the focus of active investigation.

The compound was synthesized in the 1980s at Lilly Research Laboratories (Eli Lilly and Co., Indianapolis, IN) *(2)*. Although initially developed as an antiviral agent based on its ability to inhibit DNA and RNA viruses, gemcitabine revealed a low therapeutic index because of cytotoxicity to the parental cell lines. This led to formal development as an anticancer agent with unique mechanisms of action and activity against various solid tumors *(3–8)*.

Gemcitabine is a deoxynucleoside analog with structural and metabolic similarities to cytarabine (ara-C), an agent in broad use against acute myeloblastic leukemia *(9,10)*. Both differ from the parent nucleoside by a modification at the 2′ position of the sugar moiety (Fig. 1). Furthermore, gemcitabine differs from ara-C through its different schedule dependency, greater membrane permeability, affinity for deoxycytidine kinase (dCK), longer intracellular retention, and multiple self-potentiating mechanisms of action *(6)* (*see* Chapter 11). It is believed that these differences underlie gemcitabine's greater solid tumor activity.

2. SINGLE-AGENT EFFICACY

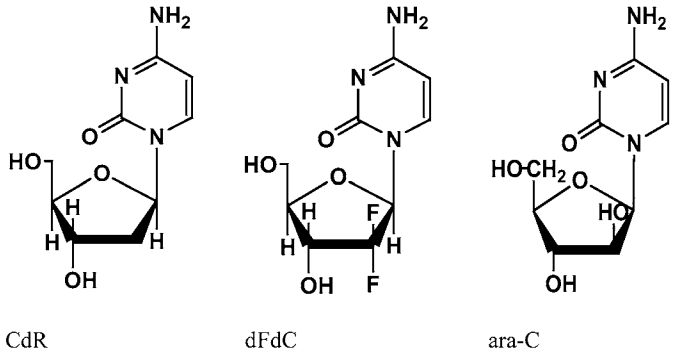
Gemcitabine has shown encouraging single-agent activity in non-small cell lung cancer (NSCLC); small cell lung cancer; and ovarian, breast, bladder, and head-and-neck squamous cell cancers (Table 1), with response rates ranging from 13% to 29%. In addition, in patients with NSCLC and pancreatic cancer, improvement of the performance status has been observed *(11–13)*. However, in the relatively chemoresistant tumor types, such as renal cancer, gastroesophageal cancer, and melanoma, gemcitabine alone was not active. Gemcitabine has been approved for first-line treatment in advanced pancreatic cancer and NSCLC.

In 1992, Rational Therapeutics initiated an analysis of gemcitabine activity in human tumor specimens obtained at surgery *(14)*. Using morphologic changes in primary culture specimens as surrogate measures of drug-induced apoptosis as previously described *(15)*, these analyses in 619 previously untreated patient specimens yielded a disease-specific profile of drug activity. Having shown that these ex vivo analyses correlated with

Table 1
Efficacy of Gemcitabine in Various Solid Tumor Types

	Phase	*Prior chemotherapy*	*No. patients (range)*	*Response (range; %)*	*Median survival (range; mo)*	*Reference*
Bladder	II	+	24–39	23–29	5–13+	*176–179*
Breast	II	+	20–42	14–29	12–15	*130–132, 180*
Esophageal cancer	II	–	17	0	5	*126*
Gastric cancer	II	–	15–26	0–4	ng	*181, 182*
Ovary	II	+	20–42	15–22	8.1	*156, 159, 183*
NSCLC	II	–	150	19	5.7	*11*
	III	–	26–49	18–19	ng–8.5	*184, 185*
SCLC	II	–	26	27	12	*186*
Renal	II	–	18–37	6–8.1	ng–12	*187, 188*
HNSCC	II	–	54	13	ng	*189*
Pancreas	II	–	32–43	9–21	5.6–8.8	*95, 190–192*
	II	+	57	10.5 [27%]	3.9	*13*
	III	–	63	5.4 [24%]	5.65	*12*

[%], clinical benefit response; ng, not given; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; HNSCC, head-and-neck squamous cell carcinoma.



CdR dFdC ara-C

Fig. 1. Structural formulas of deoxycytidine (CdR), cytarabine (ara-C), and gemcitabine (dFdC).

patient response, time to progression, and survival *(16,17)*, they used modified Z scores of the LC_{50} values to predict activity for gemcitabine by tumor type (Fig. 2). The Z-score formula allows the comparison of gemcitabine activity for diseases with widely varied LC_{50} ranges. By subtracting the disease LC_{50} result (i.e., breast, colon, etc.) from the average LC_{50} for all diseases and dividing the result by the standard deviation, the relative sensitivity or resistance is provided in standard deviation units from the mean. The formula is (Overall Mean LC_{50} – Disease LC_{50})/Standard Deviation. Results have proven concordant with the published response rates from phase II clinical trials and have provided the basis for the combination analyses.

2.1. Mechanism of Action

Gemcitabine has multiple mechanisms of action (Fig. 3). Membrane transport is mediated by the facilitated diffusion human equilibrative or concentrative nucleoside transporters (hENTs or hCNTs) dependent on the tissue or tumor (*5,18*; *see* Chapter 1, this volume) Subsequently, the prodrug is phosphorylated to its monophosphate by dCK. Subsequent phosphorylation yields the active metabolites, gemcitabine diphosphate and triphosphate (dFdCTP). The main action of gemcitabine is assumed to be competitive incorporation of dFdCTP with deoxycytidine triphosphate into DNA *(4,7,19)*, after which DNA polymerase is able to add only one more nucleotide, leading to DNA fragmentation and cell death. This so-called masked chain termination prevents exonuclease recognition and excision of gemcitabine.

Gemcitabine activity has been correlated with the extent of dFdCTP formation, its incorporation into DNA, and its inhibition of DNA synthesis *(7,19–21)*. In addition, several self-potentiating mechanisms have been described, including inhibition of ribonucleotide reductase, deoxycytidylate

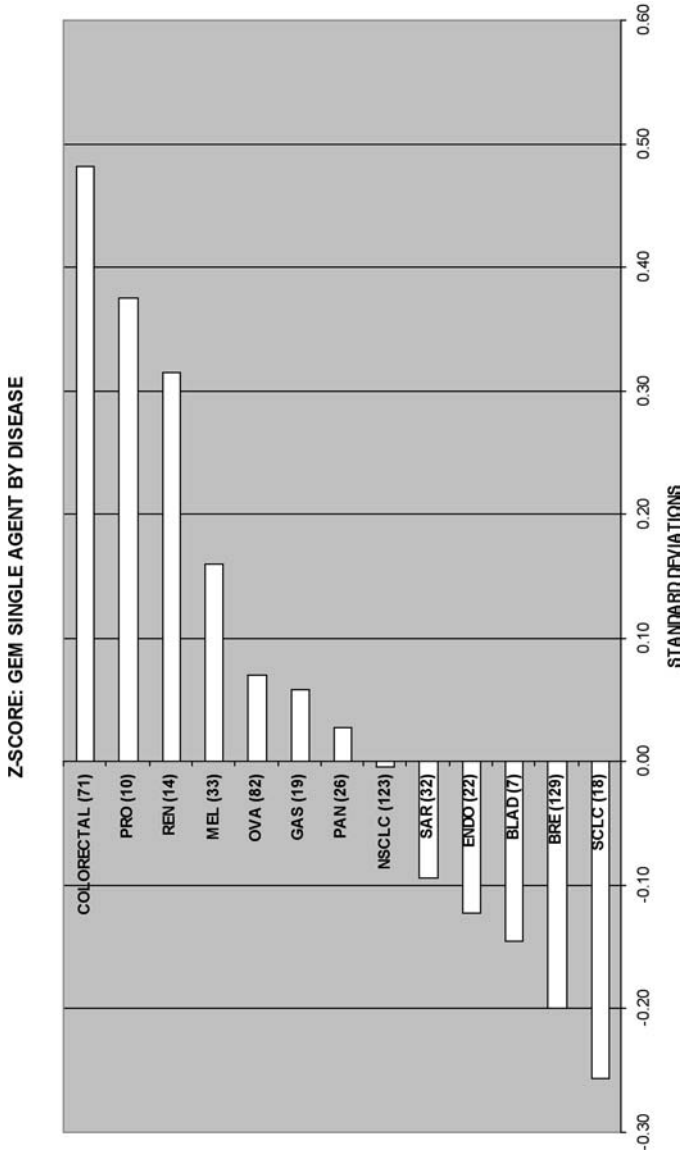


Fig. 2. Z-score analysis of gemcitabine activity in previously untreated human tumors. PRO, prostate; REN, renal; MEL, melanoma; OVA, ovary; GAS, gastric; PAN, pancreas; NSCLC, non-small cell lung cancer; SAR, soft tissue sarcoma; ENDO, endometrial; BLAD, bladder; BRE, breast; SCLC, small cell lung cancer.

deaminase and cytidine triphosphate synthetase *(22,23)*, enhancing the incorporation of dFdCTP into DNA and possibly also into RNA. The potential role of incorporation of gemcitabine into RNA has not yet been

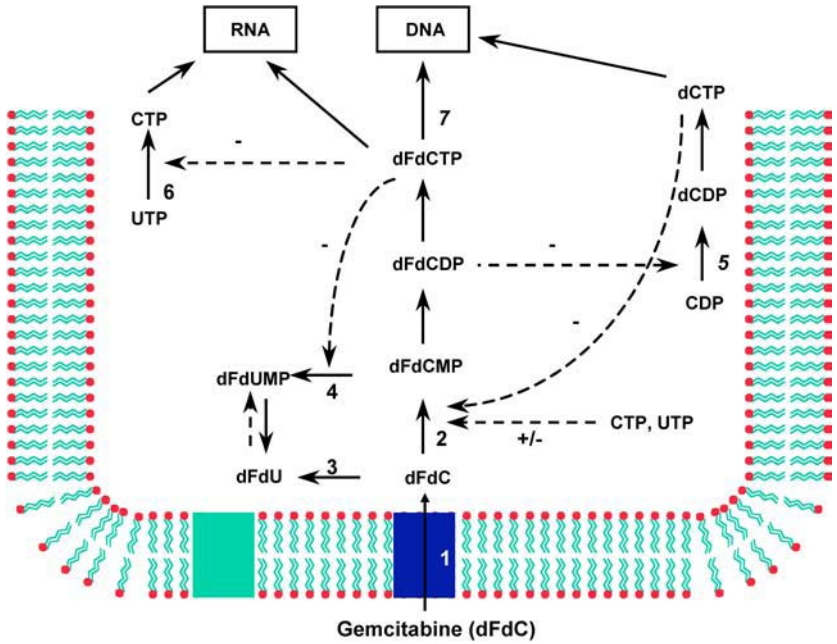


Fig. 3. Metabolism and mechanisms of action of gemcitabine: 1, facilitated diffusion human nucleoside transporters (hNT); 2, deoxycytidine kinase (dCK); 3, deoxycytidine deaminase (dCDA); 4, deoxycytidylate deaminase; 5, ribonucleotide reductase; 6, CTP-synthetase; and 7, DNA-polymerase.

elucidated but may be related to induced apoptosis, as was shown for another fluoropyrimidine, 5-FU *(24)*. Gemcitabine induces a G1/S-phase arrest *(6,25,26)* and triggers apoptosis in both human leukemia *(6,27,28)* and solid tumor cells *(25,26,29)*. Moreover, gemcitabine caused an imbalance in the (deoxy)ribonucleotide pools, which might play a role in apoptosis by influencing DNA repair. Collectively, the various effects of gemcitabine result in a unique pattern of self-potentiation.

Although these self-potentiating factors are important for the activity of gemcitabine, dCK activity and dFdCTP accumulation and incorporation into DNA seem vital to all these actions. dCK is the rate-limiting enzyme for active metabolite formation. Acquired resistance to gemcitabine has been associated with deficiency of dCK *(30–32)*; after transfection with dCK, the sensitivity to gemcitabine increased *(33)*. We found a clear relation between dCK levels and chemotherapeutic response to gemcitabine in various tumors of different histology in vivo *(34)*, indicating that dCK might be an important prognostic factor for gemcitabine sensitivity. This is supported by the favorable ratio of tumor to normal tissue for dCK *(34–36)*.

In addition, accumulation of dFdCTP has been correlated to its incorporation into DNA, its inhibition of DNA synthesis, and gemcitabine sensitivity *(7,19,21)*.

Inactivation of gemcitabine occurs through deamination of gemcitabine itself or of gemcitabine monophosphate to the metabolites 2′,2′-difluoro-2′-deoxyuridine (dFdU) or 2′,2′-difluoro-2′-deoxyuridine monophosphate by the enzymes, deoxycytidine deaminase, and deoxycytidylate deaminase, respectively (Fig. 3). dFdU is 1000-fold less active than gemcitabine *(37)*. However, plasma concentrations are maintained for a prolonged period (>24 h) at levels known to cause growth inhibition. Additional degradation of gemcitabine can occur by dephosphorylation of the nucleotides to the nucleoside. Increased nucleotidase activity has been found in gemcitabine-resistant cell lines *(30)*. In these resistant cell lines, nucleotidase activity was strongly increased and dCK activity was moderately reduced, resulting in reduced accumulation of triphosphate analogs. Furthermore, ribonucleotide reductase activity was strongly increased in the gemcitabine- selected resistant cell line.

2.2. Pharmacology

Gemcitabine antitumor activity is strongly schedule dependent. In phase I trials, several schedules with gemcitabine have been studied. The daily for 5 d schedule with a maximal tolerable dose (MTD) of 12 mg/m^2 *(38)* was too toxic and not recommended for phase II studies. In a schedule of once every 2 wk, gemcitabine was well tolerated, with MTD reached at 5700 mg/m^2 *(39)*.

The weekly schedule (d 1, 8, 15, every 4 wk) was recommended for phase II studies with gemcitabine at a dose of 800 mg/m^2, which could later be increased to 1000–1250 mg/m^2 because of lack of toxicity in most patients. The usage of prolonged gemcitabine infusions is still under investigation. Prolonged infusions required considerable dose reductions of gemcitabine, to 300 mg/m^2 at a 6-h infusion *(40)* and 180 mg/m^2 at a 24-h infusion *(41)*, with myelotoxicity dose limiting. Pharmacokinetic studies suggested that the accumulation of dFdCTP is saturated at 10–20 μ*M* gemcitabine in plasma, which was achieved at a gemcitabine infusion rate of 10 mg/m^2/min *(42)*. The maximum tolerated infusion duration at the gemcitabine infusion rate of 10 mg/m^2/min was 12–15 h *(43)*, with maximal dFdCTP levels obtained at the end of infusion. Maintaining high dFdCTP for a long period of time might optimize accumulation of dFdCTP and be favorable for clinical response to gemcitabine.

Although a study comparing fixed dose rate with standard-dose gemcitabine in 91 patients with advanced pancreatic cancer suggested benefit *(44)*, randomized trials will be necessary to compare these administration schedules formally. Plasma concentrations of gemcitabine generally reach

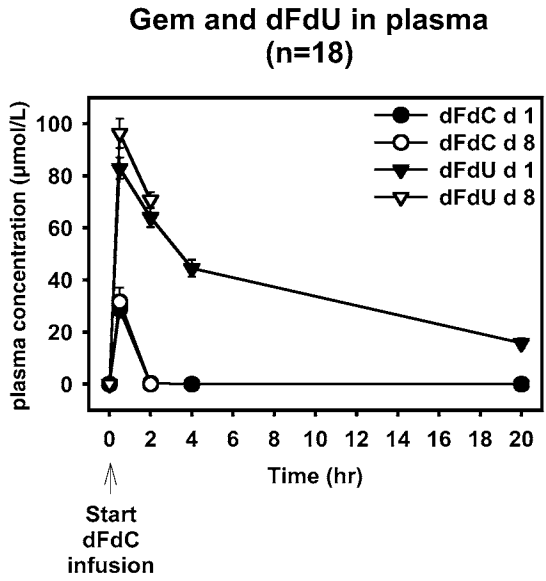


Fig. 4. Plasma levels of gemcitabine (1000 mg/m^2; (mol/L) administered directly after paclitaxel (d 1; closed circle) and as a single agent (d 8; open circle) and of its metabolite dFdU on d 1 (closed triangle) and d 8 (open triangle).

a plateau after 15–30 min during the standard 30-min infusion protocol *(45)*. Pharmacokinetics were linear over the range 53–4500 mg/m^2 *(45)* but became nonlinear at the higher dose levels *(46)*. In our pharmacokinetic studies, mean gemcitabine peak plasma concentrations ranged from 24 to 32 and to 53 µ*M* at doses of 800, 1000, and 1250 mg/m^2 *(47,48)*.

Gemcitabine is rapidly eliminated, with a median elimination half-life of 8 min (Fig. 4); its catabolite, dFdU, has a biphasic elimination, with a terminal half-life of 14 h (Fig. 4) *(45,48)*. Urinary excretion of gemcitabine predominantly occurs as dFdU *(45,49)*. Accumulation and retention of gemcitabine nucleotides by target cells are critical for the cytotoxicity of the drug. Intracellular concentrations of dFdCTP in peripheral blood mononuclear cells reached peak levels within 2–4 h after start of gemcitabine infusion (30-min infusion) and increased dose dependently until saturation at doses above 1000 mg/m^2 *(46,50)*; dFdCTP elimination was biphasic *(45)*. Gemcitabine elimination occurs largely through deamination in the blood, liver, kidney, and other tissues *(45)*. Gemcitabine pharmacokinetics are age and gender dependent *(51)*. Clearance of gemcitabine decreases with increasing age. In women, gemcitabine clearance is about 30% lower than in men; however, dosage adjustment may not be necessary.

In conclusion, gemcitabine pharmacokinetics are schedule dependent. The gemcitabine weekly schedule was recommended for further evaluation. Evaluable parameters are plasma levels of gemcitabine, its deaminated product dFdU, and intracellular concentrations of dFdCTP, the active metabolite of gemcitabine.

2.3. Toxicity

Gemcitabine exhibits schedule-dependent toxicity. The MTD on an every-other-week schedule in solid tumors is 4500 mg/m^2 *(39)*; the MTD for gemcitabine administered 5 d/wk every 4 wk was 12 mg/m^2 *(38)*. The most favorable therapeutic index is achieved when the drug is given at a weekly dose of 800–1250 mg/m^2 for 2 or 3 wk, followed by 1 wk of rest *(52)*. Myelotoxicity has been dose limiting in this schedule. The US prescribing information for gemcitabine cites data from 979 patients with various malignancies who received weekly 800–1250 mg/m^2 gemcitabine as part of 22 clinical studies *(53)*. The most common grade 3 or 4 adverse effects accordingly to the World Health Organization were myelosuppression, hepatic abnormalities, and nausea and vomiting. Rare toxicities, including pulmonary toxicity *(54)*, hemolytic-uremic syndrome *(55)*, and vasculitis *(56)*, have been described. This relatively mild toxicity profile makes gemcitabine attractive for usage in combination chemotherapy.

3. GEMCITABINE COMBINATIONS: MECHANISMS OF ACTION

The introduction of gemcitabine rapidly led to studies of its combination with other cytotoxics. In cell line and preclinical models, analyses revealed additive and synergistic effects, with particular focus on modalities that directly damaged DNA, like alkylating agents, platins, and ionizing radiation. The capacity of human tumor primary cultures to predict gemcitabine activity (described above) provided the opportunity to conduct synergy studies with other chemotherapeutics, including median-drug-effect analysis of select combinations *(57)*. The results of these analyses are provided as bar graphs indicating the percentage of specimens yielding synergy (Fig. 5). The insights gained from various preclinical studies led to several successful phase II clinical trials, including the study of cisplatin and gemcitabine in relapsed breast and ovarian cancers described in sections following. The molecular basis of these favorable interactions remains an area of investigation.

3.1. Gemcitabine-Cisplatin

Gemcitabine is attractive for use in combinations with drugs that damage DNA based on its ability to inhibit DNA replication and repair. Cisplatin acts by formation of platinum-DNA (Pt-DNA) adducts, both interstrand and

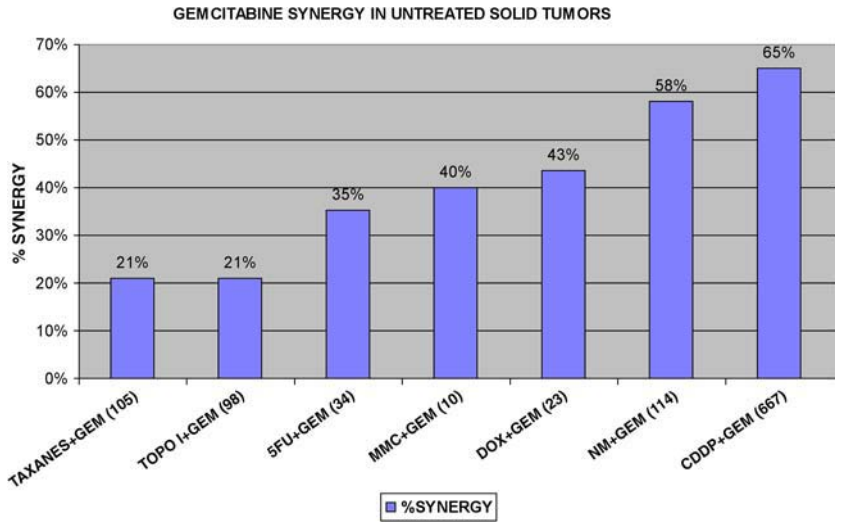


Fig. 5. Incidence of synergy for gemcitabine combinations by drug (percentage of specimens positive for synergy). Gem, gemcitabine; Topo I, topotecan; MMC, mitomycin C; Dox, doxorubicin; NM, nitrogen mustard; CDDP, cisplatin; 5FU, 5-fluorouracil.

intrastrand *(58)*. Preclinical studies of the gemcitabine-cisplatin combination clearly showed additive and synergistic effects *(3,59,60)*. The interaction might be explained by the increased gemcitabine incorporation in DNA and formation of Pt-DNA adducts. However, paradoxically, cisplatin tended to decrease the cellular dFdCTP concentration *(59,60)*. Furthermore, gemcitabine decreased the repair of Pt-DNA adducts in a cisplatin-resistant cell line.

There is no clear evidence of the best sequential schedule of administration of the two drugs. Preclinical studies showed that gemcitabine prior to cisplatin schedule was synergistic; the reversed schedule was mostly additive *(3,59,61)*. In a pharmacological study, the schedule with cisplatin prior to gemcitabine produced the best pharmacological profile *(47)* but resulted in more severe leukopenia *(62)*. Reanalysis of clinical phase II studies and a randomized study, using 4-wk schedules, suggested that the regimen with cisplatin on d 15 had highest dose intensity with the most favorable toxicity profile *(63,64)*. However, despite the better tolerance in the d 15 schedule, dose reductions were still necessary. Therefore, the 3-wk schedule is currently often used, showing comparable response rates and better compliance profile *(65)*.

3.2. Gemcitabine-Paclitaxel

Various gemcitabine-paclitaxel combinations did not show sequence-dependent cytotoxic effects in NSCLC cells; all combinations were not more than additive *(25,66)*. However, the administration of paclitaxel prior to gemcitabine seemed to be favorable compared to the reversed schedule because of the potentiating effect of paclitaxel on gemcitabine metabolism and apoptotic index *(25)*. In patients, no pharmacokinetic drug–drug interactions for the paclitaxel-gemcitabine combination were observed, but paclitaxel dose-dependently increased dFdCTP peak levels in mononuclear cells of NSCLC patients *(48)*. Increased dFdCTP levels might be related to an altered metabolism of cofactors involved in the synthesis of dFdCTP, such as adenosine triphosphate. The paclitaxel-gemcitabine combination significantly increased ribonucleotide levels *(48)*, especially adenosine triphosphate, which is the cosubstrate for dCK and together with uridine triphosphate is a major phosphate donor in the phosphorylation of gemcitabine *(67,68)*. Thus, although the paclitaxel-gemcitabine combination did not show synergistic sequence-dependent cytotoxicity, paclitaxel prior to gemcitabine seems to be favorable compared to its reversed schedule.

3.3. Other Combinations With Gemcitabine

Many other drugs have been evaluated for their potential synergistic interaction with gemcitabine. Combination of gemcitabine with oxaliplatin showed potent antitumor effects comparable to those observed for the gemcitabine-cisplatin combination *(69)*. Additive to slightly synergistic effects have been observed for docetaxel *(70)*, topotecan *(26)*, etoposide *(71)*, mitomycin C *(72)*, 5-FU *(73)*, and multitargeted antifolate *(74)*. For etoposide, the mechanism may be related to reduced repair of DNA damage *(71)*. Gemcitabine induced a decrease in deoxyuridine monophosphate concentration *(75)*, which might explain a synergistic effect with 5-FU and multitargeted antifolate. In addition, gemcitabine has been recognized as a potent radiosensitizer *(76–78)* (*see* Chapter 13), for which DNA repair, cell cycle effects, and deoxyadenosine triphosphate depletion might play a role. Because of its multiple actions, gemcitabine seems attractive for combination chemotherapy.

4. GEMCITABINE COMBINATIONS: FIRST-LINE EFFICACY

4.1. Advanced NSCLC

Currently, cisplatin is the basis for most of the effective combination chemotherapy regimens in NSCLC *(79,80)*, prolonging survival and improving quality of life. Gemcitabine, the taxanes, irinotecan, and vinorelbine have shown improved survival and quality of life compared to best supportive care in NSCLC *(11,70,81–83)*. Two-drug combinations with these new drugs demonstrated improved results compared to cisplatin alone or the

Table 2
Phase III Trials With Gemcitabine Combinations (@, Gemcitabine and Cisplatin) and Three-Drug Combinations ($) in Advanced NSCLC

Regimen		*No. of patients*	*Response (%)*	*Median survival (mo)*	*QoL*	*Reference*
Gem-Cis	@	69	41[a]	8.7	=	*193*
Cis-VP16		66	22	7.2	=	
Gem-Cis	@$	155	38[a]	8.6	=	*194*
MIC		152	26	9.6	=	
Gem-Cis			30.4[a]	9.1[a]	nd	*195*
Cis	@		11.1	7.6		
Gem-Vinor		60	22	7.3	+	*196*
Vinor		60	15	4.5		
Gem-Vinor		49	18	ng	nd	*184*
Gem		49	18			
Gem-Tax		257	35	9.8	nd	*91*
Tax-Carbo		252	28	10.4		
Gem-Cis		205	30	9.8	nd	*197*
Tax-Carbo	@	204	32	9.9		
Cis-Vinor		203	30	9.5		
Gem-Carbo		99	27	11.6[a]	nd	*198*
Cis-vinblastine		99	15	7.9		
Cis-Gem-Vinor		69	47	11.5	nd	*199*
Cis-Gem	@$	63	30	9.8		
Cis-Vinor		67	25	8.2[b]		
Cis-Tax		288	21	7.8	nd	*200*
Cis-Gem	@	288	22	8.1		

Cis-docetaxel		289	17	7.4		
Carboplatin-Tax		290	17	8.1		
Gem-ifosfamide		94	25	7.5	nd	*201*
Gem-Cis-Carbo		92	29	8.5		
Cis-Carbo-ifosfamide	$	94	23	6.0		
Cis-Gem				9.9	nd	*85*
Cis-Gem-Gef 250				9.9		
Cis-Gem-Gef 500				10.9		

Gem, gemcitabine; Cis, cisplatin; VP16, etoposide; MIC, mitomycin C + ifosfamide + cisplatin; Vinor, vinorelbine; Tax, paclitaxel; Gef, gefitinib, Iressa; nd, not determined; =, not significantly different; QoL, quality of life; mo, months.

[a]Significantly better.

[b]Significantly worse.

older platinum-based combinations using etoposide, vinblastine, or vindesine. With the newer agents, response rates seem to improve, but a survival advantage was less frequently observed. Recent or ongoing phase III trials aim to determine the optimal combination using newer agents (Table 2).

4.1.1. Gemcitabine-Cisplatin

Combinations of gemcitabine and cisplatin are theoretically attractive because of preclinical synergism and have proven to be among the most active in clinical studies (Table 2). Thrombocytopenia has generally been dose limiting.

4.1.2. Gemcitabine-Cisplatin-Gefitinib

Current research has focused on targeted therapy, and epidermal growth factor receptor inhibition is one of the promising clinical strategies. Epidermal growth factor receptor inhibitors currently under investigation include the small molecules gefitinib (Iressa, ZD1839) and erlotinib (Tarceva, OSI-774), as well as monoclonal antibodies such as cetuximab (IMC-225, Erbitux) and bevacizumab (Avastin). Agents that have only begun to undergo clinical evaluation include CI-1033, an irreversible pan-erbB tyrosine kinase inhibitor, and PKI166 and GW572016, both examples of dual kinase inhibitors (inhibiting epidermal growth factor receptor and Her2). Preclinical models have demonstrated synergy for all these agents in combination with either chemotherapy or radiotherapy *(84)*. However, serious clinical challenges persist. Gefitinib in combination with gemcitabine and cisplatin in chemotherapy-naïve patients with advanced NSCLC had a manageable and predictable safety profile but did not have improved efficacy over gemcitabine and cisplatin alone *(84,85)*. A multinational clinical trial that combined erlotinib with cisplatin and gemcitabine in 1172 patients with advanced NSCLC also failed to show benefit for the combination *(84,86)*, raising questions regarding the optimal use of these agents in combination.

4.1.3. Gemcitabine-Carboplatin and Gemcitabine-Oxaliplatin

The platinum derivatives carboplatin and oxaliplatin offer potential therapeutic advances in terms of different toxicity profiles and possibly different efficacies. Carboplatin appears to be equally efficacious compared to cisplatin in NSCLC. Carboplatin is attractive because it gives less nonhematologic toxicity, but care has to be taken for additive myelotoxicity *(87)*; oxaliplatin is relative noncrossresistant with standard platinum compounds *(88)*.

4.1.4. Gemcitabine-Taxanes

To circumvent cisplatin-induced toxicities, the combination paclitaxel or docetaxel plus gemcitabine seems attractive *(89–91)*. Combinations with paclitaxel have been studied in phase II *(89,92,93)* and phase III trials *(91,94)*. In view of the approximately equal efficacy of current regimens, quality-of-life and cost-effectiveness analysis should now be included in future phase III trials.

4.2. Pancreatic Cancer

Chemotherapy has been used for advanced pancreatic cancer but still with limited success. Because of the aggressive behavior of the disease, emphasis of current experimental chemotherapy is also focusing on clinical benefit. Single-agent gemcitabine has a significant impact on survival and clinical benefit compared to 5-FU *(12,95)*. In contrast to the common schedule in NSCLC, gemcitabine is administered in a 7-wk schedule followed by 1-wk rest. Thereafter, gemcitabine is given weekly three times every 4 wk. Retrospective evaluation of 3023 pancreatic cancer patients treated with single-agent gemcitabine yielded a symptom improvement of 18% after the four treatment cycle; a response rate of 12% and a median survival of 4.8 mo were observed in 982 and 2380 patients, respectively *(96)*. Currently, gemcitabine treatment is considered standard therapy for pancreatic cancer. Drug combinations with gemcitabine have been studied.

4.2.1. Gemcitabine-Cisplatin

Phase II trials using the gemcitabine-cisplatin combination appeared to improve treatment results regarding response rate and survival *(97,98)*. Using 1000 mg/m^2 gemcitabine (d 1, 8, and 15) and 50 mg/m^2 cisplatin (d 1 and 15) every 28 d resulted in median survival of 8.2 and 7.4 mo, respectively. Randomized studies comparing single-agent gemcitabine with the combination have been initiated.

4.2.2. Gemcitabine-5-FU

Both gemcitabine and 5-FU as single agents improved treatment of pancreatic cancer. Gemcitabine inhibits the formation of deoxynucleoside triphosphates via inhibition of ribonucleotide reductase *(99)*, reducing deoxyuridine monophosphate and deoxythymidine 5´-triphosphate, which might enhance the antitumor effect of 5-FU. Combinations with 5-FU have been tested in phase II studies with 5-FU bolus at 600 mg/m^2 *(100,101)* or 5-FU protracted infusion at 200 mg/m^2/d *(102–104)*. Objective responses ranged from 3.7% to 20% and median survival from 4.0 to 10.3 mo. However, in a randomized trial (ECOG; Eastern Cooperative Oncology Group), the combination of 5-FU and gemcitabine did not improve the median survival of patients with advanced pancreatic cancer compared to the use of gemcitabine alone *(105)*.

4.2.3. Gemcitabine-Docetaxel

The combination of both gemcitabine and docetaxel was well tolerated, with neutropenia the dose-limiting toxicity; however, so far the addition of docetaxel to gemcitabine has not shown benefit compared to gemcitabine alone *(106,107)*. Based on preclinical models, Fine *(108)* has reported phase II trial response rates approaching 50% in advanced pancreatic cancer using a combination of gemcitabine, docetaxel, and capecitabine, dubbed GTX. Confirmation in larger trials is awaited.

4.2.4. Gemcitabine-Radiotherapy

Gemcitabine has been recognized as a potent radiosensitizing drug *(76,78)*. Radiosensitization by gemcitabine can be caused by different mechanisms, such as (1) gemcitabine's inhibition of repair of chromosome damage induced by irradiation; (2) induced cell cycle redistribution, which might accumulate cells into a more radiosensitive phase of the cell cycle; (3) depletion of deoxyadenosine triphosphate; and (4) level of dCK expression. Phase I studies in advanced pancreatic cancer demonstrated that the toxicity profile of gemcitabine combined with radiation had acceptable toxicity *(109,110)*. However, the toxicity of the combination was occasionally severe, including bleeding ulceration and fistula formation *(111,112)*. A phase II study has been performed in which patients with locally advanced pancreatic cancer were treated with 300 mg/m^2 gemcitabine and 800 cgy external beam radiation (d 1, 8, and 15, every 28 d) *(111)*. If possible, treatment was continued with single-agent 1000 mg/m^2 gemcitabine. Although toxicity was severe, 7 of 22 patients (32%) responded.

4.3. Advanced Bladder Cancer

4.3.1. Gemcitabine-Cisplatin

Until recently, the multidrug combinations CMV (cisplatin, methotrexate, and vinblastine) and MVAC (methotrexate, vinblastine, adriamycin, and cisplatin) were considered standard treatment for advanced bladder cancer *(113–115)*. Phase II studies of the gemcitabine-cisplatin combination showed notable response rates in advanced bladder cancer *(116–118)*. The gemcitabine-cisplatin combination has been randomized against the MVAC standard in a multinational, multicenter, phase III study *(119)*, comparing the gemcitabine (1000 mg/m^2, d 1, 8, 15) and cisplatin (70 mg/m^2, d 2) combination with standard MVAC in 405 patients. Response rate, time to progression, and survival were comparable for both the gemcitabine-cisplatin and the MVAC combination, with a response rate of 49% and 46% and overall survival of 13.8 and 14.8 mo, respectively. In addition, the gemcitabine-cisplatin provided a better safety profile and tolerability. Although long-time follow-up of the gemcitabine-cisplatin combination remains to be completed, the better risk-benefit ratio has

changed the standard of care from MVAC to gemcitabine-cisplatin for patients with advanced bladder cancer.

4.3.2. Gemcitabine-Paclitaxel

A phase II trial using the gemcitabine-paclitaxel regimen showed results that compare favorably with those produced by the first-line regimens, with a response rate of (29/54) 54% and a median survival of 14.4 mo *(120)*. Especially in patients with compromised renal function, this regimen seems attractive.

4.4. Advanced Gastric and Esophageal Cancer

At present, there is no established standard chemotherapy for advanced gastric and esophageal cancer. Cisplatin-based regimens are among the most active in gastric and esophageal cancer *(121–123)*, resulting in modest response rates up to 45% and poor median survival times ranging from 6 to 10 mo *(124,125)*. These results have prompted the evaluation of newer agents. In gastroesophageal cancers, a gemcitabine combination has not been studied before in a phase II setting. Although gemcitabine alone is not active in this disease *(126)*, we studied the combination with cisplatin because of the encouraging results obtained in a previous schedule-finding study *(62)*. In patients with advanced gastric and esophageal cancer, toxicity was manageable, with myelosuppression the main toxicity. In advanced gastric cancer, responses were observed in 12 of 40 patients (30%), with a median survival of 11 mo *(127)*. In patients with advanced esophageal cancer, 14 of 34 patients (41%) had an objective response, with a median survival of 9.8 mo *(128)*.

4.5. Breast Cancer

Gemcitabine has shown activity as a single agent in advanced breast cancer, with response rates in the range of 14–37% *(129–132)*. Weekly schedules of 800–1250 mg/m^2 administered on d 1, 8, and 15 every 28 d have been well tolerated. Using a 6-h infusion administered weekly at an MTD of 250 mg/m^2, a response rate of 25% was reported with tolerable toxicity *(133)*. Response rates tend to favor chemonaïve over previously treated patients, suggesting some crossresistance with taxanes, anthracyclines, and alkylating agents to which relapsed patients had been exposed. Based on these favorable results, a number of combination trials in breast cancer have been undertaken (Table 3).

4.5.1. Gemcitabine-Vinorelbine

The combination of vinorelbine plus gemcitabine has been extensively investigated. In a series of phase II trials, the most widely used schedule was that of 25–30 mg/m^2 vinorelbine plus 1000–1250 mg/m^2 gemcitabine, with both drugs administered d 1 and 8 every 21 d. Response rates of

Table 3
Select Gemcitabine-Based Combinations in Breast Cancer

Regimen	*Number*	*Response rate (%)*	*Median TTP*	*Median survival*	*Reference*
Gemcitabine single agent	39	37	5.1 mo	21.1 mo	*129*
	47	29	N/A	N/A	*132*
	25	16	5.1 mo	12.6 mo	*97*
	42	14.3	N/A	15.2 mo	*131*
	44	25	1.9 mo	11.5 mo	*130*
Vinorelbine and gemcitabine	31	22	3.5 mo	9.5 mo	*136*
	25	44	17 wk	NA	*135*
	51	33.3	10.8 mo	17.8 mo	*134*
	32	43.8	5 mo	NA	*137*
	51	54	NA	NA	*138*
Doxorubicin (epirubicin) and gemcitabine		33–89	NA	NA	*139*
Liposomal doxorubicin and gemcitabine	49	52	4.5 mo	16.1 mo	*140*
Paclitaxel and gemcitabine	29	55	NA	12 mo	*144*
	45	66.7	11 mo	19 mo	*143*
	43	68	16.6 mo	NA	*142*
	267	39.3	5.4 mo	N/A	*149*
Docetaxel and gemcitabine	39	36	7 mo	12.7 mo	*147*
	53	53	7.5 mo	16.5 mo	*146*
	50	46	NA	NA	*145*
	33	79	NA	24.5 mo	*148*

Doxorubicin (epirubicin) and gemcitabine and paclitaxel		83–92	NA	NA	*151*
Cisplatin and gemcitabine	31	50	14 wk	50 wk	*17*
Carboplatin and gemcitabine	30	30	20 wk	NA	*152*
Trastuzumab and gemcitabine	61	38	5.8 mo	14.7 mo	*153*
Trastuzumab and paclitaxel and gemcitabine	42	67	9 mo	27 mo	*154*

NA, not available; TTP, time to progression.

22–54% with acceptable toxicity were observed *(134–138)*. However, with single-agent activities for each drug of 20%, it remains to be shown whether the doublet provides clinical advantage over sequential single agents.

4.5.2. Gemcitabine-Anthracycline Combinations

For gemcitabine-anthracycline combinations, both doxorubicin and epirubicin have provided response rates of 33–89% *(139)*. A 52% objective response rate was reported for the combination of liposomal doxorubicin plus gemcitabine in relapsed breast cancer patients, with 58% of patients who had previously received anthracycline therapy responding *(140)*.

4.5.3. Gemcitabine-Taxanes

Paclitaxel and docetaxel have both been successfully combined with gemcitabine in breast cancer. Paclitaxel-plus-gemcitabine combinations resulted in objective response rates of 55–71% *(141–144)*, highly similar to results with docetaxel, at 36–79% *(145–148)*. Principal toxicities have been hematologic, with fluid retention and allergic and hepatic toxicities also reported. The largest study compared paclitaxel at 175 mg/m^2 every 3 wk with the same paclitaxel dose plus gemcitabine at 1250 mg/m^2 on d 1 and 8 every 21 d. Response rates of 25.6% vs 39.3% and median time to progression of 3.5 vs 5.4 mo favored the combination arm, leading to the US Food and Drug Administration to approve this combination for relapsed breast cancer *(145–149)*. A parallel study found no significant pharmacokinetic interactions between the agents *(145–148,150)*. Based on favorable doublet results, the triplet regimens of gemcitabine plus paclitaxel with either doxorubicin or epirubicin have been explored, providing objective responses as high as 94% *(151)*. A comparison of the epirubicin-based GET (gemcitabine-epirubicin-taxol) vs FEC (fluorouracil-epirubicin-cytoxan) regimens has been undertaken by European investigators in a large phase III trial.

4.5.4. Gemcitabine-Platinum

The activity and synergy between platins and gemcitabine led to the examination of these doublets in advanced breast cancer. Cisplatin plus gemcitabine provided objective responses in 23–50% of previously treated, relapsed breast cancer patients, including patients treated following autologous bone marrow transplant relapses *(14,16)*. Carboplatin plus gemcitabine has yielded similar results *(152)*. With hematologic toxicity dose limiting, thrombocytopenia has been the most common finding. Lower dose schedules that employed cisplatin at 30 mg/m^2 with gemcitabine at 600–1000 mg/m^2 were generally well tolerated, with little neuropathy or alopecia reported. The concept of “capturing” repair-efficient

cells, although requiring further clinical confirmation, offers a theoretically attractive option for the use of this repeating-doublet schedule in heavily pretreated patients or as a consolidation regimen.

4.5.5. Gemcitabine-Trastuzumab

With the introduction of trastuzumab, combinations that employ this agent with gemcitabine alone or with taxanes have been explored. One trial combining trastuzumab at 4 mg/m^2 load followed by 2 mg/m^2 weekly combined with gemcitabine at 1200 mg/m^2 for 2 wk every 21 d provided objective responses in 23 of 61 (38%) and a median time to progression of 5.8 mo *(153)*. Adding paclitaxel to this regimen at 175 mg/m^2 on d 1 improved the overall response rate to 67% (28/42), including complete response in 10% (4/42), with a median time to progression of over 9 mo *(154)*.

The favorable toxicity profile and activity for numerous gemcitabine-based combinations has led to the application of this agent in advanced breast cancer. Current trials are examining the role of gemcitabine in the neoadjuvant setting, with future applications in the postoperative adjuvant setting under development.

4.6. Ovarian Cancer

Ovarian cancer is among the most chemoresponsive epithelial neoplasms. Favorable responses are observed with alkylating agents, platins, anthracyclines, both topoisomerase I and II inhibitors, and the antimetabolites. As a single agent, in phase II trials gemcitabine showed responses in the range of 11–22% *(155–159)*. Activity for gemcitabine is preserved even in patients with documented resistance to platinum and paclitaxel *(160,161)*, which may in part reflect the development of collateral sensitivity as previously described in cell lines *(160–162)*. The drug's favorable toxicity profile has made it an attractive option for patients with relapsed disease.

Therapy for recurrent ovarian cancer has increasingly incorporated gemcitabine into combinations with agents of known single-agent activity for this disease. A compendium of select combination trials is provided in Table 4. Gemcitabine plus paclitaxel provided a response rate of 40% and median time to progression of 5.7 mo *(163)*; gemcitabine and topotecan, in a dose-seeking trial, revealed responses in 6 and stable disease in 4 of 12 evaluable patients *(164)*. The combination of gemcitabine and doxorubicin in platinum-resistant disease was reported to provide objective response in 24% and a duration of response of 5 mo*(165)*, similar to the 34% response rate for the related gemcitabine-liposomal doxorubicin combination *(160)*.

4.6.1. Gemcitabine-Cisplatin

As platin-based therapies have become the mainstay of treatment for ovarian cancer, resistance to platins has become an important clinical chal-

Table 4
Select Gemcitabine-Based Combinations in Ovarian Cancer

Regimen	*Number*	*Response rate (%)*	*Median TTP*	*Median survival*	*Reference*
Gemcitabine single agent	40	22	NA	NA	*159*
	38	13.9	NA	6.7 mo	*155*
	27	11	NA	NA	*158*
	50	19	2.8 mo	6.2 mo	*156*
	38	11	NA	NA	*157*
	51	16	NA	NA	*161*
	41	17	NA	NA	*160*
Gemcitabine and Paclitaxel	35	40	5.7 mo	13.1 mo	*163*
Gemcitabine and Topotecan	23		NA	15.3 mo	*164*
Gemcitabine and Doxorubicin	49	24	NA	12 mo	*165*
Gemcitabine and Liposomal Doxorubicin	27	34.4	NA	NA	*160*
Gemcitabine and Cisplatin	27	70	_7.9 mo	NA	*17*
	36	42.9	NA	12 mo	*166*
	40	70.7	10.4	23.4 mo	*167*
	37	62.2	NA	27.7 mo	*168*
	37		13.4 mo	24 mo	*169*
Carboplatin and Gemcitabine	37	40.5	9 mo	24.5 mo	*171*
	26	62.5	10 mo	18+ mo	*170*
Carboplatin and Paclitaxel	21	NA	NA	NA	*172*
and Gemcitabine	57	90.4	15.5 mo	40.8 mo	*173*
	20	75	6.5 mo	NA	*174*

lenge. Preclinical observations indicating that the combination of cisplatin plus gemcitabine could reverse platinum resistance led to the clinical examination of this doublet in patients who relapsed following platinum therapy. Two phase II trials administered cisplatin at 30 mg/m^2 plus gemcitabine at 750 mg/m^2, with both drugs administered d 1 and d 8 every 21 d *(17,166)*. In one study, the overall response rate of 70% was associated with a 50% response rate in the cisplatin-resistant patients; the second study, which limited accrual to patients who were cisplatin resistant and who also overexpressed P-glycoprotein, revealed a response rate of 42% *(166)*. In chemonaive patients, a response of 71% was reported; two other groups observed similarly high responses of 62% and 57%, respectively, using cisplatin at 75–100 mg/m^2 on d 1 combined with gemcitabine at 1250 mg/m^2 on d 1 and 8 *(168,169)*. The GOG clinical trial testing this combination in platinum-resistant patients provided an objective response rate of 16%, with stable disease in an additional 54%. The median TTP of 5.4 mo compared favorably with other trials in this population *(202)*. Similar results have been observed in recurrent cancer of the cervix with the cisplatin/gemcitabine doublet, providing an objective response rate of 22% and TTP of 3.5 mo *(203)*. Related trials that combined carboplatin with an area under the curve of 4 or 5 on d 1 plus gemcitabine 1000 mg/m^2 on d 1 and 8 in the second-line setting reported objective response in 41–62%, with neutropenia and thrombocytopenia the principal toxicities *(170,171)*. Oxaliplatin-gemcitabine combinations are also under investigation.

Finally, the addition of gemcitabine to the widely used paclitaxel-carboplatin doublet has been the subject of several studies, two in the previously untreated *(172,173)* and one in the relapsed setting *(174)*, providing response rates of 75–90%, with dose-limiting myelosuppression in two studies and increased neuropathy in the third. The high activity observed has led to the incorporation of this triplet into the current GOG 182-ICON4 international clinical trial as one of the five arms *(175)*. Indeed, of the four experimental arms in the Gynecologie Oncology Group (GOG) 182-International Collaboration Ovarian Neoplasm (182-ICON)-4 clinical trial, two are gemcitabine based, indicating the important potential of this agent in the future management of advanced ovarian cancer.

5. CONCLUSIONS

Gemcitabine acts uniquely through its multiple self-potentiating mechanisms, in which dCK and dFdCTP play important roles. Its antitumor activity has been confirmed in various solid tumor types. In addition, the relatively mild toxicity profile and partially nonoverlapping toxicity with other anticancer agents, such as the platinum analogs and taxanes, make

usage in combinations attractive. Results of gemcitabine combinations with other anticancer agents or radiotherapy are encouraging but might be improved by the identification of molecular markers capable of selecting responding patients. Combinations with new therapies are currently ongoing and might benefit from the increasing knowledge of the more established agents, hopefully resulting in increased cure rates in cancer patients.

REFERENCES

1. Dumontet C, Morschhauser F, Solal-Celigny P, et al. Gemcitabine as a single agent in the treatment of relapsed or refractory low-grade non-Hodgkin's lymphoma. Br J Haematol 2001;113:772–778.
2. Hertel LW, Kroin JS, Misner JW, Tustin JM. Synthesis of 2′-deoxy-2′,2′-difluoro-D-ribose and 2′-deoxy-2′,2′-difluoro-D-ribofuranosyl nucleosides. J Org Chem 1988;53:2406–2409.
3. Braakhuis BJ, Ruiz Van Haperen V, Welters MJ, Peters GJ. Schedule-dependent therapeutic efficacy of the combination of gemcitabine and cisplatin in head and neck cancer xenografts. Eur J Cancer 1995;31A:2335–2340.
4. Heinemann V, Xu YZ, Chubb S, et al. Cellular elimination of 2′,2′-difluorodeoxycytidine 5′-triphosphate: a mechanism of self-potentiation. Cancer Res 1992;52:533–539.
5. Heinemann V, Hertel LW, Grindey GB, Plunkett W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. Cancer Res 1988;48:4024–4031.
6. Hertel LW, Boder GB, Kroin JS, et al. Evaluation of the antitumor activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). Cancer Res 1990;50:4417–4422.
7. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res 1991;51:6110–6117.
8. Merriman RL, Hertel LW, Schultz RM, et al. Comparison of the antitumor activity of gemcitabine and ara-C in a panel of human breast, colon, lung and pancreatic xenograft models. Invest New Drugs 1996;14:243–247.
9. Gale RP. Advances in the treatment of acute myelogenous leukemia. N Engl J Med 1979;300:1189–1199.
10. Rowe JM. What is the best induction regimen for acute myelogenous leukemia? Leukemia 1998;12(suppl 1):S16–S19.
11. Anderson H, Hopwood P, Stephens RJ, et al. Gemcitabine plus best supportive care (BSC) vs BSC in inoperable non-small cell lung cancer—a randomized trial with quality of life as the primary outcome. UK NSCLC Gemcitabine Group. Non-Small Cell Lung Cancer. Br J Cancer 2000;83: 447–453.
12. Burris HA, III, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 1997;15:2403–2413.
13. Rothenberg ML, Moore MJ, Cripps MC, et al. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. Ann Oncol 1996;7:347–353.
14. Nagourney RA. Gemcitabine plus cisplatin in breast cancer. Oncology (Huntingt) 2001;15:28–33.

15. Nagourney RA, Evans SS, Messenger JC, Su YZ, Weisenthal LM. 2-Chlorodeoxyadenosine activity and cross resistance patterns in primary cultures of human hematologic neoplasms. Br J Cancer 1993;67:10–14.
16. Nagourney RA, Link JS, Blitzer JB, Forsthoff C, Evans SS. Gemcitabine plus cisplatin repeating doublet therapy in previously treated, relapsed breast cancer patients. J Clin Oncol 2000;18:2245–2249.
17. Nagourney RA, Brewer CA, Radecki S, et al. Phase II trial of gemcitabine plus cisplatin repeating doublet therapy in previously treated, relapsed ovarian cancer patients. Gynecol Oncol 2003;88:35–39.
18. Mackey JR, Mani RS, Selner M, et al. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 1998;58:4349–4357.
19. Ruiz van Haperen VWT, Veerman G, Vermorken JB, Peters GJ. 2′,2′-Difluorodeoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. Biochem Pharmacol 1993;46:762–766.
20. Ruiz van Haperen VWT, Veerman G, Boven E, Noordhuis P, Vermorken JB, Peters GJ. Schedule dependence of sensitivity to 2′,2′-difluorodeoxycytidine (gemcitabine) in relation to accumulation and retention of its triphosphate in solid tumour cell lines and solid tumours. Biochem Pharmacol 1994;48:1327–1339.
21. van Moorsel CJ, Bergman AM, Veerman G, et al. Differential effects of gemcitabine on ribonucleotide pools of 21 solid tumour and leukaemia cell lines. Biochim Biophys Acta 2000;1474:5–12.
22. Peters GJ, van der Wilt CL, van Moorsel CJ, Kroep JR, Bergman AM, Ackland SP. Basis for effective combination cancer chemotherapy with antimetabolites. Pharmacol Ther 2000;87:227–253.
23. Heinemann V, Schulz L, Issels RD, Plunkett W. Gemcitabine: a modulator of intracellular nucleotide and deoxynucleotide metabolism. Semin Oncol 1995;22: 11–18.
24. Pritchard DM, Watson AJ, Potten CS, Jackman AL, Hickman JA. Inhibition by uridine but not thymidine of p53-dependent intestinal apoptosis initiated by 5-fluorouracil: evidence for the involvement of RNA perturbation. Proc Natl Acad Sci USA 1997;94:1795–1799.
25. Kroep JR, Giaccone G, Tolis C, et al. Sequence dependent effect of paclitaxel on gemcitabine metabolism in relation to cell cycle and cytotoxicity in non-small-cell lung cancer cell lines. Br J Cancer 2000;83:1069–1076.
26. Tolis C, Peters GJ, Ferreira CG, Pinedo HM, Giaccone G. Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. Eur J Cancer 1999;35:796–807.
27. Bouffard DY, Momparler RL. Comparison of the induction of apoptosis in human leukemic cell lines by 2′,2′-difluorodeoxycytidine (gemcitabine) and cytosine arabinoside. Leuk Res 1995;19:849–856.
28. Huang P, Plunkett W. Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. Cancer Chemother Pharmacol 1995;36:181–188.
29. Ferreira CG, Tolis C, Span SW, et al. Drug-induced apoptosis in lung cancer cells is not mediated by the Fas/FasL (CD95/APO1) signaling pathway. Clin Cancer Res 2000;6:203–212.
30. Dumontet C, Fabianowska-Majewska K, Mantincic D, et al. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br J Haematol 1999;106:78–85.

31. Goan YG, Zhou B, Hu E, Mi S, Yen Y. Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line. Cancer Res 1999;59:4204–4207.
32. Ruiz van Haperen VWT, Veerman G, Eriksson S, et al. Development and molecular characterization of a 2′,2′-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. Cancer Res 1994;54:4138–4143.
33. Al Madhoun AS, van der Wilt CL, Loves WJ, et al. Detection of an alternatively spliced form of deoxycytidine kinase mRNA in the 2′-2′-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cancer cell line AG6000. Biochem Pharmacol 2004;68:601–609.
34. Kroep JR, Loves WJ, van der Wilt CL, et al. Pretreatment deoxycytidine kinase levels predict in vivo gemcitabine sensitivity. Mol Cancer Ther 2002;1:371–376.
35. Spasokoukotskaja T, Arner ES, Brosjo O, et al. Expression of deoxycytidine kinase and phosphorylation of 2-chlorodeoxyadenosine in human normal and tumour cells and tissues. Eur J Cancer 1995;31A:202–208.
36. Weber G, Singhal RL, Abonyi M, et al. Regulation of deoxycytidine kinase activity and inhibition by DFDC. Adv Enzyme Regul 1993;33:39–59.
37. Bergman AM, Giaccone G, van Moorsel CJ, et al. Cross-resistance in the 2′,2′-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cancer cell line AG6000 to standard and investigational drugs. Eur J Cancer 2000;36:1974–1983.
38. O'Rourke TJ, Brown TD, Havlin K, et al. Phase I clinical trial of gemcitabine given as an intravenous bolus on five consecutive days. Eur J Cancer 1994;30A:417, 418.
39. Vermorken JB, Guastalla JP, Hatty SR, et al. Phase I study of gemcitabine using a once every 2 wk schedule. Br J Cancer 1997;76:1489–1493.
40. Pollera CF, Ceribelli A, Crecco M, Oliva C, Calabresi F. Prolonged infusion gemcitabine: a clinical phase I study at low- (300 mg/m^2) and high-dose (875 mg/m^2) levels. Invest New Drugs 1997;15:115–121.
41. Anderson H, Lund B, Bach F, Thatcher N, Walling J, Hansen HH. Single-agent activity of weekly gemcitabine in advanced non-small-cell lung cancer: a phase II study. J Clin Oncol 1994;12:1821–1826.
42. Grunewald R, Kantarjian H, Du M, Faucher K, Tarassoff P, Plunkett W. Gemcitabine in leukemia: a phase I clinical, plasma, and cellular pharmacology study. J Clin Oncol 1992;10:406–413.
43. Gandhi V, Plunkett W. Cellular and clinical pharmacology of fludarabine. Clin Pharmacokinet 2002;41:93–103.
44. Tempero M, Plunkett W, Ruiz Van Haperen V, et al. Randomized phase II comparison of dose-intense gemcitabine: 30-min infusion and fixed dose rate infusion in patients with pancreatic adenocarcinoma. J Clin Oncol 2003;21:3402–3408.
45. Abbruzzese JL, Grunewald R, Weeks EA, et al. A phase I clinical, plasma, and cellular pharmacology study of gemcitabine. J Clin Oncol 1991;9:491–498.
46. Peters GJ, Schornagel JH, Milano GA. Clinical pharmacokinetics of anti-metabolites. Cancer Surv 1993;17:123–156.
47. van Moorsel CJ, Kroep JR, Pinedo HM, et al. Pharmacokinetic schedule finding study of the combination of gemcitabine and cisplatin in patients with solid tumors. Ann Oncol 1999;10:441–448.
48. Kroep JR, Giaccone G, Voorn DA, et al. Gemcitabine and paclitaxel: pharmacokinetic and pharmacodynamic interactions in patients with non-small-cell lung cancer. J Clin Oncol 1999;17:2190–2197.

49. Edzes HT, Peters GJ, Noordhuis P, Vermorken JB. Determination of the antimetabolite gemcitabine (2´,2´-difluoro-2´-deoxycytidine) and of 2´,2´-difluoro-2´-deoxyuridine by 19F nuclear magnetic resonance spectroscopy. Anal Biochem 1993;214:25–30.
50. Grunewald R, Abbruzzese JL, Tarassoff P, Plunkett W. Saturation of 2´,2´-difluorodeoxycytidine 5´-triphosphate accumulation by mononuclear cells during a phase I trial of gemcitabine. Cancer Chemother Pharmacol 1991;27:258–262.
51. Storniolo AM, Allerheiligen SR, Pearce HL. Preclinical, pharmacologic, and phase I studies of gemcitabine. Semin Oncol 1997;24:S7.
52. Martin C, Pollera CF. Gemcitabine: safety profile unaffected by starting dose. Int J Clin Pharmacol Res 1996;16:9–18.
53. Gemcitabine US prescribing information. Eli Lilly and Company;2002. Internet communication, www.gemzar.com/hcp/for-oncology-professionals.jsp.
54. Roychowdhury DF, Cassidy CA, Peterson P, Arning M. A report on serious pulmonary toxicity associated with gemcitabine-based therapy. Invest New Drugs 2002;20:311–315.
55. Walter RB, Joerger M, Pestalozzi BC. Gemcitabine-associated hemolytic-uremic syndrome. Am J Kidney Dis 2002;40:E16.
56. Voorburg AM, van Beek FT, Slee PH, Seldenrijk CA, Schramel FM. Vasculitis due to gemcitabine. Lung Cancer 2002;36:203–205.
57. Chou TC, Talalay P. Application of the median-effect principle for the assessment of low risk carcinogens and for quantitation of synergism and antagonism of chemotherapeutic agents. In: Harrap KR, Connors TA, eds. New Avenues in Developmental Cancer Chemotherapy. New York: Academic Press, 1987:37–64.
58. Sherman SE, Lippard SJ. Structural aspects of platinum anticancer drug interactions with DNA. Chem Rev 1987;87:1153–1181.
59. Bergman AM, Ruiz Van Haperen V, Veerman G, Kuiper CM, Peters GJ. Synergistic interaction between cisplatin and gemcitabine in vitro. Clin Cancer Res 1996;2:521–530.
60. van Moorsel CJ, Pinedo HM, Veerman G, et al. Mechanisms of synergism between cisplatin and gemcitabine in ovarian and non-small-cell lung cancer cell lines. Br J Cancer 1999;80:981–990.
61. van Moorsel CJ, Pinedo HM, Veerman G, Vermorken JB, Postmus PE, Peters GJ. Scheduling of gemcitabine and cisplatin in Lewis lung tumour bearing mice. Eur J Cancer 1999;35:808–814.
62. Kroep JR, Peters GJ, van Moorsel CJ, et al. Gemcitabine-cisplatin: a schedule finding study. Ann Oncol 1999;10:1503–1510.
63. Abratt RP, Sandler A, Crino L, et al. Combined cisplatin and gemcitabine for non-small cell lung cancer: influence of scheduling on toxicity and drug delivery. Semin Oncol 1998;25:35–43.
64. Ricci S, Antonuzzo A, Galli L, et al. A randomized study comparing two different schedules of administration of cisplatin in combination with gemcitabine in advanced nonsmall cell lung carcinoma. Cancer 2000;89:1714–1719.
65. Soto PH, Cavina R, Latteri F, et al. Three-wk vs 4-wk schedule of cisplatin and gemcitabine: results of a randomized phase II study. Ann Oncol 2002;13: 1080–1086.
66. Theodossiou C, Cook JA, Fisher J, et al. Interaction of gemcitabine with paclitaxel and cisplatin in human tumor cell lines. Int J Oncol 1998;12:825–832.

67. Shewach DS, Reynolds KK, Hertel L. Nucleotide specificity of human deoxycytidine kinase. Mol Pharmacol 1992;42:518–524.
68. Ruiz van Haperen VWT, Veerman G, Vermorken JB, Pinedo HM, Peters G. Regulation of phosphorylation of deoxycytidine and 2′,2′-difluorodeoxycytidine (gemcitabine); effects of cytidine 5′-triphosphate and uridine 5′-triphosphate in relation to chemosensitivity for 2′,2′-difluorodeoxycytidine. Biochem Pharmacol 1996;51:911–918.
69. Faivre S, Raymond E, Woynarowski JM, Cvitkovic E. Supraadditive effect of 2´,2´-difluorodeoxycytidine (gemcitabine) in combination with oxaliplatin in human cancer cell lines. Cancer Chemother Pharmacol 1999;44:117–123.
70. Zoli W, Ricotti L, Dal Susino M, et al. Docetaxel and gemcitabine activity in NSCLC cell lines and in primary cultures from human lung cancer. Br J Cancer 1999;81:609–615.
71. van Moorsel CJ, Pinedo HM, Veerman G, et al. Combination chemotherapy studies with gemcitabine and etoposide in non-small cell lung and ovarian cancer cell lines. Biochem Pharmacol 1999;57:407–415.
72. Aung TT, Davis MA, Ensminger WD, Lawrence TS. Interaction between gemcitabine and mitomycin-C in vitro. Cancer Chemother Pharmacol 2000;45: 38–42.
73. Rauchwerger DR, Firby PS, Hedley DW, Moore MJ. Equilibrative-sensitive nucleoside transporter and its role in gemcitabine sensitivity. Cancer Res 2000; 60:6075–6079.
74. Tonkinson JL, Worzalla JF, Teng CH, Mendelsohn LG. Cell cycle modulation by a multitargeted antifolate, LY231514, increases the cytotoxicity and antitumor activity of gemcitabine in HT29 colon carcinoma. Cancer Res 1999;59: 3671–3676.
75. Csapo Z, Keszler G, Sasvari-Szekely M, et al. Similar changes were induced by cladribine and by gemcitabine, in the deoxypyrimidine salvage, during short-term treatments. Adv Exp Med Biol 1998;431:525–529.
76. Shewach DS, Hahn TM, Chang E, Hertel LW, Lawrence TS. Metabolism of 2´,2´-difluoro-2´-deoxycytidine and radiation sensitization of human colon carcinoma cells. Cancer Res 1994;54:3218–3223.
77. van Putten JWG, Groen HJM, Smid K, Peters GJ, Kampinga HH. End-joining deficiency and radiosensitization induced by gemcitabine. Cancer Res 2001; 61: 1585–1591.
78. McGinn CJ, Shewach DS, Lawrence TS. Radiosensitizing nucleosides. J Natl Cancer Inst 1996;88:1193–1203.
79. Non-Small Cell Lung Cancer Collaborative Group. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. BMJ 1995;311:899–909.
80. Carney DN, Hansen HH. Non-small-cell lung cancer—stalemate or progress? N Engl J Med 2000;343:1261–1262.
81. Ranson M, Davidson N, Nicolson M, et al. Randomized trial of paclitaxel plus supportive care vs supportive care for patients with advanced non-small-cell lung cancer. J Natl Cancer Inst 2000;92:1074–1080.
82. Roszkowski K, Pluzanska A, Krzakowski M, et al. A multicenter, randomized, phase III study of docetaxel plus best supportive care vs best supportive care in chemotherapy-naive patients with metastatic or non-resectable localized non-small cell lung cancer (NSCLC). Lung Cancer 2000;27:145–157.

83. The Elderly Lung Cancer Vinorelbine Italian Study Group. Effects of vinorelbine on quality of life and survival of elderly patients with advanced non-small-cell lung cancer. J Natl Cancer Inst 1999;91:66–72.
84. Langer CJ. Emerging role of epidermal growth factor receptor inhibition in therapy for advanced malignancy: focus on NSCLC. Int J Radiat Oncol Biol Phys 2004;58:991–1002.
85. Giaccone G, Herbst RS, Manegold C, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial—INTACT 1. J Clin Oncol 2004;22:777–784.
86. Gatzemeier U, Pluzanska A, Szczesna A, et al. Results of a phase III trial of erlotinib (OSI 774) combined with cisplatin and gemcitabine (GC) chemotherapy in advanced non-small cell lung cancer (NSCLC). Proc Am Soc Clin Oncol 2004;617.
87. Ng EW, Sandler AB, Robinson L, Einhorn LH. A phase II study of carboplatin plus gemcitabine in advanced non-small-cell lung cancer (NSCLC): a Hoosier Oncology Group study. Am J Clin Oncol 1999;22:550–553.
88. Page JD, Husain I, Sancar A, Chaney SG. Effect of the diaminocyclohexane carrier ligand on platinum adduct formation, repair, and lethality. Biochemistry 1990;29:1016–1024.
89. Giaccone G, Smit EF, van Meerbeeck JP, et al. A phase I–II study of gemcitabine and paclitaxel in advanced non-small-cell lung cancer patients. Ann Oncol 2000;11:109–112.
90. Georgoulias V, Kouroussis C, Androulakis N, et al. Front-line treatment of advanced non-small-cell lung cancer with docetaxel and gemcitabine: a multicenter phase II trial. J Clin Oncol 1999;17:914–920.
91. Kosmidis P, Mylonakis N, Nicolaides C, et al. Paclitaxel plus carboplatin vs gemcitabine plus paclitaxel in advanced non-small-cell lung cancer: a phase III randomized trial. J Clin Oncol 2002;20:3578–3585.
92. Douillard JY, Lerouge D, Monnier A, et al. Combined paclitaxel and gemcitabine as first-line treatment in metastatic non-small cell lung cancer: a multicentre phase II study. Br J Cancer 2001;84:1179–1184.
93. Isla D, Rosell R, Sanchez JJ, et al. Phase II trial of paclitaxel plus gemcitabine in patients with locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 2001;19:1071–1077.
94. Smit EF, van Meerbeeck JP, Lianes P, et al. Three-arm randomized study of two cisplatin-based regimens and paclitaxel plus gemcitabine in advanced non-small-cell lung cancer: a phase III trial of the European Organization for Research and Treatment of Cancer Lung Cancer Group-EORTC 08975. J Clin Oncol 2003;21:3909–3917.
95. Klein B, Sadikov E, Mishaeli M, Levin I, Figer A. Comparison of 5-FU and leucovorin to gemcitabine in the treatment of pancreatic cancer. Oncol Rep 2000;7:875–877.
96. Storniolo AM, Enas NH, Brown CA, Voi M, Rothenberg ML, Schilsky R. An investigational new drug treatment program for patients with gemcitabine: results for over 3000 patients with pancreatic carcinoma. Cancer 1999;85:1261–1268.
97. Brodowicz T, Wolfram RM, Kostler WJ, et al. Phase II study of gemcitabine in combination with cisplatin in patients with locally advanced and/or metastatic pancreatic cancer. Anticancer Drugs 2000;11:623–628.

98. Heinemann V, Wilke H, Mergenthaler HG, et al. Gemcitabine and cisplatin in the treatment of advanced or metastatic pancreatic cancer. Ann Oncol 2000;11: 1399–1403.
99. Smid K, van Moorsel CJ, Noordhuis P, Voorn DA, Peters GJ. Interference of gemcitabine triphosphate with the measurements of deoxynucleotides using an optimized DNA polymerase elongation assay. Int J Oncol 2001;19:157–162.
100. Berlin JD, Adak S, Vaughn DJ, et al. A phase II study of gemcitabine and 5-fluorouracil in metastatic pancreatic cancer: an Eastern Cooperative Oncology Group Study (E3296). Oncology 2000;58:215–218.
101. Cascinu S, Silva RR, Barni S, et al. A combination of gemcitabine and 5-fluorouracil in advanced pancreatic cancer, a report from the Italian Group for the Study of Digestive Tract Cancer (GISCAD). Br J Cancer 1999;80:1595–1598.
102. Hidalgo M, Castellano D, Paz-Ares L, et al. Phase I-II study of gemcitabine and fluorouracil as a continuous infusion in patients with pancreatic cancer. J Clin Oncol 1999;17:585–592.
103. Rauch DP, Maurer CA, Aebi S, et al. Activity of gemcitabine and continuous infusion fluorouracil in advanced pancreatic cancer. Oncology 2001;60:43–48.
104. Kurtz JE, Kohser F, Negrier S, et al. Gemcitabine and protracted 5-FU for advanced pancreatic cancer. A phase II study. Hepatogastroenterology 2000;47: 1450–1453.
105. Berlin JD, Catalano P, Thomas JP, Kugler JW, Haller DG, Benson AB III. Phase III study of gemcitabine in combination with fluorouracil vs gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297. J Clin Oncol 2002;20:3270–3275.
106. Cascinu S, Gasparini G, Catalano V, et al. A phase I-II study of gemcitabine and docetaxel in advanced pancreatic cancer: a report from the Italian Group for the Study of Digestive Tract Cancer (GISCAD). Ann Oncol 1999;10:1377–1379.
107. Ryan DP, Kulke MH, Fuchs CS, et al. A phase II study of gemcitabine and docetaxel in patients with metastatic pancreatic carcinoma. Cancer 2002;94:97–103.
108. Fine RL, Fogelman DR, Schreibman S. GTX chemotherapy for metastatic pancreatic cancer: response, survival and toxicity data. Proc Am Soc Clin Oncol 2004:23 (abstr. 4271).
109. Wolff RA, Evans DB, Gravel DM, et al. Phase I trial of gemcitabine combined with radiation for the treatment of locally advanced pancreatic adenocarcinoma. Clin Cancer Res 2001;7:2246–2253.
110. Pipas JM, Mitchell SE, Barth RJ Jr, et al. Phase I study of twice-weekly gemcitabine and concomitant external-beam radiotherapy in patients with adenocarcinoma of the pancreas. Int J Radiat Oncol Biol Phys 2001;50:1317–1322.
111. de Lange SM, van Groeningen CJ, Meijer OW, et al. Gemcitabine-radiotherapy in patients with locally advanced pancreatic cancer. Eur J Cancer 2002;38: 1212–1217.
112. McGinn CJ, Zalupski MM, Shureiqi I, et al. Phase I trial of radiation dose escalation with concurrent weekly full-dose gemcitabine in patients with advanced pancreatic cancer. J Clin Oncol 2001;19:4202–4208.
113. Harker WG, Meyers FJ, Freiha FS, et al. Cisplatin, methotrexate, and vinblastine (CMV): an effective chemotherapy regimen for metastatic transitional cell carcinoma of the urinary tract. A Northern California Oncology Group study. J Clin Oncol 1985;3:1463–1470.

114. Loehrer PJ Sr, Einhorn LH, Elson PJ, et al. A randomized comparison of cisplatin alone or in combination with methotrexate, vinblastine, and doxorubicin in patients with metastatic urothelial carcinoma: a cooperative group study. J Clin Oncol 1992;10:1066–1073.
115. Logothetis CJ, Dexeus FH, Finn L, et al. A prospective randomized trial comparing MVAC and CISCA chemotherapy for patients with metastatic urothelial tumors. J Clin Oncol 1990;8:1050–1055.
116. von der Maasse H, Andersen L, Crino L, Weinknecht S, Dogliotti L. Weekly gemcitabine and cisplatin combination therapy in patients with transitional cell carcinoma of the urothelium: a phase II clinical trial. Ann Oncol 1999;10: 1461–1465.
117. Kaufman D, Raghavan D, Carducci M, et al. Phase II trial of gemcitabine plus cisplatin in patients with metastatic urothelial cancer. J Clin Oncol 2000;18: 1921–1927.
118. Moore MJ, Winquist EW, Murray N, et al. Gemcitabine plus cisplatin, an active regimen in advanced urothelial cancer: a phase II trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 1999;17:2876–2881.
119. von der Maasse H, Hansen SW, Roberts JT, et al. Gemcitabine and cisplatin vs methotrexate, vinblastine, doxorubicin, and cisplatin in advanced or metastatic bladder cancer: results of a large, randomized, multinational, multicenter, phase III study. J Clin Oncol 2000;18:3068–3077.
120. Meluch AA, Greco FA, Burris HA III, et al. Paclitaxel and gemcitabine chemotherapy for advanced transitional-cell carcinoma of the urothelial tract: a phase II trial of the Minnie Pearl Cancer Research Network. J Clin Oncol 2001; 19:3018–3024.
121. Fuchs CS. Chemotherapy for advanced gastric cancer: where do we stand? J Clin Oncol 1997;15:3299–3300.
122. Leichman L, Berry BT. Experience with cisplatin in treatment regimens for esophageal cancer. Semin Oncol 1991;18:64–72.
123. Leichman L, Berry BT. Cisplatin therapy for adenocarcinoma of the stomach. Semin Oncol 1991;18:25–33.
124. Webb A, Cunningham D, Scarffe JH, et al. Randomized trial comparing epirubicin, cisplatin, and fluorouracil vs fluorouracil, doxorubicin, and methotrexate in advanced esophagogastric cancer. J Clin Oncol 1997;15:261–267.
125. Kulke MH. The treatment of advanced gastric cancer: in search of the right combination. J Clin Oncol 2000;18:2645–2647.
126. Sandler AB, Kindler HL, Einhorn LH, et al. Phase II trial of gemcitabine in patients with previously untreated metastatic cancer of the esophagus or gastroesophageal junction. Ann Oncol 2000;11:1161–1164.
127. de Lange SM, van Groeningen CJ, Kroep JR, et al. Phase II trial of cisplatin and gemcitabine in patients with advanced gastric cancer. Ann Oncol 2004;15: 484–488.
128. Kroep JR, Pinedo HM, Giaccone G, Van Bochove A, Peters GJ, van Groeningen CJ. Phase II study of cisplatin preceding gemcitabine in patients with advanced oesophageal cancer. Ann Oncol 2004;15:230–235.
129. Blackstein M, Vogel CL, Ambinder R, Cowan J, Iglesias J, Melemed A. Gemcitabine as first-line therapy in patients with metastatic breast cancer: a phase II trial. Oncology 2002;62:2–8.

130. Carmichael J, Possinger K, Phillip P, et al. Advanced breast cancer: a phase II trial with gemcitabine. J Clin Oncol 1995;13:2731–2736.
131. Possinger K, Kaufmann M, Coleman R, et al. Phase II study of gemcitabine as first-line chemotherapy in patients with advanced or metastatic breast cancer. Anticancer Drugs 1999;10:155–162.
132. Spielmann M, Llombart-Cussac A, Kalla S, et al. Single-agent gemcitabine is active in previously treated metastatic breast cancer. Oncology 2001;60:303–307.
133. Akrivakis K, Schmid P, Flath B, et al. Prolonged infusion of gemcitabine in stage IV breast cancer: a phase I study. Anticancer Drugs 1999;10:525–531.
134. Donadio M, Ardine M, Berruti A, et al. Gemcitabine and vinorelbine as second-line treatment in patients with metastatic breast cancer: a phase II study. Cancer Chemother Pharmacol 2003;52:147–152.
135. Lobo F, Virizuela JA, Dorta FJ, et al. Gemcitabine/vinorelbine in metastatic breast cancer patients previously treated with anthracyclines: results of a phase II trial. Clin Breast Cancer 2003;4:46–50.
136. Nicolaides C, Dimopoulos MA, Samantas E, et al. Gemcitabine and vinorelbine as second-line treatment in patients with metastatic breast cancer progressing after first-line taxane-based chemotherapy: a phase II study conducted by the Hellenic Cooperative Oncology Group. Ann Oncol 2000;11:873–875.
137. Sanal SM, Gokmen E, Karabulut B, Sezgin C. Gemcitabine and vinorelbine combination in patients with metastatic breast cancer. Breast J 2002;8:171–176.
138. Stathopoulos GP, Rigatos SK, Pergantas N, et al. Phase II trial of biweekly administration of vinorelbine and gemcitabine in pretreated advanced breast cancer. J Clin Oncol 2002;20:37–41.
139. Zielinski CC. Gemcitabine/anthracycline combinations in metastatic breast cancer. Clin Breast Cancer 2002;3(suppl 1):30–33.
140. Rivera E, Valero V, Arun B, et al. Phase II study of pegylated liposomal doxorubicin in combination with gemcitabine in patients with metastatic breast cancer. J Clin Oncol 2003;21:3249–3254.
141. Colomer R, Llombart-Cussac A, Lluch A, et al. Biweekly paclitaxel plus gemcitabine in advanced breast cancer: phase II trial and predictive value of HER2 extracellular domain. Ann Oncol 2004;15:201–206.
142. Colomer R, Llombart A, Lluch A, et al. Paclitaxel/gemcitabine administered every two weeks in advanced breast cancer: preliminary results of a phase II trial. Semin Oncol 2000;27:20–24.
143. Delfino C, Caccia G, Riva GL, et al. Gemcitabine/paclitaxel as first-line treatment of advanced breast cancer. Oncology (Huntingt) 2003;17:22–25.
144. Murad AM, Guimaraes RC, Aragao BC, Scalabrini-Neto AO, Rodrigues VH, Garcia R. Phase II trial of the use of paclitaxel and gemcitabine as a salvage treatment in metastatic breast cancer. Am J Clin Oncol 2001;24:264–268.
145. Alexopoulos A, Tryfonopoulos D, Karamouzis MV, et al. Evidence for in vivo synergism between docetaxel and gemcitabine in patients with metastatic breast cancer. Ann Oncol 2004;15:95–99.
146. Brandi M, Vici P, Lopez M, et al. Novel association with gemcitabine and docetaxel as salvage chemotherapy in metastatic breast cancer previously treated with anthracyclines: results of a multicenter phase II study. Semin Oncol 2004; 31:13–19.

147. Fountzilas G, Nicolaides C, Bafaloukos D, et al. Docetaxel and gemcitabine in anthracycline-resistant advanced breast cancer: a Hellenic Cooperative Oncology Group Phase II study. Cancer Invest 2000;18:503–509.
148. Laufman LR, Spiridonidis CH, Pritchard J, et al. Monthly docetaxel and weekly gemcitabine in metastatic breast cancer: a phase II trial. Ann Oncol 2001;12: 1259–1264.
149. O'Shaughnessy J, Nag S, Caldirello Riuz J. Gemcitabine plus paclitaxel (GT) vs paclitaxel (T) as first line treatment for anthracycline pretreated metastatic breast cancer (MBC): Interim results of a global phase II study. Proc Am Soc Clin Oncol 2003;22:7(abstr. 25).
150. Musib L, Cleverly AL, Roychowdhury DF. Gemcitabine (G) and paclitaxel (T) pharmacokinetics (PK): lack of an interaction in a D1 (T-G), d 8 G, every 21 d schedule. Proc Am Soc Clin Oncol 2003;22:0161(abstr. 644).
151. Zielinski CC. Gemcitabine, anthracycline, and taxane combinations for advanced breast cancer. Oncology (Huntingt) 2003;17:36–40.
152. Nasr FL, Chahine GY, Kattan JG, et al. Gemcitabine plus carboplatin combination therapy as second-line treatment in patients with relapsed breast cancer. Clin Breast Cancer 2004;5:117–122.
153. O' Shaughnessy JA, Vukelja S, Marsland T, Kimmel G, Ratnam S, Pippen JE. Phase II study of trastuzumab plus gemcitabine in chemotherapy-pretreated patients with metastatic breast cancer. Clin Breast Cancer 2004;5:142–147.
154. Sledge GW Jr. Gemcitabine, paclitaxel, and trastuzumab in metastatic breast cancer. Oncology (Huntingt) 2003;17:33–35.
155. Friedlander M, Millward MJ, Bell D, et al. A phase II study of gemcitabine in platinum pre-treated patients with advanced epithelial ovarian cancer. Ann Oncol 1998;9:1343–1345.
156. Lund B, Hansen OP, Theilade K, Hansen M, Neijt JP. Phase II study of gemcitabine (2´,2´-difluorodeoxycytidine) in previously treated ovarian cancer patients. J Natl Cancer Inst 1994;86:1530–1533.
157. Shapiro JD, Millward MJ, Rischin D, et al. Activity of gemcitabine in patients with advanced ovarian cancer: responses seen following platinum and paclitaxel. Gynecol Oncol 1996;63:89–93.
158. Silver DF, Piver MS. Gemcitabine salvage chemotherapy for patients with gynecologic malignancies of the ovary, fallopian tube, and peritoneum. Am J Clin Oncol 1999;22:450–452.
159. von Minckwitz G, Bauknecht T, Visseren-Grul CM, Neijt JP. Phase II study of gemcitabine in ovarian cancer. Ann Oncol 1999;10:853–855.
160. D'Agostino G, Amant F, Berteloot P, Scambia G, Vergote I. Phase II study of gemcitabine in recurrent platinum-and paclitaxel-resistant ovarian cancer. Gynecol Oncol 2003;88:266–269.
161. Markman M, Webster K, Zanotti K, Kulp B, Peterson G, Belinson J. Phase 2 trial of single-agent gemcitabine in platinum-paclitaxel refractory ovarian cancer. Gynecol Oncol 2003;90:593–596.
162. Bergman AM, Pinedo HM, Talianidis I, et al. Increased sensitivity to gemcitabine of P-glycoprotein and multidrug resistance-associated protein-overexpressing human cancer cell lines. Br J Cancer 2003;88:1963–1970.
163. Garcia AA, O'Meara A, Bahador A, et al. Phase II study of gemcitabine and weekly paclitaxel in recurrent platinum-resistant ovarian cancer. Gynecol Oncol 2004;93:493–498.

164. Sehouli J, Stengel D, Oskay G, Blohmer J, Kaubitzsch S, Lichtenegger W. Dose finding study for combination treatment with topotecan and gemcitabine of patients with recurrent ovarian cancer after failure of first-line chemotherapy with paclitaxel and platinum. Onkologie 2004;27:58–64.
165. Goff BA, Thompson T, Greer BE, Jacobs A, Storer B. Treatment of recurrent platinum resistant ovarian or peritoneal cancer with gemcitabine and doxorubicin: a phase I/II trial of the Puget Sound Oncology Consortium (PSOC 1602). Am J Obstet Gynecol 2003;188:1556–1562.
166. Rose PG, Mossbruger K, Fusco N, Smrekar M, Eaton S, Rodriguez M. Gemcitabine reverses cisplatin resistance: demonstration of activity in platinum- and multidrug-resistant ovarian and peritoneal carcinoma. Gynecol Oncol 2003;88:17–21.
167. Nogue M, Cirera L, Arcusa A, et al. Phase II study of gemcitabine and cisplatin in chemonaive patients with advanced epithelial ovarian cancer. Anticancer Drugs 2002;13:839–845.
168. Bauknecht T, Hefti A, Morack G, et al. Gemcitabine combined with cisplatin as first-line treatment in patients 60 years or older with epithelial ovarian cancer: a phase II study. Int J Gynecol Cancer 2003;13:130–137.
169. Belpomme D, Krakowski I, Beauduin M, et al. Gemcitabine combined with cisplatin as first-line treatment in patients with epithelial ovarian cancer: a phase II study. Gynecol Oncol 2003;91:32–38.
170. Du Bois BA, Luck HJ, Pfisterer J, et al. Second-line carboplatin and gemcitabine in platinum sensitive ovarian cancer—a dose-finding study by the Arbeitsgemeinschaft Gynakologische Onkologie (AGO) Ovarian Cancer Study Group. Ann Oncol 2001;12:1115–1120.
171. Papadimitriou CA, Fountzilas G, Aravantinos G, et al. Second-line chemotherapy with gemcitabine and carboplatin in paclitaxel-pretreated, platinum-sensitive ovarian cancer patients. A Hellenic Cooperative Oncology Group Study. Gynecol Oncol 2004;92:152–159.
172. Look KY, Bookman MA, Schol J, Herzog TJ, Rocereto T, Vinters J. Phase I feasibility trial of carboplatin, paclitaxel, and gemcitabine in patients with previously untreated epithelial ovarian or primary peritoneal cancer: a Gynecologic Oncology Group study. Gynecol Oncol 2004;92:93–100.
173. Micha JP, Goldstein BH, Rettenmaier MA, et al. Pilot study of outpatient paclitaxel, carboplatin and gemcitabine for advanced stage epithelial ovarian, peritoneal, and fallopian tube cancer. Gynecol Oncol 2004;94:719–724.
174. Liu FS, Ho ES, Hung MJ, Hwang SF, Lu CH, Ke YM. Triplet combination of gemcitabine, carboplatin, and paclitaxel in previously treated, relapsed ovarian and peritoneal carcinoma: an experience in Taiwan. Gynecol Oncol 2004; 94:393–397.
175. Copeland LJ, Bookman M, Trimble E. Clinical trials of newer regimens for treating ovarian cancer: the rationale for Gynecologic Oncology Group Protocol GOG 182-ICON5. Gynecol Oncol 2003;90:S1–S7.
176. Gebbia V, Testa A, Borsellino N, et al. Single agent 2′,2′-difluorodeoxycytidine in the treatment of metastatic urothelial carcinoma: a phase II study. Clin Ter 1999;150:11–15.
177. Lorusso V, Pollera CF, Antimi M, et al. A phase II study of gemcitabine in patients with transitional cell carcinoma of the urinary tract previously treated with platinum. Italian Co-operative Group on Bladder Cancer. Eur J Cancer 1998;34:1208–1212.

178. Moore MJ, Tannock IF, Ernst DS, Huan S, Murray N. Gemcitabine: a promising new agent in the treatment of advanced urothelial cancer. J Clin Oncol 1997;15: 3441–3445.
179. Stadler VM, Murphy B, Kaufman D. Phase II trial of gemcitabine plus cisplatin in metastatic urothelial cancer. Proc Am Soc Clin Oncol 16, 323. 1997.
180. Schmid P, Akrivakis K, Flath B, et al. Phase II trial of gemcitabine as prolonged infusion in metastatic breast cancer. Anticancer Drugs 1999;10:625–631.
181. Christman K, Kelsen D, Saltz L, Tarassoff PG. Phase II trial of gemcitabine in patients with advanced gastric cancer. Cancer 1994;73:5–7.
182. Sessa C, Aamdal S, Wolff I, et al. Gemcitabine in patients with advanced malignant melanoma or gastric cancer: phase II studies of the EORTC Early Clinical Trials Group. Ann Oncol 1994;5:471–472.
183. Einhorn LH, Stender MJ, Williams SD. Phase II trial of gemcitabine in refractory germ cell tumors. J Clin Oncol 1999;17:509–511.
184. Gridelli C, Cigolari S, Gallo C, et al. Activity and toxicity of gemcitabine and gemcitabine + vinorelbine in advanced non-small-cell lung cancer elderly patients: phase II data from the Multicenter Italian Lung Cancer in the Elderly Study (MILES) randomized trial. Lung Cancer 2001;31:277–284.
185. Perng RP, Chen YM, Ming-Liu J, et al. Gemcitabine vs the combination of cisplatin and etoposide in patients with inoperable non-small-cell lung cancer in a phase II randomized study. J Clin Oncol 1997;15:2097–2102.
186. Cormier Y, Eisenhauer E, Muldal A, et al. Gemcitabine is an active new agent in previously untreated extensive small cell lung cancer (SCLC). A study of the National Cancer Institute of Canada Clinical Trials Group. Ann Oncol 1994;5: 283–285.
187. De Mulder PH, Weissbach L, Jakse G, Osieka R, Blatter J. Gemcitabine: a phase II study in patients with advanced renal cancer. Cancer Chemother Pharmacol 1996;37:491–495.
188. Mertens WC, Eisenhauer EA, Moore M, et al. Gemcitabine in advanced renal cell carcinoma. A phase II study of the National Cancer Institute of Canada Clinical Trials Group. Ann Oncol 1993;4:331–332.
189. Catimel G, Vermorken JB, Clavel M, et al. A phase II study of gemcitabine (LY 188011) in patients with advanced squamous cell carcinoma of the head and neck. EORTC Early Clinical Trials Group. Ann Oncol 1994;5:543–547.
190. Carmichael J, Fink U, Russell RC, et al. Phase II study of gemcitabine in patients with advanced pancreatic cancer. Br J Cancer 1996;73:101–105.
191. Casper ES, Green MR, Kelsen DP, et al. Phase II trial of gemcitabine (2´, 2´-difluorodeoxycytidine) in patients with adenocarcinoma of the pancreas. Invest New Drugs 1994;12:29–34.
192. Ulrich-Pur H, Kornek GV, Raderer M, et al. A phase II trial of biweekly high dose gemcitabine for patients with metastatic pancreatic adenocarcinoma. Cancer 2000;88:2505–2511.
193. Cardenal F, Lopez-Cabrerizo MP, Anton A, et al. Randomized phase III study of gemcitabine-cisplatin vs etoposide-cisplatin in the treatment of locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 1999;17:12–18.
194. Crino L, Scagliotti GV, Ricci S, et al. Gemcitabine and cisplatin vs mitomycin, ifosfamide, and cisplatin in advanced non-small-cell lung cancer: a randomized phase III study of the Italian Lung Cancer Project. J Clin Oncol 1999;17: 3522–3530.

195. Sandler AB, Nemunaitis J, Denham C, et al. Phase III trial of gemcitabine plus cisplatin vs cisplatin alone in patients with locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 2000;18:122–130.
196. Frasci G, Lorusso V, Panza N, et al. Gemcitabine plus vinorelbine vs vinorelbine alone in elderly patients with advanced non-small-cell lung cancer. J Clin Oncol 2000;18:2529–2536.
197. Scagliotti GV, De Marinis F, Rinaldi M, et al. Phase III randomized trial comparing three platinum-based doublets in advanced non-small-cell lung cancer. J Clin Oncol 2002;20:4285–4291.
198. Grigorescu AC, Draghici IN, Nitipir C, Gutulescu N, Corlan E. Gemcitabine (GEM) and carboplatin (CBDCA) vs cisplatin (CDDP) and vinblastine (VLB) in advanced non-small-cell lung cancer (NSCLC) stages III and IV: a phase III randomised trial. Lung Cancer 2002;37:9–14.
199. Comella P, Frasci G, Panza N, et al. Randomized trial comparing cisplatin, gemcitabine, and vinorelbine with either cisplatin and gemcitabine or cisplatin and vinorelbine in advanced non-small-cell lung cancer: interim analysis of a phase III trial of the Southern Italy Cooperative Oncology Group. J Clin Oncol 2000;18: 1451–1457.
200. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 2002;346:92–98.
201. Sculier JP, Lafitte JJ, Lecomte J, et al. A three-arm phase III randomised trial comparing combinations of platinum derivatives, ifosfamide and/or gemcitabine in stage IV non-small-cell lung cancer. Ann Oncol 2002;13:874–882.
202. Brewer CA, Blessing JA, Nagourney RA, et al. Cisplatin plus gemcitabine in platinum-refractory ovarian or primary peritoneal cancer: a phase II study of the Gynecologic Oncology Group. Gyn Oncol 2006; in press.
203. Brewer CA, Blessing JA, Nagourney RA, et al. Cisplatin plus gemcitabine in previously treated squamous cell carcinoma of the cervix: a phase II study of the Gynecologic Oncology Group. Gyn Oncol 2006;100:385–388.

13 Nucleoside Radiosensitizers

Donna S. Shewach, PhD and Theodore S. Lawrence, MD, PhD

CONTENTS

SUMMARY

Nucleoside/nucleobase analogs (bromodeoxyuridine, iododeoxyuridine, 5-fluorouracil, fluorodeoxyuridine, difluorodeoxycytidine, fluoroadenine arabinoside, fluoromethylenedeoxycytidine) can synergistically enhance ionizing radiation-induced cell killing. These analogs are able to radiosensitize a wide variety of tumor cell types in vitro and several have proven clinical efficacy as well. They share a requirement for intracellular metabolism to phosphorylated forms. As triphosphate analogs they can serve as substrates for nucleic acid synthesis and subsequent incorporation into DNA has been correlated with radiosensitization for bromo- and iododeoxyuridine. Each of these analogs also inhibits an enzyme involved in deoxynucleotide metabolism resulting in depletion of at least one deoxynucleoside triphosphate pool. This effect appears to be responsible for radiosensitization with fluorodeoxyuridine difluorodeoxycytidine and fluoromethylenedeoxycytidine in a manner similar to hydroxyurea which elicits radiosensitization solely through its depletion of deoxynucleotides as a result of ribonucleotide

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

Acronyms and Abbreviations

BER	base excision repair
BrdUMP	5-bromo-2′-deoxyuridine 5′-monophosphate
BrdUrd	5-bromo-2′-deoxyuridine
BrdUTP	5-bromo-2′-deoxyuridine 5′-triphosphate
dATP	2′-deoxyadenosine 5′-triphosphate
dCTP	2′-deoxycytidine 5′-triphosphate
dCyd	2′-deoxycytidine
dFdCyd	2′,2′-difluoro-2′-deoxycytidine
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5′-triphosphate
dsb	double-strand break
dThd	thymidine
dTMP	thymidine 5′-mono phosphate
dTTP	thymidine 5′-triphosphate
dUMP	2′-deoxyuridine 5′-monophosphate
dUTP	2′-deoxyuridine 5′-triphosphate
dUTPase	2′-deoxyuridine 5′-triphosphatase
F-ara-A	2-fluoroadenine arabinoside
F-ara-ATP	2-fluoroadenine arabinoside 5-triphosphate
FdUDP	5-fluoro-2′-deoxyuridine 5′-diphosphate
FdUMP	5-fluoro-2′-deoxyuridine 5′-monophosphate
FdUrd	5-fluoro-2′-deoxyuridine
FMdCyd	2′-fluoromethylene-2′-deoxycytidine
5-FU	5-fluorouracil
FUDP	5-fluorouridine 5′-diphosphate
FUMP	5-fluorouridine 5′-monophosphate
FUTP	5-fluorouridine 5′-triphosphate
HU	hydroxyurea
IdUMP	5-iodo-2′-deoxyuridine 5′-monophosphate
IdUrd	5-iodo-2′-deoxyuridine
IdUTP	5-iodo-2′-deoxyuridine 5′-triphosphate
MMR	mismatch repair
MTD	maximum tolerated dose
RNA	ribonucleic acid
TK1	thymidine kinase 1
TK2	thymidine kinase 2
TS	thymidylate synthase

reductase inhibition. In addition these analogs promote accumulation of cells in S-phase which appears to be necessary for radiosensitization. Combined with data demonstrating that mismatch repair defective cells are better radiosensitized by these compounds the evidence suggests that errors in DNA replication contribute to radiosensitization. It is essential to define more completely the mechanism(s) responsible for radiosensitization with these important drugs in order to optimize antitumor efficacy and limit normal tissue toxicity.

Key Words: DNA repair; fludarabine; fluorodeoxyuridine; 5-fluorouracil; gemcitabine; radiation; radiation enhancement ratio; radiosensitization.

1. INTRODUCTION

The ability to increase the efficacy of radiotherapy by combining it with chemotherapy is an attractive approach to enhance tumor treatment for patients with solid malignancies. The advantage of using radiotherapy and chemotherapy concurrently rather than sequentially is that the two modalities have the potential to interact with each other to produce more than additive, or synergistic, cell killing. When this occurs, the chemotherapeutic agent is thought to sensitize the tumor cells to radiation. Depending on the specific drug and tumor type, this can be accomplished at a concentration of drug that is not cytotoxic when used alone; in other cases, only a cytotoxic drug concentration will enhance cell killing with radiation. For purposes of this chapter, we define radiosensitization as any combination of drug and radiation that produces synergistic cytotoxicity in vitro or synergistic antitumor activity in vivo.

Measuring the degree of radiosensitization allows comparison of the efficacy of different drugs to enhance cell killing with radiation. The two most popular methods, the sensitizer enhancement ratio (SER) and the radiation enhancement ratio (RER), are illustrated in Fig. 1. Both methods rely on cell survival data that compare survival after radiation alone with that from the combination of radiation and the drug after correction for the cytotoxicity of the drug alone. These data are usually derived using colony formation assays. The SER is then calculated from the graphical representation of these data, and is simply the amount of grays necessary to effect a specific level of cell killing for control cells divided by the amount of grays necessary to effect the same level of cell killing for drug-treated cells. The RER uses the linear quadratic equation to determine the areas under the control and drug-treated survival curves, and the ratio of these areas represents the RER *(1)*. With either method, an enhancement ratio significantly greater than 1 indicates that the drug produced radiosensitization. Although these methods are reasonably accurate when the drug alone produces minimal cytotoxicity, when there is significant cytotoxicity from drug alone (surviving fraction less than about 0.5), the nonlinear nature of the radiation cell survival curve can produce apparent synergy when drug and radiation are only additive or even antagonistic *(2–4)*.

Following the initial reports in the early 1960s of increased cell death when halogenated thymidine analogs were added to cells prior to treatment with ionizing radiation, numerous compounds have been tested as radiation sensitizers. Among the most effective have been the nucleoside/ nucleobase analogs, including 5-bromo-2′-deoxyuridine (BrdUrd) and 5-iodo-2′-

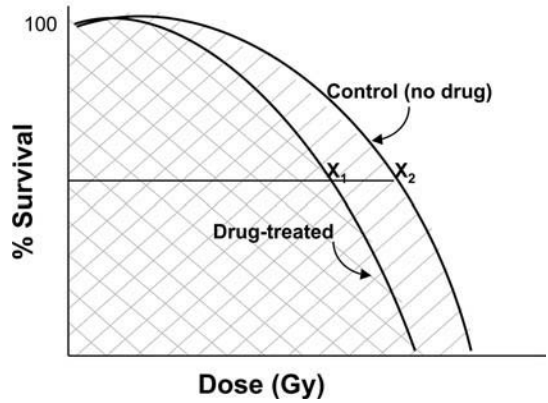


Fig. 1. Representation of SER and RER. SER is calculated as X_2/X_1 for a specific level of cell survival. RER is calculated from the ratio of the area under the control and drug-treated survival curves, shown graphically as the area represented by the single and cross-hatched lines (control) divided by the area represented by the cross-hatched lines (drug treated).

deoxyuridine (IdUrd); 5-fluoro-2′-deoxyuridine (FdUrd) and its base, 5-fluorouracil (5-FU); 2′,2′-difluoro-2′-deoxycytidine (dFdCyd); 2-fluoroadenine arabinoside (fludarabine; F-ara-A); and 2′-fluoromethylene-2′-deoxycytidine (FMdCyd). Hydroxyurea was one of the first radiosensitizers discovered and is included in this chapter because, although it is not a nucleoside, it alters the levels of endogenous deoxynucleotides. All of these drugs can be cytotoxic when administered to cells, all are antimetabolites that target deoxyribonucleic acid (DNA) replication, and consequently, all can alter the distribution of cells in the cell cycle. This redistribution must be accounted for when combining each drug with radiation as radiosensitivity is highest during mitosis, late G1 and early S phase, whereas mid-S-phase cells are more radioresistant *(5,6)*. This chapter examines these radiosensitizers, discussing both in vitro and in vivo activity, with emphasis on mechanisms that produce radiosensitization.

2. BrdUrd AND IdUrd

Because bromine and iodine have a van der Waals radius similar to that of a methyl group, replacement of the methyl group on the 5-position of thymidine with bromine or iodine results in compounds that share many similarities in biologic activity with thymidine. BrdUrd and IdUrd have long been of interest for their antitumor as well as antiviral properties *(7)*. Clinical use of these analogs has been limited by a lack of selectivity, in that they appear to be incorporated equally into proliferating normal and

tumor cells *(8)*. These limitations have been addressed primarily by administration strategies that rely on hypothesized differences between the proliferation rate of normal and tumor tissue and by regional delivery of these drugs. The halogenated thymidine analogs continue to be of interest for their radiosensitizing properties both in vitro and in vivo.

2.1. Metabolic Considerations

Both BrdUrd and IdUrd require phosphorylation for cytotoxic and radiation-sensitizing effects *(7)*. The initial and rate-limiting step in phosphorylation is thymidine kinase. Two forms of thymidine kinase have been reported: TK1 (cytosolic thymidine kinase 1) is a cytosolic enzyme with activity that increases as cells enter S phase, whereas TK2 (mitochondrial thymidine kinase 2) is a mitochondrial enzyme with broader substrate specificity *(9–11)*.[1] BrdUrd and IdUrd are excellent substrates for mammalian thymidine kinase (TK1) with K_m values similar to those reported for dThd *(9)*. Once the monophosphates are formed, they are readily converted to their corresponding triphosphates, which are the major cellular metabolites. Both BrdUTP and IdUTP are excellent substrates for DNA replication and are readily incorporated into DNA instead of their endogenous competitor, thymidine 5′ triphosphate (dTTP) *(8,12)*. The resulting incorporation of 5-bromodeoxyuridine-5′-monophosphate (BrdUMP) and 5-iododeoxyuridine-5′-monophosphate (IdUMP) into internal linkages in DNA leads to cytotoxicity. IdUrd is a more potent cytotoxic agent compared to BrdUrd *(13,14)*.

2.2. Mechanisms of Radiosensitization

Although it has been suggested since the 1960s that DNA incorporation is also the critical event for radiosensitization by BrdUrd and IdUrd *(13)*, the mechanism underlying the enhanced response to radiation still is not completely understood. Several investigators have demonstrated that BrdUMP and IdUMP incorporation in DNA correlates with radiosensitization in vitro *(15–20)* as well as in an animal model *(14)*. Radiosensitization occurred after incorporation of the analog into either one or both strands of DNA, with increasing exposure to the thymidine analog correlating with greater radiosensitization *(21,22)*. Radiosensitization was linearly related to the extent of substitution of the halogenated analog for thymidine *(15,18,23)*.

Several mechanisms have been suggested to account for the radiosensitizing property of BrdUrd/IdUrd resulting from their incorporation into DNA. Investigators have demonstrated that incorporation of the thymidine analogs into DNA results in DNA double-strand breaks (dsbs), which has correlated with radiosensitization *(19,20,24)*. Although the mechanism of dsb formation is not completely clear, it has been suggested that the presence of the halogenated uracil analogs in DNA during irradiation produces

uracilyl radicals capable of extracting a hydrogen atom from a deoxyribose, resulting in a DNA strand break *(25)*. This would increase the number of dsbs per gray of radiation delivered *(26)*. In addition, several studies have demonstrated that the presence of BrdUrd or IdUrd inhibits the repair of the dsbs formed after ionizing radiation *(20)*. This may be caused by direct inhibition of repair processes by 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP) or 5-iodo-2′-deoxyuridine 5′-triphosphate (IdUTP). However, the fact that BrdUTP and IdUTP are rapidly accumulated in cells yet radiosensitization increases with increasing length of incubation prior to irradiation suggests that factors other than simply the levels of analog triphosphates are necessary for radiosensitization. Increasing the duration of incubation will increase the amount of analog or other lesions in DNA *(15,17,18,23)*, and such lesions may inhibit certain types of repair.

To explain the synergy in cell killing with the combination of BrdUrd or IdUrd and ionizing radiation, it is clear that the inhibition of repair by the triphosphates could result in synergistic cytotoxicity. Radiation-induced DNA dsbs that would normally be repaired will persist then in the presence of analog triphosphate. Accounting for synergy based on increased numbers of DNA dsbs must be considered carefully because an additive increase in dsbs from the combined drug and radiation treatments would only be expected to produce additive cytotoxicity. However, if the combination of BrdUrd or IdUrd with irradiation produces more than an additive increase in dsbs, then synergistic cytotoxicity would be predicted. This explanation is consistent with the theory of strand breakage caused by the reactivity of the uracilyl radical generated by irradiation in the vicinity of the halogenated analog in DNA. Alternatively, a combination of inhibition of dsb repair with an increased number of DNA dsbs would also explain the synergistic cytotoxicity.

In addition to evaluating the number of DNA dsbs and their efficiency of repair, studies have examined the role of specific DNA repair processes on radiosensitization by nucleoside analogs. As mismatch repair (MMR) is a primary pathway for excision and repair of misincorporated nucleotides in DNA *(27)*, determining whether this process plays a role in the incorporation or retention of halogenated thymidine analogs into DNA is important. Mismatch repair is carried out by a complex of proteins responsible for the identification, excision, and repair of the incorrect nucleotide pair *(27)*. In mammalian cells, mismatch repair is characterized by proteins related to the MutHLS system of repair in bacteria. Recognition of mismatches is carried out by the heterodimeric complexes MutSα (MSH2 and MSH6) and MutSβ (MSH2 and MSH3). The identification of single base:base mismatches is primarily a function of MutSα, whereas MutSβ participates mainly in recognition of insertion-deletion loops. Once the mismatch is identified, MutS complexes are assisted by the MutL heterodimer of MLH1 and PMS1

to excise and repair the mismatch. Deficiency of mismatch repair is associated with hereditary nonpolyposis colorectal cancer and is most frequently associated with inactivation of MLH1 or MSH2 *(28)*.

To evaluate the role of mismatch repair in radiosensitization with BrdUrd and IdUrd, studies have been carried out in cell lines that lack expression of a mismatch repair protein. Deficiency of MLH1 because of its inactivation in HCT116 colorectal cancer cells was associated with increased incorporation of IdUrd and BrdUrd into cellular DNA compared to the MMR-proficient subline *(29)*. As expected, this increased incorporation was associated with higher cytotoxicity as well as greater radiosensitization with the thymidine analogs. Similarly, studies in embryonic fibroblasts with a genetic deletion of MSH2 demonstrated that they retained higher levels of IdUrd in their DNA, which was associated with greater cytotoxicity and radiosensitization *(30)*. The authors suggested that the lower incorporation in MMR-proficient cells was caused by excision of the halogenated thymidine analog from DNA and supported this with an article showing that IdUMP opposite a mismatched deoxyguanosine 5′-monophosphate residue in DNA was able to bind MutSα *(31)*

Studies evaluated another repair pathway, base excision repair (BER), for its role in repairing DNA damage induced by IdUrd *(32)*. Chinese hamster ovary cells deficient in this pathway because of lack of XRCC1 expression exhibited greater cytotoxicity and radiosensitization with IdUrd, although the amount of IdUrd in DNA was not altered. BER is involved primarily in repair of DNA single-strand breaks rather than dsbs, suggesting a role for this pathway in the cytotoxic and radiosensitizing properties of IdUrd. As DNA repair pathways become better defined at the molecular level, study of the contribution of each pathway to radiosensitization with nucleoside analogs will be central to our understanding of the mechanism by which these drugs enhance radiation-induced cell killing.

One aspect that has received little attention with respect to radiosensitization has been the ability of BrdUrd and IdUrd metabolites to alter endogenous deoxynucleotide pools. Cells treated with BrdUrd or IdUrd have shown decreases in dTTP and deoxycytidine triphosphate (dCTP) pools, which may be caused by feedback inhibition of thymidine kinase or ribonucleotide reductase by the corresponding triphosphates *(9,33–35)*. Although these effects on dTTP and dCTP have been discussed with respect to their role in mutagenesis with BrdUrd and IdUrd *(33,34)*, their potential role in radiosensitization has not been evaluated. This is a difficult issue to resolve because both the feedback inhibition and incorporation into DNA depend on the level of the 5′-triphosphate analog. Certainly, other drugs that decrease dTTP as their major effect, such as 5-FU and FdUrd, also act as radiation sensitizers (discussed in Section 3), so there is support for the notion that effects of BrdUrd and IdUrd on pyrimidine

deoxyribonucleoside triphosphate (dNTP) pools contribute to their radiosensitizing ability. At least, the ability of BrdUrd and IdUrd to decrease dTTP pools would be considered advantageous to radiosensitization because it would promote incorporation of the halogenated nucleotides into DNA. Potential mechanisms by which imbalances in nucleotide pools would result in radiosensitization are discussed in Section 8.

2.3. Clinical Aspects

Clinical trials with BrdUrd or IdUrd and radiotherapy have been evaluated in patients with head-and-neck cancer *(36)*, malignant gliomas *(37–39)*, soft tissue sarcomas *(40,41)*, intrahepatic cancer *(42)*, and cervical cancer *(43)*. Findings of improved progression-free survival *(44)* in phase I/II studies spurred a larger trial of BrdUrd and radiotherapy in patients with high-grade gliomas *(45)*. Incorporation of the halogenated thymidine analog into tumor cell DNA increased with the duration of infusion and achieved values that have been shown to radiosensitize tumor cells in vitro and in vivo *(36,38,43)*. Unfortunately, randomized trials assessing the potential of intravenous BrdUrd to improve the outcome of treatment of primary gliomas and of brain metastases have been negative. In most clinical studies, the dose-limiting systemic toxicity has been hematological, which is not surprising given the rapid proliferation of normal bone marrow elements.

The key to success of any radiosensitizer is selectivity of radiosensitization in tumor compared to normal tissue. The two main approaches that have been used to obtain selectivity are the use of regional infusion *(37,42,46)* and differential tumor/normal tissue kinetics. In the latter case, investigators have used concurrent analog infusion and radiation in settings in which the tumor was anticipated to proliferate more rapidly than the surrounding normal tissue, such as brain *(47)*. Patient studies combining regional therapy with a kinetic approach have demonstrated increased incorporation in tumor compared to normal liver *(48)*. Animal models have demonstrated that, although initially there was relatively high incorporation of these analogs into normal intestine, the analogs were eliminated faster from normal tissue *(14,49)*.

Based on these findings, a clinical trial in cervical carcinoma was developed in which radiotherapy began 3 d after infusion of BrdUrd *(43)*. This study also utilized an alternating schedule of drug and radiation treatment because the radiation dose-limiting tissue (small intestine) proliferated more rapidly than the tumor *(43)*. Biopsies taken during that study demonstrated that incorporation of BrdUrd was higher in DNA from tumor cells compared to rectal mucosa. In addition, BrdUrd-containing rectal mucosal cells had migrated from the crypts to the surface at the time of radiotherapy, suggesting that proper sequencing of therapy can spare normal tissue toxicity. With in vitro studies suggesting that some selectivity is observed

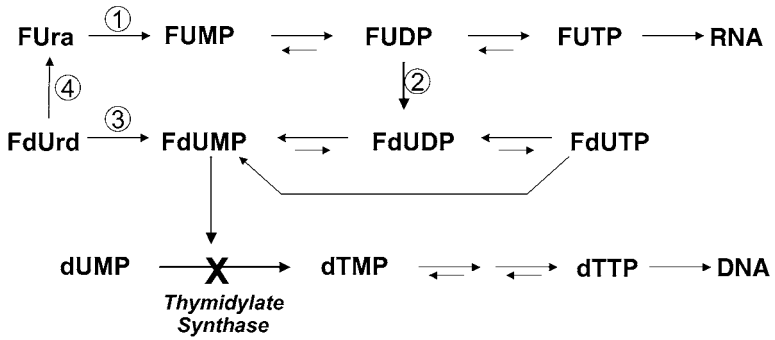


Fig. 2. Metabolism of 5-fluorouracil (5-FU). 1, orotic acid phosphoribosyltransferase; 2, ribonucleotide reductase; 3, thymidine kinase; 4, thymidine phosphorylase. See text for details.

in tumor cells with deficiencies of DNA repair pathways *(29,30,32)*, better selectivity may also result by utilizing these radiosensitizers in patients whose tumors have such a repair defect.

3. 5-FU AND FdUrd

5-FU continues to be the mainstay of treatment for colorectal and other gastrointestinal (GI) tumors. It has been widely used in different infusion schedules *(50)* and is commonly administered with reduced folate *(51)* and other chemotherapeutic agents *(52)* to improve efficacy. The related nucleoside FdUrd has been used primarily in hepatic regional chemotherapy in which there is greater than 90% first-pass extraction *(53)*. Both 5-FU and FdUrd have been used clinically as radiosensitizers.

Although 5-FU and FdUrd are halogenated uracil analogs that share many of the same metabolic pathways as BrdUrd and IdUrd, their mechanisms of action for both cytotoxicity and radiosensitization differ considerably. This is likely because of the difference in size of the halogen at the 5-position of the uracil base *(54)*. Because the van der Waals radius of bromine and iodine approximates that of a methyl group, BrdUrd and IdUrd have actions within the cell that are similar to thymidine *(7)*. In contrast, fluorine is comparable in size to a hydrogen atom, and thus this substitution at the 5-position of the base makes such derivatives more similar to uracil/uridine. Because the primary target for both 5-FU and FdUrd is thymidylate synthase (TS), they are considered together in this chapter.

3.1. Metabolic Considerations

For cytotoxicity and radiosensitization, 5-FU requires intracellular conversion to phosphorylated analogs (Fig. 2). After transport into the cell

(55), 5-FU is initially converted to a nucleotide via orotic acid phosphoribosyltransferase to form FUMP *(56,57)*. This metabolite is rapidly converted to the corresponding di- and triphosphate, with FUTP as the most abundant phosphorylated derivative. FUTP is incorporated into ribonucleic acid (RNA), which can interfere with RNA processing and function and contributes to the cytotoxicity of 5-FU *(56)*. In addition, FUDP can be converted to its deoxy analog by the enzyme ribonucleotide reductase. The resulting FdUDP is then converted to the triphosphate FdUTP and the action of 2′-deoxyuridine 5′-triphosphate (dUTPase) readily converts this compound to FdUMP, a potent inhibitor of TS, as discussed below. FdUMP is considered the metabolite responsible in large part for the antitumor activity of 5-FU, as inhibition of TS by FdUMP has correlated with clinical response *(58)*. 5-FU can be degraded initially through the action of dihydropyrimidine dehydrogenase, a ubiquitous enzyme that follows a circadian pattern of activity in vivo *(59)*. Inhibitors of this enzyme have been developed to increase the efficacy of 5-FU, although selective effects against tumors compared to normal tissues have not been demonstrated *(60,61)*.

Intracellular metabolism of FdUrd has a more straightforward activation pathway. Following its transport into the cell *(62)*, FdUrd is readily phosphorylated by thymidine kinase to FdUMP *(57)*. Although FdUrd can be metabolized to 5-FU by thymidine phosphorylase, the low concentrations of FdUrd used result in little FUTP *(57)*. This monophosphate can then be converted to its di- and triphosphate derivatives, but the action of dUTPase maintains FdUMP as a major metabolite. Thus, 5-FU and FdUrd both produce FdUMP, whereas significant levels of FUTP are only observed with 5-FU. FdUMP can be converted to FdUTP, although relatively low levels of this nucleotide are present in cells because of the action of dUTPase *(63,64)*. Despite the low levels of FdUTP, there is evidence that some incorporation of this fraudulent nucleotide occurs, which may also contribute to the biologic activity of 5-FU and analogs *(65,66)*. However, the extent of the incorporation is far less than that for BrdUrd or IdUrd, and thus mechanistically these analogs differ dramatically. In contrast to the avid replication of DNA in the presence of BrdUrd and IdUrd, DNA synthesis is inhibited in cells incubated with FdUrd or 5-FU, resulting in accumulation of cells in early S phase *(67)*.

The primary target for FdUMP is the inhibition of TS, the enzyme responsible for conversion of deoxyuridine monophosphate (dUMP) to dTMP. FdUMP in the presence of 5,10-methylenetetrahydrofolate forms a tight-binding ternary complex with TS, causing prolonged enzyme inhibition, resulting in lowered dTMP and elevated dUMP pools *(68)*. Ultimately, this produces lowered dTTP pools, but because the cells are still able to produce thymidylate through the salvage thymidine kinase

pathway, dTTP is generally lowered by only 50–60% *(69)*. The elevation in dUMP results in increased dUTP, which can be incorporated into DNA *(63)*. FdUrd and 5-FU have also been noted to affect purine pools, with a marked increase in deoxyadenosine 5′-triphosphate (dATP) in murine cells *(70–72)* as well as human cells *(73)* (our unpublished data, 1995), which likely contributes to the inhibition of DNA synthesis and may be an important determinant of cell death with these drugs *(74)*.

3.2. Mechanisms of Radiosensitization

The ability of 5-FU or FdUrd to enhance ionizing radiation-induced cell death has been studied in a variety of cell lines. Initial reports indicated that, when added postirradiation, 5-FU decreased cell survival *(75)*. Considering the time required for intracellular conversion of 5-FU to its active metabolites, it seemed that treatment with 5-FU prior to irradiation would be a more effective mode of radiosensitization. Subsequent studies with FdUrd compared pre- and postirradiation drug exposure and demonstrated that increasing the duration of incubation with FdUrd prior to irradiation resulted in greater radiosensitization than postirradiation exposure *(69)*. The addition of 5-FU after irradiation increased primarily the β-component of the cell survival curve that had been fit to the linear quadratic equation *(75)*, whereas preirradiation exposure increased both α- and β-components *(69)*.

Cell cycle position at the time of irradiation appears to play an important role in radiosensitization with FdUrd and 5-FU. Several studies have noted that the accumulation of cells in early S phase, because of the effects on deoxynucleotide pools produced by these analogs, may actually be a key element of radiosensitization *(67,76)*. In a study in which FdUrd-treated cells were separated by physical methods into four fractions ranging from late G1 up to early S phase and then irradiated, investigators observed that early- to mid-S-phase cells were radiosensitized to a significant degree *(67)*. Cells unable to progress into S phase with FdUrd treatment, because of either overexpression of wild-type p53 or lack of cyclin E activation, could not be radiosensitized by FdUrd *(77,78)*. As cells in mid-S phase are typically less sensitive to radiation-induced DNA damage than cells in late G1 or G2/M *(5,79)*, these data demonstrate that radiosensitization with these analogs is not simply caused by redistribution to a more radiosensitive phase of the cell cycle.

The inhibition of TS by FdUMP has long been recognized as an important target for the anticancer properties of 5-FU and FdUrd both in vitro and in vivo, and the accumulated data indicate that it is also the main target for radiosensitization by these drugs *(57,58,68)*. Although a role for FUTP in the cytotoxicity of 5-FU has been established *(80)*, the majority of the radiosensitization with 5-FU can be accounted for by TS inhibition *(81)*. In view of the ability of 5-FU and FdUrd to cause accumulation of cells in

early to mid-S phase as well as inhibit TS, investigators have studied whether the biochemical changes that resulted from TS inhibition or the lack of TS activity in early S phase produced the observed radiosensitization. Although low enzymatic activity in cells deficient in TS did not reproducibly produce radiosensitization when those cells were synchronized in early S phase, an altered dATP:dTTP ratio did correlate with radiosensitization *(82)*, similar to that observed with FdUrd, suggesting that the biochemical changes associated with FdUMP inhibition of TS are essential elements of radiosensitization. It has been hypothesized that radiation-induced DNA damage is not repaired in cells that are progressing into S phase in the presence of FdUMP *(67)*. Dysregulation of the cell cycle because of altered checkpoint controls, a common trait of cancer cells, would promote S-phase progression in the presence of DNA-damaging agents, resulting in radiosensitization *(83)*.

The nature of the DNA damage produced by FdUMP-mediated inhibition of TS has been the subject of considerable debate. Recognition of the erroneous incorporation of FdUMP or dUMP in DNA causes cells to attempt to repair these lesions *(84)*. It has been proposed that this initiates a futile repair cycle because, as cells attempt to repair these lesions, they continue to incorporate uracil-containing nucleotides from the precursor pool *(65)*, eventually producing dsbs that result in DNA fragmentation *(65,85,86)*. Although the ability of 5-FU or FdUrd to produce DNA dsbs suggests a mechanism for radiosensitization, this is not a requirement as noncytotoxic concentrations of FdUrd that did not produce dsbs could induce radiosensitization, apparently through inhibiting the rate of DNA repair after irradiation *(69)*. Furthermore, although cells overpressing dUTPase were relatively resistant to the cytotoxic effects of FdUrd, they showed a similar ability to be radiosensitized *(87)*.

Accounting for 5-FU and FdUrd-mediated radiosensitization based on their effects on TS and initiation/repair of DNA dsbs is well supported by the available data. The relationship of more recently reported effects of 5-FU and FdUrd to radiosensitization remains to be explored. For example, it is known that TS expression can be regulated by the uninhibited protein, and whether interference with this biological feedback loop affects radiosensitization is not known *(88)*. A novel mechanism for cytotoxicity independent of DNA dsbs that involves Fas-mediated apoptosis has been demonstrated for 5-FU, although whether this is an important pathway for radiosensitization is not known *(89)*. Reports demonstrated a multitude of signaling and repair proteins associated at the sites of DNA dsbs that may also mediate radiosensitization *(90)*. As the complex biologic interactions resulting from 5-FU and FdUrd exposure become apparent, subsequent effects on radiosensitization should be evaluated.

3.3. Clinical Aspects

Radiosensitization with 5-FU (often administered with leucovorin) in patients has been studied in many different clinical trials, with the most promising effects in patients with anal, rectal, esophageal, pancreatic, or stomach cancer *(91)*. Randomized trials have shown a benefit in locoregional control or survival when systemically administered 5-FU was added to radiotherapy *(92–96)*. In addition, when radiotherapy was combined with 5-FU and other chemotherapeutic drugs in patients with cervical cancer, there was improved survival, and with head-and-neck cancer, improvements in locoregional control, organ preservation, and survival were reported *(97–99)*. As both 5-FU and radiation therapy have the potential to produce mucositis, this radiochemotherapy combination increases normal tissue toxicity but still produces substantial benefit.

Innovative formulations have been developed that address some of the major limitations regarding the clinical administration of 5-FU. Capecitabine is an oral formulation that can be absorbed from the GI tract and then is metabolized by several enzymes to 5-FU. The final step in this conversion process is mediated by thymidine phosphorylase, an enzyme that is present at higher levels in tumor compared to normal tissue, which may allow preferential uptake of 5-FU in tumors *(100)*. Uracil:tegafur is also an oral formulation with the added benefit of preventing degradation of 5-FU by dihydropyrimidine dehydrogenase *(101)*. Combinations of these oral formulations of 5-FU with radiation therapy have produced promising results in patients with colorectal cancer *(101,102)*.

Regional infusion of 5-FU and FdUrd has been evaluated in patients with hepatic tumors to increase drug levels selectively at the tumor site and improve response *(91)*. FdUrd is amenable to such therapy because it is efficiently extracted through the hepatic artery into hepatic tissue. When combined with conformal radiotherapy, hepatic artery-delivered FdUrd produced long-term freedom from tumor progression and survival benefits in patients with unresectable hepatobiliary cancer *(103)*. However, in a large randomized trial of patients with colorectal cancer who received portal vein infusion of 5-FU with perioperative radiotherapy, there was not a clear benefit for 5-FU, although the results suggested a small benefit for the subset of patients with rectal cancer *(104)*. These results establish an important role for the halogenated pyrimidines in clinically beneficial radiosensitization.

4. GEMCITABINE

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdCyd) is a deoxycytidine (dCyd) analog with broad activity as a chemotherapeutic agent in a variety of solid malignancies *(105)*. It was first approved for use in patients

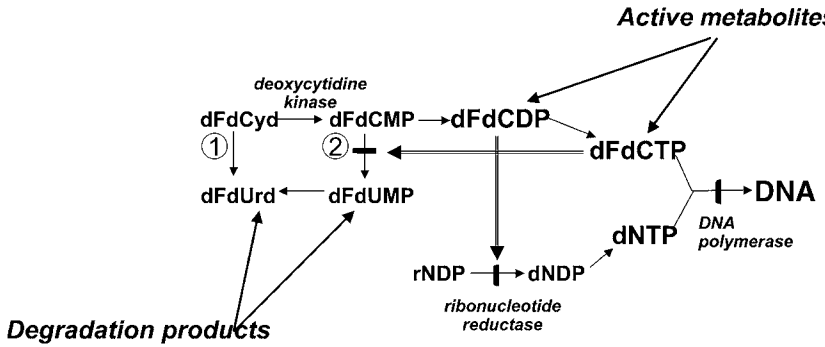


Fig. 3. Metabolism of gemcitabine. 1, deoxycytidine deaminase; 2, deoxycytidylate deaminase. See text for details.

with pancreatic cancer, for whom it was shown to provide a small but significant survival benefit compared to 5-FU and was noted for its ability to improve symptoms and decrease the need for narcotic analgesics *(106,107)*. dFdCyd has also been approved for use in non-small cell lung cancer, for which it is a common component of multiagent chemotherapy *(108,109)*. Demonstration of its excellent radiosensitizing properties in vitro and in animal models has encouraged numerous clinical trials.

An important distinction between dFdCyd and the halogenated thymidine and uracil analogs is that the halogen (fluorine) substitution occurs on the sugar ring instead of the base. As with other analogs discussed in this chapter, the fluorines permit the drug to be metabolized along pathways utilized by the endogenous dCyd, but the metabolites are not as bulky as bromine and iodine in the thymidine analogs. With the deoxyribose sugar, dFdCyd has primarily DNA-directed effects, as do BrdUrd and IdUrd, but lacks the strong RNA effects of 5-FU.

4.1. Metabolic Considerations

As discussed for the other nucleoside/nucleobase analogs, dFdCyd requires intracellular phosphorylation for its antitumor activity (Fig. 3) *(110)*. dFdCyd is a substrate for several nucleoside transporters, with highest affinity for the Na^+-dependent concentrative transporters *(111,112)*. Once inside the cell, dFdCyd is an excellent substrate for dCyd kinase, and subsequent phosphorylation allows accumulation of two active metabolites: the 5′-diphosphate, gemcitabine diphosphate (dFdCDP), and the 5′-triphosphate, gemcitabine triphosphate (dFdCTP) *(113,114)*. Although dFdCDP accounts for less than 10% of the total phosphorylated dFdCyd in the cell *(113)*, it is a potent, mechanism-based inhibitor of ribonucleotide reductase with measurable effects on dNTP pools at clinically achievable

concentrations *(115)*. In leukemia cells, this inhibition results primarily in reduction of pyrimidine deoxynucleotide pools *(116)*, whereas purine deoxyribonucleotide pools are more affected in solid tumor cells *(117,118)*. The decrease in deoxynucleotide pools results primarily in an inhibition of DNA synthesis *(119)*. dFdCTP is also an inhibitor of and substrate for DNA polymerases, and incorporation of this analog into DNA correlates strongly with cytotoxicity *(120–122)*. When incorporated into template DNA, dFdCMP increases misincorporation in the opposing nucleotide *(123)*. Although the majority of the dFdCyd-mediated effects are DNA directed, some incorporation into RNA occurs as well; whether this contributes to cytotoxicity or radiosensitization is not known *(124)*. dFdCyd can be degraded rapidly through deamination at either the nucleoside or the 5′-monophosphate level *(125)*. dFdCTP can inhibit its own degradation through inhibition of dCMP deaminase and thus is able to potentiate its own activity *(126)*.

4.2. Mechanisms of Radiosensitization

Initial reports demonstrated that dFdCyd could enhance radiation-induced cytotoxicity in breast cancer and colon cancer cells, even at noncytotoxic concentrations *(118,127)*. Radiosensitization was highest when exposure to dFdCyd preceded irradiation, requiring at least a 4-h incubation with dFdCyd prior to irradiation *(118)*. Radiosensitization increased with duration of dFdCyd exposure and drug concentration *(117,118)*. dFdCyd is remarkable for its ability to enhance radiation-induced cell killing at concentrations that, by themselves, are not cytotoxic. Because of the excellent intracellular retention of phosphorylated dFdCyd in tumor cells, a brief (2-h) exposure to higher concentrations of dFdCyd could produce significant radiosensitization 24–48 h later *(128)*. Radiosensitization with dFdCyd is characterized by a change in both the α and β-components of the cell survival curve when fitted to a linear quadratic model *(118,127,129)*.

As with the uracil analog radiosensitizers, cell cycle appears to play an important role in radiosensitization with dFdCyd *(91,130)*. For most cell lines, incubation with dFdCyd promotes accumulation of cells in early S phase *(118,128)*. S-phase accumulation is time dependent and is a characteristic of some, but not all, cell lines *(131,132)*. Treatment of synchronized cell populations with dFdCyd demonstrated that radiosensitization was highest for cells in S phase *(133)*. One cell line that was not radiosensitized by dFdCyd did not show S-phase accumulation *(134)*. These results suggested that expression of wild-type p53 may prevent cells from entering S phase with dFdCyd, thus blocking radiosensitization. However, subsequent studies in matched wild-type and null or mutant p53 cell lines have demonstrated that p53 expression does not prevent radiosensitization with dFdCyd *(135,136)*. Although induction of p53 expression can produce a

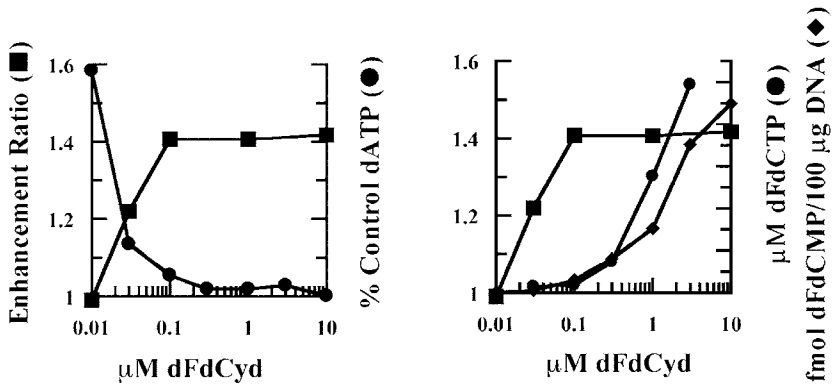


Fig. 4. Radiosensitization with dFdCyd correlates with dATP depletion. HT29 colon cancer cells were incubated with gemcitabine for 4 h prior to irradiation. Cells were then plated for survival in a colony formation assay for determination of RER. Levels of dATP, dFdCTP, and dFdCMP were measured by high-performance liquid chromatographic analysis.

G1 block, this occurs slowly with dFdCyd and does not prevent the initial accumulation of cells in S phase *(136)*.

Studies demonstrated that radiosensitization with dFdCyd correlated with depletion of the dATP pool but not dFdCTP accumulation or its incorporation into DNA (Fig. 4) *(117,118,128)*. Consistent with this finding, a correlation was observed between dCyd kinase activity, the rate-limiting step in the phosphorylation of dFdCyd, and radiosensitization *(137)*. The importance of dATP depletion is further substantiated by the finding that radiosensitization with dFdCyd can be mimicked by hydroxyurea, a nonnucleoside inhibitor of ribonucleotide reductase. At radiosensitizing concentrations, hydroxyurea depletes dATP pools in a fashion similar to that of dFdCyd *(119)*. Furthermore, cells that are radiosensitized by dFdCyd are radiosensitized by hydroxyurea, whereas a single cell line that was not radiosensitized by dFdCyd was not radiosensitized by hydroxyurea *(134)*. Thus, although incorporation of dFdCMP into DNA is required for cytotoxicity, it appears that the inhibition of ribonucleotide reductase by dFdCDP is the pathway that leads to radiosensitization with this analog.

dFdCyd differs markedly from other nucleoside/nucleobase radiosensitizers in that it does not increase induction of DNA dsbs or inhibit their repair *(128,138)*. However, an effect on DNA integrity was demonstrated in a study in which addition of dFdCyd prior to irradiation increased residual chromosomal aberrations *(139)*. When the two major pathways by which DNA dsbs are repaired in mammalian cells were evaluated, it was demonstrated that dFdCyd did not affect nonhomologous end joining *(140)* but appeared to target homologous recombination *(141)*.

We have proposed a hypothesis for radiosensitization with dFdCyd based on strong correlative data suggesting that dATP depletion and accumulation of cells in S phase are important for radiosensitization. It is known that an imbalance in dNTP pools, because of depletion or elevation of a single pool, can produce errors of replication in DNA *(142–144)*. We propose that dATP produces misincorporation events in DNA that, if left unrepaired, result in enhanced sensitivity to radiation. This hypothesis would then predict that cells deficient in the repair of DNA misincorporation events would be better radiosensitized by dFdCyd. We evaluated this hypothesis in matched mismatch repair-proficient and -deficient HCT-116 cells *(145,146)*. As predicted, the HCT-116 (hMLH1-deficient) cell line was better radiosensitized than the mismatch repair-proficient sublines *(147)*. Although the HCT-116 mismatch repair-deficient cell lines could be radiosensitized at dFdCyd concentrations at or below the 50% inhibitory concentration (IC_{50}), the HCT-116 mismatch repair-proficient lines required at least IC_{96} of dFdCyd to produce an increase in radiation-induced cell killing. These data suggest that mismatch repair proficiency prevents radiosensitization with dFdCyd, but that this can be overcome at excessively high doses of dFdCyd. Further data are needed to support this hypothesis, such as identifying mismatches in cellular DNA with radiosensitizing concentrations of dFdCyd.

These data with dFdCyd demonstrate that, as observed for BrdUrd and IdUrd, radiosensitization was increased in mismatch repair-deficient cells. However, mechanistically dFdCyd differs from the halogenated thymidine analogs in that incorporation of dFdCMP in DNA was higher in mismatch repair-proficient cells *(147)*, whereas BrdUMP and IdUMP incorporation was higher in DNA in mismatch repair-deficient cells *(29)*. The specificity of mismatch repair for recognizing and excising nucleotide analogs from DNA is an important area for further investigation.

The actual mechanism by which these lesions produce cell death is still uncertain. The role of apoptosis was evaluated in a study of HT29 colon cancer cells, UMSCC-6 head-and-neck cancer cells, and A549 lung cancer cells, which differ substantially in the ability to undergo radiation-induced apoptosis. It was hypothesized that if dFdCyd produced radiosensitization by potentiating preexisting death pathways, then only the apoptotic-prone HT29 cells would show a substantial increase in apoptosis when treated with the combination of dFdCyd and radiation, and that UMSCC-6 cells and A549 cells would be radiosensitized through nonapoptotic mechanisms. Radiosensitization of HT29 cells (enhancement ratio 1.81 ± 0.16) was accompanied by an increase in apoptosis and by caspase activation, and inhibition of this activation by the caspase inhibitor Z-Asp-Glu-Val-Asp-fluoromethylketone (DEVD) significantly decreased radiosensitization (to 1.36 ± 0.24; $p < 0.05$). In contrast, UMSCC-6 cells and A549 cells were

modestly radiosensitized (enhancement ratio 1.47 ± 0.24 and 1.31 ± 0.04, respectively) via a nonapoptotic mechanism. These findings *(143)*, along with those from other studies *(134,136,148)*, suggest that although apoptosis can contribute significantly to dFdCyd-mediated radiosensitization, the role of apoptosis in dFdCyd-mediated radiosensitization depends on the cell line rather than representing a general property of the drug.

4.3. Clinical Considerations

dFdCyd is an excellent example of a drug for which the clinical application was modeled after the in vitro schedule determined to be optimal. In a clinical trial in patients with unresectable head-and-neck cancer, dFdCyd was administered at low dose to patients once weekly approx 4–6 h prior to radiotherapy, with concurrent standard radiotherapy in daily 2-gy fractions for 7 wk *(149)*. These studies demonstrated that dFdCyd is an excellent radiosensitizer in vivo, with nearly 80% of all patients achieving complete pathologic regression of their tumors. This is approximately twice the expected complete response rate based on historical controls treated with standard radiotherapy only. Pharmacologic studies in tumor biopsies taken 2 h after drug infusion demonstrated excellent uptake and phosphorylation of dFdCyd to concentrations that could inhibit ribonucleotide reductase. However, toxicity was high, with mucosal toxicity that resulted in swallowing dysfunction in most patients *(150)*. Current studies have attempted to decrease normal tissue toxicity by utilizing gemcitabine twice per week, as suggested by preclinical studies *(151)*, during the last 2 wk of radiotherapy when radiotherapy portals address only the gross tumor.

A number of phase I trials using dFdCyd combined with radiotherapy in patients with pancreatic cancer have produced promising results. Based on in vitro results showing that a single low dose of dFdCyd could radiosensitize tumor cells for up to 48 h, two clinical trials used twice-weekly dFdCyd with standard daily radiotherapy *(152,153)*. Maximum tolerated doses (MTDs) were 40–50 mg/m^2, and several partial responses were observed along with radiographic evidence of response. Dose-limiting toxicities were mainly nausea and vomiting or hematologic. With the rationale that these relatively low doses of dFdCyd may control local but not metastatic disease, a novel trial of weekly full-dose dFdCyd (1000 mg/m^2) with escalating daily radiotherapy demonstrated an MTD of 36 gy in 2.4-gy fractions *(154)*. The number of partial responders along with lack of excess regional failures encourages future trials based on this design.

Greater toxicity was observed in two clinical trials using higher doses of radiotherapy. Based on animal studies suggesting that dFdCyd administered at least 24 h prior to radiotherapy would maximize tumor response and allow normal tissue to recover the ability to synthesize DNA faster than tumor tissue *(155–157)*, dFdCyd was administered to patients 3 d prior to

beginning daily radiotherapy *(158)*. However, this resulted in unacceptably high hematologic and GI toxicity. In a separate study, 300 mg/m^2 dFdCyd weekly with weekly 8 gy produced GI ulcerations in more than one-third of the patients *(159)*. These studies demonstrated that the dose per fraction as well as the volume irradiated can affect toxicity and suggest that the use of conformal radiotherapy to the gross tumor is the best method for intensifying chemoradiation. The addition of dFdCyd to 5-FU with concurrent radiotherapy resulted in severe GI toxicity, as might be expected from combining three agents capable of producing GI toxicity *(160)*. In contrast, the combination of dFdCyd and cisplatin with radiation showed some promise *(161)*.

Phase I trials of dFdCyd in patients with non-small cell lung cancer have produced promising clinical results. Response rates were 66% and 88% with once or twice weekly dFdCyd, respectively, and standard radiotherapy *(162,163)*. High toxicity observed in an early trial of dFdCyd with concurrent radiotherapy was attributed to using full doses of each modality with a large radiation field *(164)*.

Taken together, these studies suggest that dFdCyd with concurrent radiotherapy has significant activity, but dose and scheduling must be optimized to decrease normal tissue toxicity. Minimizing the volume of normal tissue in the radiation field *(165)* or adding radioprotective agents such as amifostine *(166)* may improve the selectivity of this approach. Development of an appropriate animal model may help as well. However, to date all animal models have demonstrated only an additive interaction between dFdCyd and radiotherapy, in which therapeutic doses of each modality when used individually were required to demonstrate any increase in efficacy in combination *(151,155,156,167)*.

5. FLUDARABINE

Development of fludarabine (F-ara-A) as an antitumor agent grew out of prior work on the antitumor activity of arabinofuranosyl adenine (ara-A). Although ara-A showed good antitumor activity in animal models, results in patients were less impressive, likely because of the rapid deamination of ara-A by the ubiquitous adenosine deaminase. Fludarabine was developed as a deaminase-resistant analog that showed superior activity to ara-A, and it is now commonly used to treat patients with chronic lymphocytic leukemia *(168)*. Early reports demonstrated that ara-A sensitized cells to ionizing radiation *(169,170)*, which encouraged similar studies with F-ara-A *(171)*. As ara-A is no longer used in patients, this section focuses on interactions between the therapeutically active F-ara-A and ionizing radiation.

5.1. Metabolic Considerations

After its facilitated transport into the cell, F-ara-A is phosphorylated initially by dCyd kinase to its monophosphate; subsequently, other cellular kinases convert it to its major metabolite, F-ara-ATP, the 5′-triphosphate derivative *(168)*. F-ara-ATP has several actions on enzymes of DNA replication: it is an inhibitor of and substrate for mammalian DNA polymerases *(12,172)* and DNA primase *(173,174)*, and it acts as an inhibitor of DNA ligase *(175)*. In addition, F-ara-ATP is an allosteric inhibitor of ribonucleotide reductase in a manner analogous to that of the endogenous dATP *(12,172,176,177)*. Cytotoxicity correlated with incorporation of F-ara-ATP into DNA, where it acted as a chain terminator *(178)*. The ability of F-ara-ATP to inhibit ribonucleotide reductase and thus reduce its endogenous competitor for DNA synthesis likely enhances its incorporation into DNA. This incorporation can result in apoptosis in tumor cells *(179)*.

5.2. Mechanism of Radiosensitization

Studies have demonstrated that F-ara-A is an excellent radiation sensitizer in a variety of animal and human tumor cells. Similar to the results with other nucleoside analog radiation sensitizers, F-ara-A was initially found to be most effective when delivered to animal tumors prior to irradiation *(171,180)*. Radiosensitization of animal tumors was F-ara-A dose dependent and was evident when radiation was administered in a single high dose or on a fractionated schedule *(181)*. Analysis of cell cycle parameters in tumors treated with F-ara-A and radiation suggested that F-ara-A induced specific loss of S-phase cells, followed by redistribution of remaining cells to a more radiosensitive phase of the cell cycle *(182)*. Although F-ara-A was shown to inhibit repair of cisplatin and alkylation adducts in DNA *(183,184)*, other studies in vitro have demonstrated that F-ara-A did not inhibit the ability of cells to rejoin dsbs *(185)*, similar to results with dFdCyd. To our knowledge, studies have not yet determined whether radiosensitization with F-ara-A is related to DNA incorporation or inhibition of ribonucleotide reductase.

Normal tissue sensitization has been observed with F-ara-A because F-ara-A was shown to decrease the tolerance of rat spinal cord to radiation *(186)*. However, studies comparing the sensitivity of human fetal lung fibroblasts and human squamous carcinoma cells demonstrated that there was less radiosensitization in the fibroblasts *(185)*. Furthermore, the combination of F-ara-A and radiation was synergistic in the tumor cells but only additive in the fibroblasts. Thus, in these studies it was possible to attain some selectivity of F-ara-A radiosensitization in tumor cells, encouraging clinical studies.

5.3. Clinical Considerations

To date, there has been only one report of F-ara-A administered as a radiation sensitizer in patients with malignancies. In a phase I trial in patients with locally advanced head-and-neck carcinoma, all patients were treated with 70 gy in 2-gy fractions over 7 wk *(187)*. F-ara-A was added only during the last 2 wk of radiotherapy, and it was given 3–4 h prior to irradiation to maximize sensitization. The results demonstrated a MTD of 15 mg/m^2, with lymphocytopenia and neutropenia as dose-limiting toxicities. Survival in this group was similar to historical controls. Thus, F-ara-A is tolerable when administered during the last 2 wk of standard radiotherapy to this group of patients, although further studies are necessary to determine efficacy.

6. FLUOROMETHYLENEDEOXYCYTIDINE

FMdCyd was synthesized as an anticancer drug that would act as a mechanism-based inhibitor of ribonucleotide reductase *(188)*. After uptake, tumor cells metabolize FMdCyd to its mono-, di-, and triphosphate forms *(189)*. FMdCyd diphosphate binds to the catalytic site of ribonucleotide reductase and results in enzyme inactivation *(190)*. Although dFdCDP is also a mechanism-based inhibitor of ribonucleotide reductase, there appear to be differences in the mechanism of enzyme inactivation. For example, no chromophore was generated with inactivation by dFdCDP, whereas binding of FMdCyd diphosphate to the enzyme resulted in rapid formation of a new chromophore. In addition, FMdC diphosphate inactivated R1 as well as R2 subunits of ribonucleotide reductase, presumably by alkylation, which cannot be accomplished with dFdCDP. The triphosphate of FMdCyd is a substrate for DNA polymerases, and its incorporation into DNA is thought to be an important event in cytotoxicity *(191)*. FMdCyd exhibited good antitumor activity in a variety of human tumor xenografts in nude mice *(192–194)*.

Initial studies demonstrated that FMdCyd could enhance the toxicity of ionizing radiation in HeLa cells in vitro when the drug was added 1 h prior to irradiation *(195)*. Subsequent studies in colon carcinoma and cervical carcinoma cells demonstrated that radiosensitization increased with increasing dose and duration of incubation preceding irradiation *(196)*. Radiosensitization could be accomplished at minimally toxic concentrations of FMdCyd, similar to results with dFdCyd. FMdCyd also induced a precipitous decline in the dATP pool, with a dose-dependent increase in the dCTP and dTTP pools. Initially, deoxyguanosine 5′-triphosphate levels increased, but then declined at higher FMdCyd concentrations. It is not known whether the dNTP pool changes or DNA incorporation or a combination of both effects mediate radiosensitization by FMdCyd.

Flow cytometry studies demonstrated that FMdCyd alone produced an accumulation of WiDr colon cells in early S phase, similar to the cell cycle redistribution observed with other nucleoside analogs as discussed earlier *(197)*. FMdCyd followed by ionizing radiation produced an accumulation of cells in G2/M at 24 h after treatment. When the S and G2/M accumulation was abrogated by the addition of pentoxifylline, radiosensitization was also enhanced *(197)*.

Studies in animal models demonstrated that FMdCyd acts as a radiosensitizer in vivo as well. When human xenografts in nude mice were treated with fractionated radiation, the addition of FMdCyd further enhanced the tumor growth delay, with some animals achieving long-term remission *(198)*. As with dFdCyd, radiosensitization was observed at doses of FMdCyd that, by themselves, had a substantial chemotherapeutic effect.

Phase I clinical trials with FMdCyd have demonstrated that it is well tolerated in patients with solid tumors *(199,200)*. As with other anti metabolites, dose-limiting toxicity was hematologic, manifested primarily as neutropenia. Although FMdCyd is relatively resistant to deamination by cytidine deaminase *(201)*, the primary metabolite in patients was the deamination product fluoromethylene deoxyuridine. However, in contrast to the rapidly deaminated dFdCyd, FMdCyd had a longer half-life of 2–4 h, with measurable elimination of FMdCyd in urine. Further studies are necessary to determine its efficacy as a chemotherapeutic agent and whether it has radiosensitizing antitumor activity in patients.

7. HYDROXYUREA

Hydroxyurea is a small molecule which has a primary action in mammalian cells of inhibition of *de novo* biosynthesis of deoxynucleotides by ribonucleotide reductase, resulting in strong inhibition of DNA synthesis *(202)*. As hydroxyurea targets cells that are actively replicating DNA, it has been used clinically in a variety of myeloproliferative diseases, such as chronic leukemias and polycythemia vera, and it has been useful in treating sickle cell disease *(203,204)*. Clinical success with hydroxyurea has encouraged development of other inhibitors of ribonucleotide reductase *(205)*. Studies have shown that hydroxyurea can inhibit replication of human immunodeficiency virus, and it is currently under evaluation clinically as a component of multidrug therapy for patients with acquired immunodeficiency syndrome *(206)*. Although it is not a nucleoside analog, hydroxyurea is important to include in this chapter because it acts as a radiation sensitizer through alteration of the endogenous deoxynucleotide pools in cells.

7.1. Metabolic Considerations

Hydroxyurea inhibits ribonucleotide reductase through its action at the tyrosyl residue on the catalytic subunit of the enzyme. Formation of a free radical at tyrosine 122 is essential to initiate reduction of the ribonucleotide substrate *(207)*. Hydroxyurea destroys the tyrosyl radical so that the enzyme is unable to abstract the hydroxyl at the 2′-position of the ribose *(208)*.

Although it would be expected that inhibition of ribonucleotide reductase would decrease the levels of all four dNTP pools, it has been demonstrated that pool alteration is cell specific *(209)*. This may be because dNTPs can also be synthesized through the salvage of deoxynucleosides and subsequent phosphorylation by the endogenous kinase pathways, which are not affected by hydroxyurea *(210)*. In addition, the regulation of ribonucleotide reductase by endogenous nucleotides, which differ in amount between cell types, may alter effects from inhibiting the *de novo* biosynthesis pathway *(211,212)*. Furthermore, once synthesis is inhibited, the rate of decay for each dNTP may be different *(209)*. Studies in patients with chronic myelogenous leukemia undergoing treatment with hydroxyurea or another inhibitor of ribonucleotide reductase demonstrated that this pattern of effects on dNTPs occurs *in vivo* as well *(213,214)*. Analysis of dNTP pools in the leukemia cells during therapy showed a strong decrease in the dATP pools with lesser effects on deoxyguanosine 5′-triphosphate, while dCTP and dTTP pools increased.

7.2. Mechanism of Radiosensitization

Evidence that hydroxyurea was most effective at killing cells in S phase *(215)* encouraged evaluation of its effects on repair of ionizing radiation damage. Studies in Chinese hamster ovary cells demonstrated that the combination of hydroxyurea and ionizing irradiation resulted in significantly enhanced cell killing compared to either modality alone *(6,216,217)*. Studies in HeLa cells demonstrated that hydroxyurea could alter the repair of DNA single-strand breaks induced by ionizing radiation, although only a cytotoxic concentration was used *(218)*. Hydroxyurea has also been used in combination with nucleoside analogs because it was proposed that reduction of endogenous deoxynucleotide pools would improve incorporation of the nucleoside analogs into DNA *(209)*. Based on this rationale, the combination of hydroxyurea with cytosine arabinoside was shown to inhibit repair of DNA single-strand breaks induced by ionizing radiation *(218,219)*. A noncytotoxic concentration of hydroxyurea added simultaneously with IdUrd synchronized cells in S phase, produced higher IdUTP levels, and resulted in greater incorporation of IdUMP into DNA *(220)*. Each drug alone enhanced radiation-induced cell killing, and the combination produced a significant enhancement of radiosensitization.

This enhancement increased with longer exposure to hydroxyurea. These studies have fueled numerous clinical investigations.

7.3. Clinical Considerations

The appealing idea of treating rapidly growing tumors with S-phase-specific drugs that would enhance sensitivity to ionizing radiation resulted in numerous clinical trials employing hydroxyurea alone or combined with other chemotherapeutic and radiosensitizing agents *(204,221)*. In randomized trials in patients with cervical cancer treated with radiotherapy, the addition of hydroxyurea significantly prolonged the disease-free interval *(222,223)*. However, the addition of hydroxyurea during radiotherapy followed by treatment with BCNU and 6-thioguanine did not change overall survival for patients with glioblastoma *(204,224,225)*.

Hydroxyurea has been a component of several studies of multiagent intensive chemo- and radiotherapy for head-and-neck cancer, in which it has improved control of local disease *(99,226)*. As expected, when combined with other antimetabolites such as 5-FU, there was substantial mucositis and myelosuppression. These toxicities were controlled somewhat by a 1-wk on/1-wk off cycle. However, toxicity remains difficult with these regimens, and improving quality of life as well as control of metastatic disease continue to be high priorities in these studies. More recent regimens have used a 5-d continuous infusion instead of oral hydroxyurea *(227)*, based on preclinical studies in which longer duration of exposure to hydroxyurea enhanced cytotoxicity *(228)*. In view of studies demonstrating that cells can regain the ability to synthesize dNTPs even during continuous hydroxyurea incubation of cells in culture *(119)*, continuous infusion of hydroxyurea in patients may provide improved enzyme inhibition compared to oral dosing.

8. CONCLUDING REMARKS

The nucleoside/nucleobase analogs discussed in this chapter can produce a dramatic increase in radiation-induced cell killing in vitro, and they have proven clinical efficacy as well. The malignancies in which radiosensitizers have had the most impact when used alone (or with a reduced folate cofactor) include anal, rectal, gastric, and pancreatic cancer, and they play a role (in combination with cisplatin) in the treatment of esophageal, head-and-neck, and cervical cancers. These improvements extend from organ conservation (in the case of anal and laryngeal cancers) to local control (all of the above cancers) to survival (esophageal, head-and-neck, cervix, pancreatic, and gastric cancers). As radiation and antimetabolites are known for their ability to produce mucositis, radiochemotherapy with nucleoside/nucleobase compounds is associated with

increased toxicity to the mucosa within the radiation field, and reduction of this normal tissue toxicity remains an important goal.

Comparison of the mechanisms mediating radiosensitization for the nucleoside/nucleobase discussed in this chapter reveals important common features. For example, the majority of the studies indicated that the nucleoside radiosensitizers and hydroxyurea are more effective when administered prior to irradiation. This likely reflects the necessity for metabolism to a nucleotide form for the nucleoside/nucleobase analogs and the requirement for a time-dependent alteration in dNTP pools for hydroxyurea. In addition, most studies demonstrated that the degree of radiosensitization increases with longer duration of prior drug exposure. There may be several explanations for this optimal sequence of administration. The nucleoside/nucleobase radiosensitizers require intracellular phosphorylation for activity, and this can require several hours to achieve steady-state levels. In addition, it requires several hours for the nucleosides/nucleobases and hydroxyurea to achieve their effects on DNA incorporation or dNTP pool alteration. Furthermore, cell cycle studies have demonstrated that these radiosensitizers work best when cells have accumulated in S phase, also a time-dependent process. We have previously suggested that, for FdUrd/5-FU, inappropriate activation of cyclin-dependent kinases in cells with mutant p53 promotes S-phase accumulation and DNA replication prior to repair, resulting in radiosensitization *(91)*. In general, drug exposure prior to radiation allows cells time for the requisite metabolism and progression of cells lacking checkpoint controls into S phase, which leads to DNA damage or inhibition of repair.

There is an impressive amount of work done on the characteristics of radiosensitization by the nucleoside/nucleobase analogs, such as optimal sequence of drug/radiation treatment, cell cycle redistribution, and effects on DNA strand breakage and repair. However, less is known about the actual lesions that may be necessary for radiosensitization. Perhaps the most specific hypothesis has been proposed for BrdUrd and IdUrd, in which the irradiation of the halogenated uracil may produce a uracilyl radical that can cleave deoxyribose, resulting in strand breakage. For the other drugs discussed in this chapter, the mechanism of strand breakage is not clear, or the specific DNA damage that leads to radiosensitization is not known.

We suggest that the dNTP pool alteration, a feature common to all of the radiosensitizers discussed here, is a component that contributes to radiosensitization. We propose that the dNTP alterations in cells that are replicating DNA in S-phase lead to errors in nucleotide incorporation into DNA. Without further DNA damage, these misincorporation events would normally be repaired correctly and not result in cytotoxicity. However, following ionizing radiation, we propose that these lesions are either not

recognized or not repaired correctly. If these errors are not corrected prior to irradiation, then we suggest they will result in enhanced cell death (radiosensitization). There is ample evidence for imbalanced dNTP pools producing incorrect nucleotide incorporation in DNA *(142–144)*. If such mismatched nucleotides are the lesions necessary for radiosensitization by these agents, then it would follow that cells that are not capable of repairing these errors, that is, mismatch repair-deficient cells, would be better radiosensitized. New evidence that is now accumulating demonstrates that defective mismatch repair enhances radiosensitization by BrdUrd, IdUrd, and dFdCyd *(29,30,147)*.

Although it has been argued that the role of MMR is to reduce BrdUrd and IdUrd incorporation into DNA, it is also expected to correct errors that would result from the BrdUrd- and IdUrd-mediated decrease in pyrimidine dNTPs. For dFdCyd, we have demonstrated that MMR does not decrease incorporation of the fraudulent nucleotide into DNA *(147)*, and preliminary data suggest that MMR can repair the incorrect nucleotides in DNA that likely resulted from the altered dNTP pools *(229)*. Regardless of the mechanism, the number of radiosensitizers that have dNTP pool alteration as their major cellular effect suggests that this is an important mechanism to radiosensitization. Future studies should evaluate other drugs that alter dNTP pools for their ability to produce radiosensitization.

The nucleoside/nucleobase radiosensitizers and hydroxyurea have an established role in radiochemotherapy treatment of malignancy. Continued studies to determine the mechanism by which they act are important to improve efficacy as well as selectivity in patients.

REFERENCES

1. Fertil, B., Dertinger, H., Courdi, A., and Malaise, E. P. Mean inactivation dose: a useful concept for intercomparison of human cell survival curves. Radiat. Res., 99, 73–84, 1984.
2. Steel, G. G., and Peckham, M. J. Exploitable mechanisms in combined radiotherapy-chemotherapy: the concept of additivity. Int. J. Radiat. Oncol. Biol. Phys., 5, 85–91, 1979.
3. Chou, T. -C., and Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enz. Reg., 22, 27–55, 1984.
4. Greco, W. R., Park, H. S., and Rustum, Y. M. Application of a new approach for the quantitation of drug synergism to the combination of *cis*-diamminedichloroplatinum and 1—D-arabinofuranosylcytosine. Cancer Res., 50, 5318–5327, 1990.
5. Terasima, T., and Tolmach, L. J. Variations in several responses of HeLa cells to X-irradiation during divison cycle. Biophysical J., 3, 11–33, 1963.
6. Sinclair, W. K., and Morton, R. A. X-ray sensitivity during cell generation cycle of cultured Chinese hamster cells. Radiat. Res., 29, 450–474, 1966.

7. Prusoff, W., and Goz, B. Halogenated pyrimidine deoxyribonucleosides. In A. C. Sartorelli and D. G. Johns (eds.), Handbook of Experimental Pharmacology, vol. 38, pp. 272–347. New York: Springer-Verlag, 1975.
8. Goz, B. The effects of incorporation of 5-halogenated deoxyuridines into the DNA of eukaryotic cells. Pharmacol. Rev., 29, 249–272, 1978.
9. Lee, L. -S., and Cheng, Y. Human deoxythymidine kinase II: substrate specificity and kinetic behavior of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia. Biochemistry, 15, 3686–3690, 1976.
10. Lee, L. -S., and Cheng, Y. Human deoxythymidine kinase. I. Purification and general properties of the cytoplasmic and mitochondrial isozymes derived from blast cells of acute myelocytic leukemia. J. Biol. Chem., 251, 2600–2604, 1976.
11. Eriksson, S., Kierdaszuk, B., Munch-Petersen, B., Oberg, O., and Johansson, N. G. Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. Biochem. Biophys. Res. Commun., 176, 586–592, 1991.
12. Parker, W. B., Bapat, A. R., Shen, J.-X., Townsend, A. J., and Cheng, Y. Interaction of 2-halogenated dATP analogs (F, Cl, and Br) with human DNA polymerases, DNA primase, and ribonucleotide reductase. Mol. Pharmacol., 34, 485–491, 1988.
13. Djordjevic, B., and Szybalski, W. Genetics of human cell lines. III. Incorporation of 5-bromo- and 5-iododeoxyuridine into the deoxyribonucleic acid of human cells and its effect on radiation sensitivity. J. Exp. Med., 112, 509–531, 1960.
14. Lawrence, T. S., Davis, M. A., Maybaum, J., et al. The potential superiority of bromodeoxyuridine to iododeoxyuridine as a radiation sensitizer in the treatment of colorectal cancer. Cancer Res., 52, 3698–3704, 1992.
15. Dewey, W. C., and Humphrey, R. M. Increase in radiosensitivity to ionizing radiation related to replacement of thymidine in mammalian cells with 5-bromodeoxyuridine. Radiat. Res., 26, 538-&, 1965.
16. McLaughlin, P. W., Lawrence, T. S., Seabury, H., et al. Bromodeoxyuridine-mediated radiosensitization in human glioma: the effect of concentration, duration, and fluoropyrimidine modulation. Int. J. Radiat. Oncol. Biol. Phys., 30, 601–607, 1994.
17. Mancini, W. R., Stetson, P. L., Lawrence, T. S., Wagner, L. M., Greenberg, H. S., and Ensminger, W. D. Variability of 5-bromo-2′-deoxyuridine incorporation into DNA of human glioma cell lines and modulation with fluoropyrimidines. Cancer Res., 51, 870–874, 1991.
18. Lawrence, T. S., Davis, M. A., Maybaum, J., Stetson, P. L., and Ensminger, W. D. The effect of single vs double-strand substitution on halogenated pyrimidine-induced radiosensitization and DNA strand breakage in human tumor cells. Radiat. Res., 123, 192–198, 1990.
19. Kinsella, T. J., Dobson, P. A., Mitchell, J. B., and Fornace, A. J. Enhancement of X-ray induced DNA damage by pretreatment with halogenated pyrimidine analogs. Int. J. Radiat. Oncol. Biol. Phys., 13, 733–739, 1987.
20. Iliakis, G., Kurtzman, S., Pantelias, G., and Okayasu, R. Mechanism of radiosensitization by halogenatedpyrimidines: effect of BrdU on radiation induction of DNA and chromosome damage and its correlation with cell killing. Radiat. Res., 119, 286–304, 1989.

21. Lawrence, T. S., Davis, M. A., Maybaum, J., Stetson, P. L., and Ensminger, W. D. The dependence of halogenated pyrimidine incorporation and radiosensitization on the duration of drug exposure. Int. J. Radiat. Oncol. Biol. Phys., 18, 1393–1398, 1990.
22. Tishler, R. B., and Geard, C. R. Correlation of sensitizer enhancement ratio with bromodeoxyuridine concentration and exposure time in human cervical-carcinoma cells treated with low-dose rate irradiation. Int. J. Radiat. Oncol. Biol. Phys., 22, 495–498, 1992.
23. Fornace, A. J., Dobson, P. A., and Kinsella, T. J. Enhancement of radiation damage in cellular DNA following unifilar substitution with iododeoxyuridine. Int. J. Radiat. Oncol. Biol. Phys., 18, 1990.
24. Dillehay, L. E., Thompson, L. H., and Carrano, A. V. DNA-strand breaks associated with halogenated pyrimidine incorporation. Mutat. Res., 131, 129–136, 1984.
25. Zimbrick, J. D., Ward, J. F., and Myers, L. S., Jr. Studies on the chemical basis of cellular radiosensitizatioin by 5-bromouracil substitution in DNA. II. Pulse- and steady-state radiolysis of bromouracil-substituted and unsubstituted DNA. Int. J. Radiat. Biol., 16, 525–534, 1969.
26. Lawrence, T. S., Davis, M. A., and Normolle, D. P. Effect of bromodeoxyuridine on radiation-induced DNA damage and repair based on DNA fragment size using pulsed-field gel electrophoresis. Radiat. Res., 144, 282–287, 1995.
27. Marti, T. M., Kunz, C., and Fleck, O. DNA mismatch repair and mutation avoidance pathways. J. Cell. Physiol., 191, 28–41, 2002.
28. Toft, N. J., and Arends, M. J. DNA mismatch repair and colorectal cancer. J. Pathol., 185, 123–129, 1998.
29. Berry, S. E., Garces, C., Hwang, H.-S., et al. The mismatch repair protein, hMLH1, mediates 5-substituted halogenated thymidine analogue cytotoxicity, DNA incorporation, and radiosensitization in human colon cancer cells. Cancer Res., 59, 1840–1845, 1999.
30. Berry, S. E., Davis, T. W., Schupp, J. E., Hwang, H.-S., de Wind, N., and Kinsella, T. J. Selective radiosensitization of drug-resistant MutS homologue-2 (MSH2) mismatch repair-deficient cells by halogenated thymidine (dThd) analogues: Msh2 mediates dThd analogue DNA levels and the differential cytotoxicity and cell cycle effects of the dThd analogues and 6-thioguanine. Cancer Res., 60, 5773–5780, 2003.
31. Berry, S. E., Loh, T., Yan, T., and Kinsella, T. J. Role of MutSα in the recognition of iododeoxyuridine in DNA. Cancer Research 63, 5490–5495. 2003.
32. Taverna, P., Hwang, H.-S., Schupp, J. E., et al. Inhibition of base excision repair potentiates iododeoxyuridine-induced cytotoxicity and radiosensitization. Cancer Res., 63, 838–846, 2003.
33. Meuth, M., and Green, H. Induction of a deoxycytidineless state in cultured mammalian cells by bromodeoxyuridine. Cell, 2, 109–112, 1974.
34. Ashman, C. R., and Davidson, R. L. Bromodeoxyuridine mutagenesis in mammalian cells is related to deoxyribonucleotide pool imbalance. Mol. Cell. Biol., 1, 254–260, 1981.
35. Shewach, D. S., Ellero, J., Mancini, W. R., and Ensminger, W. D. Decrease in TTP pools mediated by 5-bromo-2′-deoxyuridine exposure in a human glioblastoma cell line. Biochem. Pharmacol., 43, 1579–1585, 1992.

36. Epstein, A. H., Lebovics, R. S., Goffman, T., et al. Treatment of locally advanced cancer of the head and neck with 5′-iododeoxyuridine and hyperfractionated radiation-therapy—measurement of cell labeling and thymidine replacement. J.N.C.I., 86, 1775–1780, 1994.
37. Greenberg, H. S., Chandler, W. F., Diaz, R. F., et al. Intra-arterial bromodeoxyuridine radiosensitization and radiation in treatment of malignant astrocytomas. J. Neurosurg., 69, 500–505, 1988.
38. Sullivan, F. J., Herscher, L. L., Cook, J. A., et al. National-Cancer-Institute (Phase-II) study of high-grade glioma treated with accelerated hyperfractionated radiation and iododeoxyuridine—results in anaplastic astrocytoma. Int. J. Radiat. Oncol. Biol. Phys., 30, 583–590, 1994.
39. Phillips, T. L., Levin, V. A., Ahn, D. K., et al. Evaluation of bromodeoxyuridine in glioblastoma-multiforme—a Northern California Cancer Center phase-II study. Int. J. Radiat. Oncol. Biol. Phys., 21, 709–714, 1991.
40. Goffman, T., Tochner, Z., and Glatstein, E. Primary-treatment of large and massive adult sarcomas with iododeoxyuridine and aggressive hyperfractionated irradiation. Cancer, 67, 572–576, 1991.
41. Robertson, J. M., Sondak, V. K., Weiss, S. A., Sussman, J. J., Chang, A. E., and Lawrence, T. S. Preoperative radiation-therapy and iododeoxyuridine for large retroperitoneal sarcomas. Int. J. Radiat. Oncol. Biol. Phys., 31, 87–92, 1995.
42. Chang, A. E., Collins, J. M., Speth, P. A. J., et al. A phase-I study of intraarterial iododeoxyuridine in patients with colorectal liver metastases. J. Clin. Oncol., 7, 662–668, 1989.
43. Eisbruch, A., Robertson, J. M., Johnston, C. M., et al. Bromodeoxyuridine alternating with radiation for advanced uterine cervix cancer: a phase I and drug incorporation study. J. Clin. Oncol., 17, 31–40, 1999.
44. Levin, V. A., Prados, M. R., Wara, W. M., et al. Radiation-therapy and bromodeoxyuridine chemotherapy followed by procarbazine, lomustine, and vincristine for the treatment of anaplastic gliomas. Int. J. Radiat. Oncol. Biol. Phys., 32, 75–83, 1995.
45. Prados, M. D., Scott, C., Sandler, H., et al. A phase 3 randomized study of radiotherapy plus procarbazine, CCNU, and vincristine (PCV) with or without BUdR for the treatment of anaplastic astrocytoma: a preliminary report of RTOG 9404. Int. J. Radiat. Oncol. Biol. Phys., 45, 1109–1115, 1999.
46. Ensminger, W. D., Walker, S. C., Stetson, P. L., et al. Clinical-pharmacology of hepatic arterial infusions of 5-bromo-2′-deoxyuridine. Cancer Res., 54, 2121–2124, 1994.
47. Prados, M. D., Scott, C. B., Rotman, M., et al. Influence of bromodeoxyuridine radiosensitization on malignant glioma patient survival: a retrospective comparison of survival data from the northern California Oncology Group (NCOG) and Radiation Therapy Oncology Group trials (RTOG) for glioblastoma multiforme and anaplastic astrocytoma. Int. J. Radiat. Oncol. Biol. Phys., 40, 653–659, 1998.
48. Knol, J. A., Walker, S. C., Robertson, J. M., et al. Incorporation of 5-bromo-2′-deoxyuridine into colorectal liver metastases and liver in patients receiving a 7-d hepatic arterial infusion. Cancer Res., 55, 3687–3691, 1995.
49. Lawrence, T. S., Davis, M. A., Stetson, P. L., Maybaum, J., and Ensminger, W. D. Kinetics of bromodeoxyuridine elimination from human colon-cancer cells in-vitro and in-vivo. Cancer Res., 54, 2964–2968, 1994.

50. Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. Meta-analysis Group in Cancer. J. Clin. Oncol., 16, 301–308, 1998.
51. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. Advanced Colorectal Cancer Meta-Analysis Project. J. Clin. Oncol., 10, 896–903, 1992.
52. Saltz, L. B., Cox, J. V., Blanke, C., et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. N. Engl. J. Med., 343, 905–914, 2000.
53. Ensminger, W. D. Intrahepatic arterial infusion of chemotherapy: pharmacologic principles. Semin. Oncol., 29, 119–125, 2002.
54. Abeles, R. H., and Alston, T. A. Enzyme-inhibition by fluoro compounds. J. Biol. Chem., 265, 16,705–16,708, 1990.
55. Wohlhueter, R. M., Mcivor, R. S., and Plagemann, P. G. W. Facilitated transport of uracil and 5-fluorouracil, and permeation of orotic-acid into cultured mammalian-cells. J. Cell. Physiol., 104, 309–319, 1980.
56. Ardalan, B., and Glazer, R. An update on the biochemistry of 5-fluorouracil. Cancer Treat. Rev., 8, 157–167, 1981.
57. Myers, C. E. The pharmacology of the fluoropyrimidines. Pharmacol. Rev., 33, 1–15, 1981.
58. Longley, D. B., Harkin, D. P., and Johnston, P. G. 5-Fluorouracil: mechanisms of action and clinical strategies. Nat. Rev. Cancer, 3, 330–338, 2003.
59. Harris, B. E., Song, R., Soong, S. J., and Diasio, R. B. Relationship between dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels with evidence for circadian variation of enzyme activity and plasma drug levels in cancer patients receiving 5-fluorouracil by protracted continuous infusion. Cancer Res., 50, 197–201, 1990.
60. Baccanari, D. P., Davis, S. T., Knick, V. C., and Spector, T. 5-Ethynyluracil (776C85): a potent modulator of the pharmacokinetics and antitumor efficacy of 5-fluorouracil. Proc. Natl. Acad. Sci. U. S. A., 90, 11064–11068, 1993.
61. Spector, T., Cao, S., Rustum, Y. M., Harrington, J. A., and Porter, D. J. Attenuation of the antitumor activity of 5-fluorouracil by (R)-5-fluoro-5,6-dihydrouracil. Cancer Res., 55, 1239–1241, 1995.
62. Lang, T. T., Selner, M., Young, J. D., and Cass, C. E. Acquisition of human concentrative nucleoside transporter 2 (hCNT2) activity by gene transfer confers sensitivity to fluoropyrimidine nucleosides in drug-resistant leukemia cells. Mol. Pharmacol., 60, 1143–1152, 2001.
63. Ingraham, H. A., Tseng, B. Y., and Goulian, M. Nucleotide levels and incorporation of 5-fluorouracil and uracil into DNA of cells treated with 5-fluorodeoxyuridine. Mol. Pharmacol., 21, 211–216, 1982.
64. Canman, C. E., Lawrence, T. S., Shewach, D. S., Tang, H.-Y., and Maybaum, J. Resistance to fluorodeoxyuridine-induced DNA damage and cytotoxicity correlates with an elevation of deoxyuridine triphosphatase activity and failure to accumulate deoxyuridine triphosphate. Cancer Res., 53, 5219–5223, 1993.
65. Ingraham, H. A., Tseng, B. Y., and Goulian, M. Nucleotide levels and incorporation of 5-fluorouracil and uracil into DNA of cells treated with 5-fluorodeoxyuridine. Mol. Pharmacol., 21, 211–216, 1982.
66. Kufe, D. W., Major, P. P., Egan, E. M., and Loh, E. 5-Fluoro-2′-deoxyuridine incorporation in L1210 DNA. J. Biol. Chem., 256, 8885–8888, 1981.

67. Davis, M. A., Tang, H. Y., Maybaum, J., and Lawrence, T. S. Dependence of fluorodeoxyuridine-mediated radiosensitization on S-phase progression. Int. J. Radiat. Oncol. Biol. Phys., 67, 509–517, 1995.
68. Danenberg, P. V. Thymidylate synthetase—target enzyme in cancer chemotherapy. Biochim. Biophys. Acta, 473, 73–92, 1977.
69. Bruso, C. E., Shewach, D. S., and Lawrence, T. S. Fluorodeoxyuridine-induced radiosensitization and inhibition of DNA double strand break repair in human colon cancer cells. Int. J. Radiat. Oncol. Biol. Phys., 19, 1411–1417, 1990.
70. Tattersall, M. H. N., and Harrap, K. R. Changes in the deoxyribonucleotide triphosphate pools of mouse 5178Y lymphoma cells following exposure to methotrexate or 5-fluorouracil. Cancer Res., 33, 3086–3090, 1973.
71. Chong, L., and Tattersall, M. H. N. 5,10-Dideazatetrahydrofolic acid reduces toxicity and deoxyadenosine triphosphate pool expansion in cultured L1210 cells treated with inhibitors of thymidylate synthase. Biochem. Pharmacol., 49, 819–827, 1995.
72. Yoshioka, A., Tanaka, S., Hiraoka, O., et al. Deoxyribonucleotide triphosphate imbalance. J. Biol. Chem., 262, 8235–8241, 1987.
73. Cheng, Y., and Nakayama, K. Effects of 5-fluoro-2′-deoxyuridine on DNA metabolism in HeLa cells. Mol. Pharmacol., 23, 171–174, 1983.
74. Houghton, J. A., Tillman, D. M., and Harwood, F. G. Ratio of 2′-deoxyadenosine-5′-triphosphate thymidine-5′-triphosphate influences the commitment of human colon carcinoma cells to thymineless death. Clin. Cancer Res., 1, 723–730, 1995.
75. Buchholz, D. J., Lepek, K. J., Rich, T. A., and Murray, D. 5-Fluorouracil-radiation interactions in human colon adenocarcinoma cells. Int. J. Radiat. Oncol. Biol. Phys., 32, 1053–1058, 1995.
76. McGinn, C. J., Miller, E. M., Lindstrom, M. J., Kunugi, K. A., Johnston, P. G., and Kinsella, T. J. The role of cell cycle redistribution in radiosensitization: implications regarding the mechanism of fluorodeoxyuridine radiosensitization. Int. J. Radiat. Oncol. Biol. Phys., 30, 851–859, 1994.
77. Naida, J. D., Davis, M. A., and Lawrence, T. S. The effect of activation of wild-type p53 function on fluoropyrimidine-mediated radiosensitization. Int. J. Radiat. Oncol. Biol. Phys., 41, 675–680, 1998.
78. Lawrence, T. S., Davis, M. A., and Loney, T. L. Fluoropyrimidine-mediated radiosensitization depends on cyclin E-dependent kinase activation. Cancer Res., 56, 3203–3206, 1996.
79. Tang, H. Y., Davis, M. A., Strickfaden, S. M., Maybaum, J., and Lawrence, T. S. Influence of cell-cycle phase on radiation-induced cytotoxicity and DNA-damage in human colon-cancer (Ht29) and Chinese-hamster ovary cells. Radiat. Res., 138, S109–S112, 1994.
80. Kufe, D. W., and Major, P. P. 5-Fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. J. Biol. Chem., 256, 9802–9805, 1981.
81. Lawrence, T. S., Davis, M. A., and Maybaum, J. Dependence of 5-fluorouracil-mediated radiosensitization on DNA-directed effects. Int. J. Radiat. Oncol. Biol. Phys., 29, 519–523, 1994.
82. Hwang, H. S., Davis, T. W., Houghton, J. A., and Kinsella, T. J. Radiosensitivity of thymidylate synthase-deficient human tumor cells is affected by progression through the G(1) restriction point into S-phase: implications for fluoropyrimidine radiosensitization. Cancer Res., 60, 92–100, 2000.

83. Lawrence, T. S., Blackstock, A. W., and McGinn, C. The mechanism of action of radiosensitization of conventional chemotherapeutic agents. Semin. Radiat. Oncol., 13, 13–21, 2003.
84. Schuetz, J. D., Wallace, H. J., and Diasio, R. B. DNA-repair following incorporation of 5-fluorouracil into DNA of mouse bone-marrow cells. Cancer Chemother. Pharmacol., 21, 208–210, 1988.
85. Ingraham, H. A., Dickey, L., and Goulian, M. DNA fragmentation and cytotoxicity from increased cellular deoxyuridylate. Biochemistry, 25, 3225–3230, 1986.
86. Canman, C. E., Tang, H.-Y., Normolle, D. P., Lawrence, T. S., and Maybaum, J. Variations in patterns of DNA damage induced in human colorectal tumor cells by 5-fluorodeoxyuridine: implications for mechanisms of resistance and cytotoxicity. Proc. Natl. Acad. Sci. U. S. A., 89, 10,474–10,478, 1992.
87. Lawrence, T. S., Davis, M. A., Chang, E. Y., Canman, C. E., Maybaum, J., and Radany, E. H. Lack of dependence of 5-fluorodeoxyuridine-mediated radiosensitization on cytotoxicity. Radiat. Res., 143, 281–285, 1995.
88. Chu, E., Voeller, D. M., Jones, K. L., et al. Identification of a thymidylate synthase ribonucleoprotein complex in human colon-cancer cells. Mol. Cell. Biol., 14, 207–213, 1994.
89. Tillman, D. M., Petak, I., and Houghton, J. A. A Fas-dependent component in 5-fluorouracil/leucovorin-induced cytotoxicity in colon carcinoma cells. Clin. Cancer Res., 5, 425–430, 1999.
90. Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M., and Bonner, W. M. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Current Biol., 10, 886–895, 2000.
91. McGinn, C. J., Shewach, D. S., and Lawrence, T. S. Radiosensitizing nucleosides. J.N.C.I., 4, 1193–1203, 1996.
92. Bartelink, H., Roelofsen, F., Eschwege, F., et al. Concomitant radiotherapy and chemotherapy is superior to radiotherapy alone in the treatment of locally advanced anal cancer: results of a phase III randomized trial of the European organization for research and treatment of cancer radiotherapy and gastrointestinal cooperative groups. J. Clin. Oncol., 15, 2040–2049, 1997.
93. Browman, G. P., Cripps, C., Hodson, D. I., Eapen, L., Sathya, J., and Levine, M. N. Placebo-controlled randomized trial of infusional fluorouracil during standard radiotherapy in locally advanced head and neck-cancer. J. Clin. Oncol., 12, 2648–2653, 1994.
94. Radiation-therapy combined with adriamycin or 5-fluorouracil for the treatment of locally unresectable pancreatic-carcinoma. Cancer, 56, 2563–2568, 1985.
95. Morris, M., Eifel, P. J., Lu, J. D., et al. Pelvic radiation with concurrent chemotherapy compared with pelvic and para-aortic radiation for high-risk cervical cancer. N. Engl. J. Med., 340, 1137–1143, 1999.
96. Merlano, M., Benasso, M., Corvo, R., et al. Five-year update of a randomized trial of alternating radiotherapy and chemotherapy compared with radiotherapy alone in treatment of unresectable squamous cell carcinoma of the head and neck. J.N.C.I., 88, 583–589, 1996.
97. Wendt, T. G., Grabenbauer, G. G., Rodel, C. M., et al. Simultaneous radiochemotherapy vs radiotherapy alone in advanced head and neck cancer: a randomized multicenter study. J. Clin. Oncol., 16, 1318–1324, 1998.
98. Denis, F., Garaud, P., Bardet, E., et al. Final results of the 94=01 French Head and Neck Oncology and Radiotherapy Group randomized trial comparing radiotherapy

alone with concomitant radiochemotherapy in advanced-stage oropharynx carcinoma. J. Clin. Oncol., 22, 69–76, 2004.
99. Vokes, E. E., Kies, M. S., Haraf, D. J., et al. Concomitant chemoradiotherapy as primary therapy for locoregionally advanced head and neck cancer. J. Clin. Oncol., 18, 1652–1661, 2000.
100. Villalona-Calero, M. A., Weiss, G. R., Burris, H. A., et al. Phase I and pharmacokinetic study of the oral fluoropyrimidine capecitabine in combination with paclitaxel in patients with advanced solid malignancies. J. Clin. Oncol., 17, 1915–1925, 1999.
101. Minsky, B. D. UFT plus oral leucovorin calcium (Orzel) and radiation in combined modality therapy: a comprehensive review. Int. J. Cancer, 96, 1–10, 2001.
102. Corvo, R., Pastrone, I., Scolaro, T., Marcenaro, M., Berretta, L., and Chiara, S. Radiotherapy and oral capecitabine in the preoperative treatment of patients with rectal cancer: rationale, preliminary results and perspectives. Tumori, 89, 361–367, 2003.
103. Robertson, J. M., Lawrence, T. S., Andrews, J. C., Walker, S., Kessler, M. L., and Ensminger, W. D. Long-term results of hepatic artery fluorodeoxyuridine and conformal radiation therapy for primary hepatobiliary cancers. Int. J. Radiat. Oncol. Biol. Phys., 37, 325–330, 1997.
104. Miller, R., Dewar, E. P., Kapadia, C. R., et al. Randomized clinical trial of adjuvant radiotherapy and 5-fluorouracil infusion in colorectal cancer (AXIS). Br. J. Surg., 90, 1200–1212, 2003.
105. Kaye, S. B. Gemcitabine: current status of phase I and II trials. J. Clin. Oncol., 12, 1527–1531, 1994.
106. Moore, M., Andersen, J., Burris, H., et al. A randomized trial of gemcitabine (Gem) vs 5FU as first-line therapy in advanced pancreatic cancer. Proc. Am. Soc. Clin. Oncol., 14, 199, 1995.
107. Burris, H. A. I., Moore, M. J., Green, M. R., et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J. Clin. Oncol., 15, 2403–2413, 1997.
108. Abratt, R., Bezwoda, W. R., Falkson, G., Goedhals, L., Hacking, D., and Rugg, T. Efficacy and safety profile of gemcitabine in non-small-cell lung cancer: a phase II study. J. Clin. Oncol., 12, 1535–1540, 1994.
109. Abratt, R. P., Bezwoda, W. R., Goedhals, L., and Hacking, D. J. Weekly gemcitabine with monthly cisplatin: effective chemotherapy for advanced non-small-cell lung cancer. J. Clin. Oncol., 15, 744–749, 1997.
110. Plunkett, W., Huang, P., Searcy, C. E., and Gandhi, V. Gemcitabine: preclinical pharmacology and mechanisms of action. Semin. Oncol., 23, 3–15, 1996.
111. Burke, T., Lee, S., Ferguson, P. J., and Hammond, J. R. Interaction of 2′,2′-difluorodeoxycytidine (gemcitabine) and formycin B with the Na^+-dependent and -independent nucleoside transporters of Ehrlich ascites tumor cells. J. Pharmacol. Exp. Ther., 286, 1333–1340, 1998.
112. Hammond, J. R., Lee, S., and Ferguson, P. J. [H-3]Gemcitabine uptake by nucleoside transporters in a human head and neck squamous carcinoma cell line. J. Pharmacol. Exp. Ther., 288, 1185–1191, 1999.
113. Heinemann, V., Hertel, L. W., Grindey, G. B., and Plunkett, W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. Cancer Res., 48, 4024–4031, 1988.

114. Shewach, D. S., Reynolds, K. K., and Hertel, L. Nucleotide specificity of human deoxycytidine kinase. Mol. Pharmacol., 42, 518–524, 1992.
115. Baker, C. H., Banzon, J., Bollinger, J. M., et al. 2′-Deoxy-2′-methylenecytidine and 2′-deoxy-2′,2′-difluorocytidine 5′-diphosphates: Potent mechanism-based inhibitors of ribonucleotide reductase. J. Med. Chem., 34, 1879–1884, 1991.
116. Heinemann, V., Xu, Y.-Z., Chubb, S., et al. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2′,2′-difluorodeoxycytidine. Mol. Pharmacol., 38, 567–572, 1990.
117. Lawrence, T. S., Chang, E. Y., Hahn, T. M., Hertel, L. W., and Shewach, D. S. Radiosensitization of pancreatic cancer cells by 2′,2′-difluoro-2′-deoxycytidine. Int. J. Radiat. Oncol. Biol. Phys., 34, 867–872, 1996.
118. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W., and Lawrence, T. S. Metabolism of 2′,2′-difluoro-2′-deoxycytidine and radiation sensitization of human colon carcinoma cells. Cancer Res., 54, 3218–3223, 1994.
119. Ostruszka, L. J., and Shewach, D. S. The role of DNA synthesis inhibition in the cytotoxicity of 2′,2′-difluoro-2′-deoxycytidine. Cancer Chemother. Pharmacol., 52, 325–332, 2003.
120. Huang, P., Chubb, S., Hertel, L. W., Grindey, G. B., and Plunkett, W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res., 51, 6110–6117, 1991.
121. Ross, D. D., and Cuddy, D. P. Molecular effects of 2′,2′-difluorodeoxycytidine (gemcitabine) on DNA replication in intact HL-60 cells. Biochem. Pharmacol., 48, 1619–1630, 1994.
122. Jiang, H. Y., Hickey, R. J., Abdel-Aziz, W., and Malkas, L. H. Effects of gemcitabine and araC on in vitro DNA synthesis mediated by the human breast cell DNA synthesome. Cancer Chemother. Pharmacol., 45, 320–328, 2000.
123. Schy, W. E., Hertel, L. W., Kroin, J. S., Bloom, L. B., Goodman, M. F., and Richardson, F. C. Effect of a template-located 2′,2′-difluorodeoxycytidine on the kinetics and fidelity of base insertion by Klenow (3′-5′ exonuclease-) fragment. Cancer Res., 53, 4582–4587, 1993.
124. Ruiz van Haperen, V. W. T., Veerman, G., Vermorken, J. B., and Peters, G. J. 2′,2′-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. Biochem. Pharmacol., 46, 762–766, 1993.
125. Abbruzzese, J. L., Grunewald, R., Weeks, E. A., et al. A phase I clinical, plasma, and cellular pharmacology study of gemcitabine. J. Clin. Oncol., 9, 491–498, 1991.
126. Heinemann, V., Xu, Y.-Z., Chubb, S., Sen, A., Hertel, L. W., Grindey, G. B., and Plunkett, W. Cellular elimination of 2′,2′-difluorodeoxycytidine 5′-triphosphate: a mechanism of self-potentiation. Cancer Res., 52, 533–539, 1992.
127. Rockwell, S., and Grindey, G. B. Effect of 2′,2′-difluorodeoxycytidine on the viability and radiosensitivity of EMT6 cells in vitro. Oncol. Res., 4, 151–155, 1992.
128. Lawrence, T. S., Chang, E. Y., Hahn, T. M., and Shewach, D. S. Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2′,2′-difluoro-2′-deoxycytidine (gemcitabine). Clin. Cancer Res., 6, 777–782, 1997.
129. Rosier, J. F., Beauduin, M., Bruniaux, M., et al. The effect of 2′-2 ′ difluorodeoxycytidine (dFdC, gemcitabine) on radiation-induced cell lethality in two

human head and neck squamous carcinoma cell lines differing in intrinsic radiosensitivity. Int. J. Radiat. Biol., 75, 245–251, 1999.
130. Shewach, D. S., and Lawrence, T. S. Gemcitabine and radiosensitization in human tumor cells. Invest. New Drugs, 14, 257–263, 1996.
131. Tolis, C., Peters, G. J., Ferreira, C. G., Pinedo, H. M., and Giaccone, G. Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. Eur. J. Cancer, 35, 706–807, 1999.
132. Cappella, P., Tomasoni, D., Faretta, M., et al. Cell cycle effects of gemcitabine. Int. J. Cancer, 93, 401–408, 2001.
133. Latz, D., Fleckenstein, K., Eble, M., Blatter, J., Wannenmacher, M., and Weber, K. J. Radiosensitizing potential of gemcitabine (2′,2′-difluoro-2′-deoxycytidine) within the cell cycle in vitro. Int. J. Radiat. Oncol. Biol. Phys., 41, 875–882, 1998.
134. Ostruszka, L. J., and Shewach, D. S. The role of cell cycle progression in radiosensitization by 2′,2′-difluoro-2′-deoxycytidine. Cancer Res., 60, 6080–6088, 2000.
135. Chen, M., Hough, A. M., and Lawrence, T. S. The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization. Cancer Chemother. Pharmacol., 45, 369–374, 2000.
136. Robinson, B. W., and Shewach, D. S. Radiosensitization by gemcitabine in p53 wild-type and mutant MCF-7 breast carcinoma cell lines. Clin. Cancer Res., 7, 2581–2589, 2001.
137. Gregoire, V., Rosier, J. F., De Bast, M., et al. Role of deoxycytidine kinase (dCK) activity in gemcitabine's radioenhancement in mice and human cell lines in vitro. Radiother. Oncol., 63, 329–338, 2002.
138. Gregoire, V., Beauduin, M., Bruniaux, M., DeCoster, B., Octave Prignot, M., and Scalliet, P. Radiosensitization of mouse sarcoma cells by fludarabine (F-ara-A) or gemcitabine (dFdC), two nucleoside analogues, is not mediated by an increased induction or a repair inhibition of DNA double-strand breaks as measured by pulsed-field gel electrophoresis. Int. J. Radiat. Biol., 73, 511–520, 1998.
139. Rosier, J. F., Michaux, L., Ameye, G., et al. The radioenhancement of two human head and neck squamous cell carcinomas by 2′-2′ difluorodeoxycytidine (gemcitabine; dFdC) is mediated by an increase in radiation-induced residual chromosome aberrations but not residual DNA DSBs. Mut. Res. Fundam. Mol. Mech. Mutagen., 527, 15–26, 2003.
140. van Putten, J. W. G., Groen, H. J. M., Smid, K., Peters, G. J., and Kampinga, H. H. End-joining deficiency and radiosensitization induced by gemcitabine. Cancer Res., 61, 1585–1591, 2001.
141. Wachters, F. M., van Putten, J. W. G., Maring, J. G., Zdzienicka, M. Z., Groen, H. J. M., and Kampinga, H. H. Selective targeting of homologous DNA recombination repair by gemcitabine. Int. J. Radiat. Oncol. Biol. Phys., 57, 553–562, 2003.
142. Kunz, B. A. Genetic effects of deoxyribonucleotide imbalances. Environ. Mutagen., 4, 695–725, 1982.
143. Bebenek, K., Roberts, J. D., and Kunkel, T. A. The effects of dNTP pool imbalances on frameshift fidelity during DNA replication. J. Biol. Chem., 267, 3589–3596, 1992.
144. Martomo, S. A., and Mathews, C. K. Effects of biological DNA precursor pool asymmetry upon accuracy of DNA replication in vitro. Mutat. Res. Fundam. Mol. Mech. Mutagen., 499, 197–211, 2002.

145. Koi, M., Umar, A., Chauhan, D. P., et al. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res., 54, 4308–4312, 1994.
146. Jacob, S., Aguado, M., Fallik, D., and Praz, F. The role of the DNA mismatch repair system in the cytotoxicity of the topoisomerase inhibitors camptothecin and etoposide to human colorectal cancer cells. Cancer Res., 61, 6555–6562, 2001.
147. Robinson, B. W., Im, M. I., Ljungman, M., Praz, F., and Shewach, D. S. Enhanced radiosensitization with gemcitabine in mismatch repair-deficient HCT-116 cells. Cancer Res., 63, 6935–6941, 2003.
148. Lawrence, T. S., Davis, M. A., Hough, A., and Rehemtulla, A. The role of apoptosis in 2′,2′-difluoro-2′-deoxycytidine (gemcitabine)-mediated radiosensitization. Clin. Cancer Res., 7, 314–319, 2001.
149. Eisbruch, A., Shewach, D. S., Bradford, C. R., et al. Radiation concurrent with gemcitabine for locally advanced head and neck cancer: a phase I trial and intracellular drug incorporation study. J. Clin. Oncol., 19, 792–799, 2001.
150. Eisbruch, A., Lyden, T., Bradford, C. R., et al. Objective assessment of swallowing dysfunction and aspiration after radiation concurrent with chemotherapy for head-and-neck cancer. Int. J. Radiat. Oncol. Biol. Phys., 53, 23–28, 2002.
151. Fields, M. T., Eisbruch, A., Normolle, D., et al. Radiosensitization produced in vivo by once- vs. twice-weekly 2′,2′-difluoro-2′-deoxycytidine (gemcitabine). Int. J. Radiat. Oncol. Biol. Phys., 47, 785–791, 2000.
152. Blackstock, A. W., Bernard, S. A., Richards, F., et al. Phase I trial of twice-weekly gemcitabine and concurrent radiation in patients with advanced pancreatic cancer. J. Clin. Oncol., 17, 2208–2212, 1999.
153. Pipas, J. M., Mitchell, S. E., Barth, R. J., et al. Phase I study of twice-weekly gemcitabine and concomitant external-beam radiotherapy in patients with a denocarcinoma of the pancreas. Int. J. Radiat. Oncol. Biol. Phys., 50, 1317–1322, 2001.
154. McGinn, C. J., Zalupski, M., Shureiqi, I., et al. Phase I trial of radiation dose escalation with concurrent weekly full-dose gemcitabine in patients with advanced pancreatic cancer. J. Clin. Oncol., 19, 4202–4208, 2001.
155. Mason, K. A., Milas, L., Hunter, N. R., et al. Maximizing therapeutic gain with gemcitabine and fractionated radiation. Int. J. Radiat. Oncol. Biol. Phys., 44, 1125–1135, 1999.
156. Milas, L., Fujii, T., Hunter, N., et al. Enhancement of tumor radioresponse in vivo by gemcitabine. Cancer Res., 59, 104–114, 1999.
157. Gregoire, V., Beauduin, M., Rosier, J. F., et al. Kinetics of mouse jejunum radiosensitization by 2′,2′-difluorodeoxycytidine (gemcitabine) and its relationship with pharmacodynamics of DNA synthesis inhibition and cell cycle redistribution in crypt cells. Br. J. Cancer, 76, 1315–1321, 1997.
158. Wolff, R. A., Evans, D. B., Gravel, D. M., et al. Phase I trial of gemcitabine combined with radiation for the treatment of locally advanced pancreatic adenocarcinoma. Clin. Cancer Res., 7, 2246–2253, 2001.
159. Lange, S. M., van Groeningen, C. J., Meijer, O. W. M., et al. Gemcitabine-radiotherapy in patients with locally advanced pancreatic cancer. Eur. J. Cancer, 38, 1212–1217, 2002.
160. Talamonti, M. S., Catalano, P. J., Vaughn, D. J., et al. Eastern Cooperative Oncology Group phase I trial of protracted venous infusion fluorouracil plus weekly gemcitabine with concurrent radiation therapy in patients with locally

advanced pancreas cancer: a regimen with unexpected early toxicity. J. Clin. Oncol., 18, 3384–3389, 2000.
161. Muler, J. H., McGinn, C. J., Normolle, D., et al. Phase I trial using a time-to-event continual reassessment strategy for dose escalation of cisplatin combined with gemcitabine and radiation therapy in pancreatic cancer. J. Clin. Oncol., 22, 238–243, 2004.
162. Trodella, L., Granone, P., Valente, S., et al. Phase I trial of weekly gemcitabine and concurrent radiotherapy in patients with inoperable non-small-cell lung cancer. J. Clin. Oncol., 20, 804–810, 2002.
163. Blackstock, A. W., Lesser, G. J., Fletcher-Steede, J., et al. Phase I study of twice-weekly gemcitabine and concurrent thoracic radiation for patients with locally advanced non-small-cell lung cancer. Int. J. Radiat. Oncol. Biol. Phys., 51, 1281–1289, 2001.
164. Goor, C., Scalliet, P., van Meerbeek, J., et al. A phase II study combining gemcitabine with radiotherapy in stage III NSCLC. Ann. Oncol., 7, 101, 1996.
165. McGinn, C. J., and Zalupski, M. M. Radiation therapy with once-weekly gemcitabine in pancreatic cancer: current status of clinical trials. Int. J. Radiat. Oncol. Biol. Phys., 56, 10–15, 2003.
166. Yavuz, A. A., Aydin, F., Yavuz, M. N., Ilis, E., and Ozdemir, F. Radiation therapy and concurrent fixed dose amifostine with escalating doses of twice-weekly gemcitabine advanced pancreatic cancer. Int. J. Radiat. Oncol. Biol. Phys., 51, 974–981, 2001.
167. Joschko, M. A., Webster, L. K., Groves, J., et al. Enhancement of radiation-induced regrowth delay by gemcitabine in a human tumor xenograft model. Radiat. Oncol. Invest., 5, 62–71, 1997.
168. Gandhi, V., and Plunkett, W. Cellular and clinical pharmacology of fludarabine. Clin. Pharmacokinet., 41, 93–103, 2002.
169. Iliakis, G., and Bryant, P. E. Effects of the nucleoside analogs α-ara-A, β-ara-A and β-ara-C on cell-growth and repair of both potentially lethal damage and DNA double strand breaks in mammalian-cells in culture. Anticancer Res., 3, 143–149, 1983.
170. Mustafi, R., Heaton, D., Brinkman, W., and Schwartz, J. L. Enhancement of X-ray toxicity in squamous-cell carcinoma cell-lines by DNA-polymerase inhibitors. Int. J. Radiat. Biol., 65, 675–681, 1994.
171. Kim, J. H., Alfieri, A. A., Kim, S. H., and Fuks, Z. The potentiation of radiation response on murine tumor by fludarabine phosphate. Cancer Lett., 31, 69–76, 1986.
172. Tseng, W. C., Derse, D., Cheng, Y., Brockman, R. W., and Bennett, L. L. In vitro biological activity of 9-β-D-arabinofuranosyl-2-fluoroadenine and the biochemical actions of its triphosphate on DNA polymerases and ribonucleotide reductase from HeLa cells. Mol. Pharmacol., 21, 474–477, 1982.
173. Parker, W. B., and Cheng, Y. C. Inhibition of DNA primase by nucleoside triphosphates and their arabinofuranosyl analogs. Mol. Pharmacol., 31, 146–151, 1987.
174. Catapano, C. V., Perrino, F. W., and Fernandes, D. J. Primer RNA chain termination induced by 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate—a mechanism of DNA synthesis inhibition. J. Biol. Chem., 268, 7179–7185, 1993.
175. Yang, S. W., Huang, P., Plunkett, W., Becker, F. F., and Chan, J. Y. H. Dual mode of inhibition of purified DNA ligase I from human cells by 9-β-D-arabinofuranosyl-2-fluoroadenine triphosphate. J. Biol. Chem., 267, 2345–2349, 1992.
176. Chang, C.-H., and Cheng, Y. Effects of deoxyadenosine triphosphate and 9-β-D-arabinofuranosyladenine 5′-triphosphate on human ribonucleotide reductase

from Molt-4F cells and the concept of "self-potentiation." Cancer Res., 40, 3555–3558, 1980.
177. White, E. L., Shaddix, S. C., Brockman, R. W., and Bennett, L. L. Comparison of the actions of 9-β-D-arabinofuranosyl-2-fluoroadenine and 9-β-D-arabinofuranosyladenine on target enzymes from mouse tumor cells. Cancer Res., 42, 2260–2264, 1982.
178. Huang, P., Chubb, S., and Plunkett, W. Termination of DNA synthesis by 9-β-D-arabinofuranosyl-2-fluoroadenine. A mechanism for cytotoxicity. J. Biol. Chem., 265, 16,617–16,625, 1990.
179. Huang, P., and Plunkett, W. Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. Cancer Chemother. Pharmacol., 36, 181–188, 1995.
180. Gregoire, V., Hunter, N., Brock, W. A., Milas, L., Plunkett, W., and Hittelman, W. N. Fludarabine improves the therapeutic ratio of radiotherapy in mouse-tumors after single-dose irradiation. Int. J. Radiat. Oncol. Biol. Phys., 30, 363–371, 1994.
181. Gregoire, V., Hunter, N., Milas, L., Brock, W. A., Plunkett, W., and Hittelman, W. N. Potentiation of radiation-induced regrowth delay in murine tumors by fludarabine. Cancer Res., 54, 468–474, 1994.
182. Gregoire, V., Van, N. T., Stephens, C., et al. The role of fludarabine-induced apoptosis and cell cycle synchronization in enhanced murine tumor radiation response in vivo. Cancer Res., 54, 6201–6209, 1994.
183. Li, L., Liu, X. M., Glassman, A. B., et al. Fludarabine triphosphate inhibits nucleotide excision repair of cisplatin-induced DNA adducts in vitro. Cancer Res., 57, 1487–1494, 1997.
184. Yamauchi, T., Nowak, B. J., Keating, M. J., and Plunkett, W. DNA repair initiated in chronic lymphocytic leukemia lymphocytes by 4-hydroperoxycyclophosphamide is inhibited by fludarabine and clofarabine. Clin. Cancer Res., 7, 3580–3589, 2001.
185. Laurent, D., Pradier, O., Schmidberger, H., Rave-Frank, M., Frankenberg, D., and Hess, C. F. Radiation rendered more cytotoxic by fludarabine monophosphate in a human oropharynx carcinoma cell line than in fetal lung fibroblasts. J. Cancer Res. Clin. Oncol., 124, 485–492, 1998.
186. Gregoire, V., Ruifrok, A. C. C., Price, R. E., et al. Effect of intra-peritoneal fludarabine on rat spinal-cord tolerance to fractionated-irradiation. Radiother. Oncol., 36, 50–55, 1995.
187. Gregoire, V., Ang, K. K., Rosier, J. F., et al. A phase I study of fludarabine combined with radiotherapy in patients with intermediate to locally advanced head and neck squamous cell carcinoma. Radiother. Oncol., 63, 187–193, 2002.
188. McCarthy, J. R., Matthews, D. P., Stemerick, D. M., et al. Stereospecific method to E-terminal and Z-terminal fluoro olefins and its application to the synthesis of 2′-deoxy-2′-fluoromethylene nucleosides as potential inhibitors of ribonucleoside diphosphate reductase. J. Am. Chem. Soc., 113, 7439–7440, 1991.
189. Takahashi, T., Nakashima, A., Kanazawa, J. J., et al. Metabolism and ribonucleotide reductase inhibition of (E)-2 ′-deoxy-2 ′-(fluoromethylene)cytidine, MDL 101,731 in human cervical carcinoma HeLa S-3 cells. Cancer Chemother. Pharmacol., 41, 268–274, 1998.
190. van der Donk, W. A., Yu, G., Silva, D. J., and Stubbe, J. Inactivation of ribonucleotide reductase by (E)-2′-fluoromethylene-2′-deoxycytidine 5′-diphosphate: a paradigm for nucleotide mechanism-based inhibitors. Biochem., 35, 8381–8391, 1996.

191. Zhou, Y., Achanta, G., Pelicano, H., Gandhi, V., Plunkett, W., and Huang, P. Action of (E)-2′-deoxy-2′-(fluoromethylene)cytidine on DNA metabolism: incorporation, excision, and cellular response. Mol. Pharmacol., 61, 222–229, 2002.
192. Bitonti, A. J., Dumont, J. A., Bush, T. L., et al. Regression of human breast tumor xenografts in response to (E)-2′-deoxy-2′-(fluoromethylene)cytidine, and inhibitor of ribonucleoside diphosphate reductase. Cancer Res., 54, 1485–1490, 1994.
193. Piepmeier, J. M., Rabidou, N., Schold, S. C., Bitonti, A. J., Prakash, N. J., and Bush, T. L. In vitro and in vivo inhibition of glioblastoma and neuroblastoma with MDL 101,731, a ribonucleoside diphosphate reductase inhibitor. Cancer Res., 56, 359–361, 1996.
194. Miwa, M., Eda, H., Ura, M., et al. High susceptibility of human cancer xenografts with higher levels of cytidine deaminase to a 2 ′-deoxycytidine antimetabolite, 2′-deoxy-2 ′-methylidenecytidine. Clin. Cancer Res., 4, 493–497, 1998.
195. Snyder, R. D. Effect of 2′-deoxy-2′-(fluoromethylene) cytidine on the ultraviolet and x-ray-sensitivity of HeLa-cells. Oncol. Res., 6, 177–182, 1994.
196. Coucke, P. A., Decosterd, L. A., Li, Y. X., et al. The ribonucleoside diphosphate reductase inhibitor (E)-2′-deoxy(fluoromethylene)cytidine as a cytotoxic radiosensitizer in vitro. Cancer Res., 59, 5219–5226, 1999.
197. Li, Y. X., Sun, L. Q., Weber-Johnson, K., Paschoud, N., and Coucke, P. A. Potentiation of cytotoxicity and radiosensitization of (E)-2-deoxy-2′-(fluoromethylene) cytidine by pentoxifylline in vitro. Int. J. Cancer, 80, 155–160, 1999.
198. Sun, L.-Q., Li, Y.-X., Guillou, L., and Coucke, P. A. (E)-2′-Deoxy-2′-(fluoromethylene) cytidine potentiates radioresponse of two human solid tumor xenografts. Cancer Res., 58, 5411–5417, 1998.
199. Rodriguez, G. I., Jones, R. E., Orenberg, E. K., Stoltz, M. L., and Brooks, D. J. Phase I clinical trials of tezacitabine [(E)-2 ′-deoxy-2 ′-(fluoromethylene)cytidine] in patients with refractory solid tumors. Clin. Cancer Res., 8, 2828–2834, 2002.
200. Masuda, N., Negoro, S., Takeda, K., et al. Phase I and pharmacologic study of oral (E)-2′-deoxy-2′-(fluoromethylene) cytidine: on a daily × 5-d schedule. Invest. New Drugs, 16, 245–254, 1998.
201. Eda, H., Ura, M., Ouchi, K. F., Tanaka, Y., Miwa, M., and Ishitsuka, H. The antiproliferative activity of DMDC is modulated by inhibition of cytidine deaminase. Cancer Res., 58, 1165–1169, 1998.
202. Reichard, P. Interactions between deoxyribonucleotide and DNA synthesis. Annu. Rev. Biochem., 57, 349–374, 1988.
203. Yarbro, J. W. Mechanism of action of hydroxyurea. Semin. Oncol., 19, 1–10, 1992.
204. Donehower, R. C. An overview of the clinical experience with hydroxyurea. Semin. Oncol., 19, 11–19, 1992.
205. Nocentini, G. Ribonucleotide reductase inhibitors: new strategies for cancer chemotherapy. Crit. Rev. Oncol./Hematol,, 22, 89–126, 1996.
206. Lori, F., Malykh, A., Cara, A., et al. Hydroxyurea as an inhibitor of human immunodeficiency virus-type 1 replication. Science, 266, 801–805, 1994.
207. Stubbe, J. Ribonucleotide reductases. Adv. Enzymol., 63, 349–419, 1990.
208. Jordan, A., and Reichard, P. Ribonucleotide reductases. Annu. Rev. Biochem., 67, 71–98, 1998.
209. Fox, R. M. Changes in deoxynucleoside triphosphate pools induced by inhibitors and modulators of ribonucleotide reductase. Pharmacol. Ther., 30, 31–42, 1985.

210. Arner, E. S. J., and Eriksson, S. Mammalian deoxyribonucleoside kinases. Pharmacol. Ther., 67, 155–186, 1995.
211. Moore, E. C., and Hurlbert, R. B. Regulation of mammalian deoxyribonucleotide biosynthesis by nucleotides as activators and inhibitors. J. Biol. Chem., 241, 4802–4809, 1966.
212. Nutter, L. M., and Cheng, Y. C. Nature and properties of mammalian ribonucleoside diphosphate reductase. Pharmacol. Ther., 26, 191–207, 1984.
213. Gandhi, V., Kantarjian, H., Talpaz, M., Robertson, L. E., and O'Brien, S. Cellular pharmacodynamics and plasma pharmacokinetics of parenterally infused hydroxyurea during a phase I clinical trial in chronic myelogenous leukemia. J. Clin. Oncol., 16, 2321–2331, 1998.
214. Giles, F. J., Fracasso, P. M., Kantarjian, H. M., et al. Phase I and pharmacodynamic study of Triapine, a novel ribonucleotide reductase inhibitor, in patients with advanced leukemia. Leuk. Res., 27, 1077–1083, 2003.
215. Sinclair, W. K. Hydroxyurea—differential lethal effects on cultured mammalian cells during cell cycle. Science, 150, 1729-&, 1965.
216. Sinclair, W. K. The combined effect of hydroxyurea and x-rays on Chinese hamster cells in vitro. Cancer Res., 28, 198–206, 1968.
217. Sinclair, W. K. X-ray survival and DNA synthesis in Chinese hamster cells. I. The effect of inhibitors added before x-irradiation. Proc. Natl. Acad. Sci. U. S. A, 58, 115–122, 1967.
218. Ward, J. F., Joner, E. I., and Blakely, W. F. Effects of inhibitors of DNA strand break repair on HeLa cell radiosensitivity. Cancer Res., 44, 59–63, 1984.
219. Fram, R. J., and Kufe, D. W. Inhibition of DNA excision repair and the repair of x-ray-induced DNA damage by cytosine arabinoside and hydroxyurea. Pharmacol. Ther., 31, 165–176, 1985.
220. Kuo, M.-L., Kunugi, K. A., Lindstrom, M. J., and Kinsella, T. J. The interaction of hydroxyurea and iododeoxyuridine on the radiosensitivity of human bladder cancer cells. Cancer Res., 55, 2800–2805, 1995.
221. Kinsella, T. J. Radiosensitization and cell kinetics: clinical implications for S-phase-specific radiosensitizers. Semin. Oncol., 19, 41–47, 1992.
222. Hreshchyshyn, M. M., Aron, B. S., Boronow, R. C., et al. Hydroxyurea or placebo combined with radiation to treat stages IIIB and IV cervical cancer confined to the pelvis. Int. J. Radiat. Oncol. Biol. Phys., 5, 317–322, 1979.
223. Piver, M., Khalil, M., and Emrich, L. J. Hydroxyurea plus pelvic irradiation vs placebo plus pelvic irradiation in nonsurgically staged stage IIIB cervical cancer. J. Surg. Oncol., 42, 120–125, 1989.
224. Prados, M. D., Larson, D. A., Lamborn, K., et al. Radiation therapy and hydroxyurea followed by the combination of 6-thioguanine and BCNU for the treatment of primary malignant brain tumors. Int. J. Radiat. Oncol. Biol. Phys., 40, 57–63, 1998.
225. Beitler, J. J., Anderson, P., Haynes, H., et al. Phase I clinical trial of parenteral hydroxyurea in combination with pelvic and para-aortic external radiation and brachytherapy for patients with advanced squamous cell cancer of the uterine cervix. Int. J. Radiat. Oncol. Biol. Phys., 52, 637–642, 2002.
226. Argiris, A., Haraf, D. J., Kies, M. S., and Vokes, E. E. Intensive concurrent chemoradiotherapy for head and neck cancer with 5-fluorouracil- and hydroxyurea-based regimens: reversing a pattern of failure. Oncologist, 8, 350–360, 2003.

227. Beitler, J. J., Anderson, P., Haynes, H., et al. Phase I clinical trial of parenteral hydroxyurea in combination with pelvic and para-aortic external radiation and brachytherapy for patients with advanced squamous cell cancer of the uterine cervix. Int. J. Radiat. Oncol. Biol. Phys., 52, 637–642, 2002.
228. Wadler, S., Horowitz, R., Rao, J., Mao, X., Schlesinger, K., and Schwartz, E. L. Interferon augments the cytotoxicity of hydroxyurea without enhancing its activity against the M2 subunit of ribonucleotide reductase: effects in wild-type and resistant human colon cancer cells. Cancer Chemother. Pharmacol., 38, 522–528, 1996.
229. Robinson, B. W., and Shewach, D. S. Gemcitabine enhances the mutation rate in mismatch repair deficient but not in mismatch repair proficient HCT116 cells. Proc. Am. Assoc. Cancer Res., 44, 1173, 2003.

14 NONMEM Population Models of Cytosine Arabinoside and Fludarabine Phosphate in Pediatric Patients With Leukemia

Vassilios I. Avramis, PhD

CONTENTS

SUMMARY

Computer software *NONMEM* for nonlinear mixed-effects modeling was used to determine the population pharmacokinetics (PPK) and pharmacodynamics (PPD) of intravenously administered nucleoside analogs cytosine arabinoside (ara-C) and fludarabine phosphate (F-ara-A) in pediatric

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

patients with leukemias. A description of the major *NONMEM* tasks as well as the statistical models has been given for these drugs. Two pharmacokinetic-pharmacodynamic (PK-PD) models have been developed for ara-C and one for F-ara-A. These *NONMEM* population analyses present a novel approach for determining the PK parameters in many patients; these parameters are superimposable to the ones derived from the classical two-stage PK approach. However, by minimizing errors, population analyses explained the significant variabilities in the elimination of these drugs from the central compartment and the variable high peak plasma concentrations in younger leukemia patients. Moreover, the influence of the covariate product (TR *AGE* × *SA*) demonstrated an increase in the PK parameter values with increasing patient age (*AGE*) and surface area (*SA*). The evaluation of the intercompartmental clearance of ara-C demonstrated statistically significant linear relationships with the covariate product (*AGE* × *SA)*. These results explained the lower drug plasma concentration in the older children as compared to the infant and younger patients with leukemias. In addition to the classical population pharmacokinetic and population pharmacodynamic parameters of half-lives of elimination, clearance, and volumes of distribution, these analyses in pediatric patients determined the effects of deamination by cytidine deaminase on the clearance of ara-C from the circulation. Moreover, the K_m of activation of these drugs to their respective phosporylated anabolites in patients, and under treatment conditions, was determined by the Michaelis-Menten equation, which was fused into the INPUT PK-PD subroutine. Overall, *NONMEM* population PK-PD analyses are complicated and time consuming and require a rich database from many patients. However, they produce the best possible PK-PD analyses for a drug and its metabolites, the parameters of which give better understanding of the biochemical pathways in plasma and in leukemic blasts simultaneously, which can then be applied to many other patients in future clinical trials.

Key Words: Acute lymphoblastic leukemia, ALL; apparent volume of distribution, V_d; cytosine arabinoside, ara-C; fludarabine phosphate, F-ara-A; interindividual variance, ω^2; nonlinear mixed-effects modeling, *NONMEM*; total body clearance, CL; value of minimum objective function, OBF; vector of intraindividual effects, ε_{ij}; vector of random interindividual effects, η_i.

1. INTRODUCTION

NONMEM software was produced by researchers in the Schools of Medicine and Pharmacy of the University of California at San Francisco (UCSF) and is marketed by GloboMax (Hanover, MD). The project is concerned with the development of data analysis techniques and exportable software for fitting nonlinear mixed-effects models (NONMEM). The data analyses may be described by regression models with mixed effects (i.e., both fixed and random effects, any of which may enter the model nonlinearly). Data of this sort arise frequently in clinical pharmacology projects, among other scientific fields. These techniques are particularly useful when

the data are population pharmacokinetic/pharmacodynamic (PK/PD) origin data, when there are only a few PK/PD measurements from some individuals sampled from the population, or when the regression design varies considerably between individual subjects. This project is a continuously evolving one, with the first version released in 1979 and version III released in 1989 and written in ANSI FORTRAN 77.

Currently, the UCSF *NONMEM* version V is licensed and distributed by GloboMax® LLC (Hanover, MD). The inputs to the program consist of data files, control information, and user-coded subroutines. The program (version V) consists of three parts. The *NONMEM* program itself is a very general (non-interactive) regression program that can be used to fit many different types of data. *PREDPP* is a powerful package of subroutines that can be used by *NONMEM* to compute predictions for population PK data. Last, *NM-TRAN* is a non-interactive pre-processor that allows control and other needed inputs to *NONMEM/PREDPP* to be specified in a user-friendly manner, with quick and comprehensive detection of a variety of errors that may have been introduced by the user in the data file. The *NM-TRAN* package allows the user to select the desirable PK model by selecting the appropriate model in the *ADVAN/TRANS* subroutines of *PREDPP* as indicated by the two-stage PK analysis (STS) of the PK data. *See* the appendix for guidelines to get started.

2. MAJOR *NONMEM* TASKS

There are six major *NONMEM* tasks that may be undertaken in solving any given PD data problem. In the first step, the simulation step, data are simulated under the user-specified PK model. The key advantage of this pharmacokinetic approach is that the large PK data are examined as if they were from one source, even though later the program allows the user to examine the individual patient's data as they "fit" into the population.

In the second step, the initial estimation step, initial estimates of model parameters are computed. Initial estimates may also be specified and the estimates of the fixed-effect parameters may be constrained by the user, and this is often highly recommended.

In the third step, the estimation step, final estimates of the model parameters—fixed-effect parameters and variance-covariance components—are obtained. For this purpose, an objective function (k_{el}, V_d, clearance *CL*, etc.) in the model parameters is minimized, and the final estimate is taken to be the minimum point (as a vector).

In the covariance step, an estimate for the covariance matrix of the estimate obtained in the estimation step is computed. The accuracy of this covariate estimate increases as the number of the statistically independent observations increases. The original impetus of the *NONMEM* project was

to develop an ability to analyze large quantities of PK data arising from routine patient care. Both intra- and inter-individual variabilities can be described by the "constant coefficient of variation" (CCV) model because the frequency distributions of the individual parameters are skewed (non-normal distribution).

Routinely, the improvement of the fit of the PK data, as a result of the incorporation of covariates in the regression model, is determined based on the value of the "minimum objective function" (Likelihood Ratio Test), the magnitude of the standard errors of the parameter estimates, the plots of weighted residuals against the predicted PK drug concentrations, and decreases in the estimates of interindividual variabilities. The difference in the values of the objective function (Log Likelihood Difference) of a reduced model with $n - 1$ parameters and a full model (n parameters) can be approximated by the chi-square (χ^2) distribution with q degrees of freedom, where q is the number of parameters with values that are fixed in the reduced $(n - 1)$ model.

In the fifth step, the Tables step, all data items of selected parameters are tabulated. Last, in the sixth step, the Scatterplot step, the actual dependent variable data (i.e., PK drug concentrations) can be scatterplotted against the predicted data or any other type of data.

3. POPULATION PHARMACOKINETIC ANALYSIS OF CYTOSINE ARABINOSIDE

A population PK analysis of cytosine arabinoside (ara-C) has been performed utilizing the computer program *NONMEM* by the Project Group of UCSF. The NONMEM analysis of nucleoside analog drugs has been accomplished in other studies by using the *PREDPP* and *NM-TRAN* packages of *NONMEM*, version IV, level 2, in this laboratory. Similarly, population PK of fludarabine phosphate has been done in pediatric patients with acute lymphoblastic leukemia (ALL). These analyses have yielded important information on the pharmacology of these drugs, where classical PK analyses (STS) have been shown to be inadequate to describe the significant variability among patients. This methodology has been used retrospectively to analyze all of the pediatric patient data concerning ara-C and its catabolite uracil arabinoside (ara-U) data *(1)*.

The main advantage of *NONMEM* analysis is that it considers data from all patients as one set, and the population PK parameter estimates are obtained, as stated in this section, using a nonlinear mixed (fixed and random) effects regression model. In addition, *NONMEM* analysis requires a few data points per individual patient, and it correlates covariates such as age, weight, surface area, liver function, and creatinine levels with PK parameters.

Specifically, the plasma concentrations of the drug are described by the equation:

$$C_{ij} = F_{ij}(\theta, x_{ij}) + G_{ij}(\theta, x_{ij})\eta_i + H_{ij}(\theta, x_{ij})\varepsilon_{ij} \quad (1)$$

where, F_{ij} is the structural PK model that predicts plasma drug concentrations for each time point j of every patient i, θ is the vector of fixed effects such as dose and sampling times, x_{ij} is the vector of all values of fixed effects associated with a particular observation *Yij*, *Yij* represents the value of the *j*th observation for an individual subject i, η_i is the vector of random interindividual effects, ε_{ij} is the vector of intraindividual effects, and G_{ij} and H_{ij} are matrices of partial derivatives of F with respect to η_i and ε_{ij}, respectively, evaluated at their expected values. The parameters η_i and ε_{ij} are assumed to be randomly distributed with expected values $E(\eta_i) = 0$ and $E(\varepsilon_{ij}) = 0$, respectively.

The data have been analyzed by the one- or two-compartment open model to determine the parameters that describe the data best.

In the event of intramuscular or subcutaneous administration of the drug, all further population PK analyses were performed using the *ADVAN2*, *TRANS2* subroutines of the *NONMEM-PREDPP* load module as if they included absorption of the drug from the injection site.

For drugs administered orally or intramuscularly, the structural PK model F will be described by the equation

$$F = \frac{F_1 X_0 k_a (e^{-(CL/Vd)t} - e^{-kat})}{(V_d k_a - CL)} \quad (2)$$

where, K_a is the absorption rate constant, F_1 is the average relative drug bioavailability of drug after oral, intramuscular, or subcutaneous administration compared to intravenous administration, CL is the systemic clearance, and V_d is the apparent volume of distribution of the drug.

If the PK parameter distribution of the drug is skewed (usually they are), then the inter-individual variability of the PK parameters will be described by the following equations:

$$K_{a,i} = k_{a,pop}(1 + \eta_{ka,i}) \quad (3)$$

$$CL_i = CL_{pop}\,(1 + \eta_{CL,I}) \quad (4)$$

$$V_{d,i} = V_{d,pop}(1 + \eta_{Vdi}) \quad (5)$$

Intraindividual variability may also be described by the CCV model as shown in Eq. 6.

$$\text{Observed } C_{ij} = \text{Predicted } C_{ij}(1 + \varepsilon_{ij}) \quad (6)$$

4. CYTOSINE ARABINOSIDE

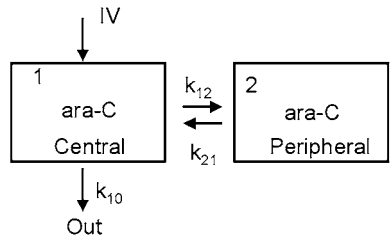
Ara-C is considered one of the most active antimetabolites for inducing complete remissions (CR) in acute myelogenous leukemia (AML) and in ALL in adult and pediatric patients *(2–5)*. In combination with other antineoplastic drugs, such as purine nucleoside analogs, anthracyclines, asparaginases, ara-C treatment leads to CR in the majority (60–70%) of patients. Ara-C is a pro-drug and must be activated intracellularly to its triphosphate anabolite to exert its cytotoxic effect. Only a small portion of the administered drug is converted intracellularly to its active anabolite, cytosine arabinoside triphosphate (ara-CTP). The majority of the dose is deaminated to ara-U in plasma or blood, a reaction that is catalyzed by cytidine deaminase (Cyt DA). Numerous inhibitors of Cyt DA have been tested with limited success in efforts to enhance the parent drug's activity against leukemias.

Pharmacokinetic (PK) studies in pediatric patients with AML or ALL have shown that ara-C is rapidly eliminated from plasma, obeying a two-compartment open model after low- or high-dose ara-C (HDara-C). The average peak plasma concentration is much higher in pediatric patients than in adult patients. The plasma concentrations of ara-U are five- to eightfold higher than ara-C, and ara-U is eliminated mono-exponentially from the central circulation with a faster half-life than that of ara-C. However, the in vivo relationship between ara-C and its metabolite ara-U has not been made clear in either pediatric or adult patients. It is known that infant patients with ALL tend to accumulate much higher concentrations of ara-C than older children, primarily because of the reduced deamination capacity in the infant organism. The average ratio of ara-U to ara-C in infant patients is usually 1:2, indicating that their capacity to deaminate or eliminate the parent drug is limited, and that its clearance is reduced significantly.

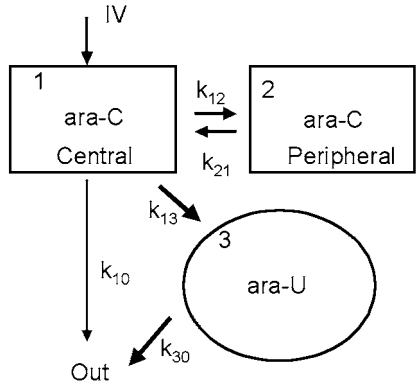
5. *NONMEM* PK MODEL CHARACTERISTICS

The ara-C and ara-U PK data were obtained from four separate clinical studies using HDara-C (3 g/m^2 over 3 h, every 12 h times four and repeat on day 8). In one clinical study, ara-C was administered as a loading bolus (LB) followed immediately by a continuous infusion (CI) of the drug (LB plus CI). Quite a few patients were treated with both the HDara-C and the LB plus CI infusion regimens at various times of their leukemia treatment. Therefore, we had a considerable number of patients from these four clinical trials who had been treated repeatedly with the drug *(1)*.

The two-compartment open model with first-order elimination was chosen as the best PK model to describe adequately the kinetics of ara-C in the pediatric patient population. However, a third compartment was added to represent ara-U in the mammalian body. This model simultaneously described the combined PK models of the parent drug ara-C and its metabolite ara-U. The



Schema I. PK model I.



Schema II. PK model II.

ara-C PK data were best modeled by a two-compartment open model, and simultaneously, ara-U PK data were modeled by the one-compartment open model I.

For model 1, the concentration–time PK data of ara-C were best described, pharmacokinetically, by a two-compartment open model with first order of elimination (Schema I). The *ADVAN3/TRANS4* subroutines of the *PREDPP/NONMEM* program were used for the best-fit population model.

Model 2 allowed for simultaneous description of the PK of ara-C and its metabolite ara-U by a two-compartment open model with first order of elimination for the parent drug while those of ara-U were analyzed by a one-compartment open model with first order of elimination of ara-U from the central compartment (Schema II). The metabolic conversion of ara-C to ara-U was modeled as a first-order kinetic process despite the fact that ara-C is converted to ara-U by the mammalian enzyme Cyt DA. This simplification was permitted by the final solution of this complex PK model because there are vast amounts of Cyt DA in the body of patients, with the exception those children younger than 12 months, in whom this enzyme is of limited capacity or availability.

The *CL* of ara-C from the central compartment (k_{total} rate constant) was described by two processes: (1) deamination of ara-C to ara-U (k_{13}) and (2) renal elimination of the parent drug (ara-C) from the central compartment (k_{10}). The following differential equations describing the complete PK model were entered in the *ADVAN6* subroutine of *NONMEM*:

$$dA(1)/dt = \quad k_{12}A(1) + k_{21}A(2) \quad k_{10}A(1) \quad k_{13}A(1) \tag{7}$$

$$dA(2)/dt = k_{12}A(1) \quad k_{21}A(2) \tag{8}$$

$$dA(3)/dt = k_{13}A(1) \quad k_{30}A(3) \tag{9}$$

where, $A(1)$ is the amount of ara-C in compartment 1, $A(2)$ is the amount of ara-C in compartment 2, and $A(3)$ is the amount of the metabolite ara-U in compartment 3. The microconstants, k_{12}, k_{21}, k_{13}, k_{10}, and k_{30} are the first-order rate constants of transfer or elimination of the parent drug ara-C and its catabolite ara-U within the various compartments of the PK model.

6. STATISTICAL MODELS FOR ara-C AND ara-U NONMEM POPULATION ANALYSES

The interindividual variability in PK parameters was expressed as the variance ω^2 of the random error η_i with a mean value of zero, and it was described by the constant coefficient of variation (CCV) model. This is the most appropriate statistical model for skewed parameter distributions. Therefore, an individual patient's clearance was described by the equation

$$CL_i = CL(1 + \eta_{CL,i}) \tag{10}$$

where, CL_i represents the clearance of ara-C in patient *i*, *CL* is the average patient population value of ara-C clearance, and $\eta_{CL,i}$ represents the deviation of the individual's ara-C clearance from the average population value of this parameter.

Intraindividual variability of ara-C in models depicted in Schemas I and II was expressed as the variance σ^2 of random effects ε_{ij} and was described by a combined additive and CCV error model:

$$C_{ij} = f + f\varepsilon_{1,ij} + \varepsilon_{2,ij} \tag{11}$$

where, C_{ij} is the ara-C plasma concentration of patient i at time *j*, *f* is the structural PK model, $\varepsilon_{1,ij}$ is the residual error for the CCV portion of the model with variance σ_1^2, and $\varepsilon_{2,ij}$ is the residual error for the additive portion of the model with variance σ_2^2.

The two variance terms (additive and proportional) describing the intraindividual variability of the ara-C PK data were estimated in the *NONMEM* analyses of the PK model depicted in Schema I. In the model depicted

in Schema II, these two variance terms were fixed to the values obtained from the *NONMEM* analyses of the model in Schema I. This was perceived as necessary to reduce the estimation time, which is considerably longer when the PK models are expressed as differential equations and when the drug concentrations of ara-C and ara-U are analyzed simultaneously. Finally, the intra-individual variability of ara-U PK data was described nicely by the CCV statistical model.

6.1. NONMEM Analyses of ara-C (Model I)

The combined ara-C data obtained after HDara-C and after the LB plus CI modes of administration were analyzed by a stepwise regression model using the two-compartment open model (Schema I) with first-order elimination of the parent drug from the central compartment. Initially, the data were analyzed using the simple regression model (basic PK model) in the absence of covariates. The variance-covariance (OMEGA) matrix of the basic PK model was constrained to be diagonal. This basic model calculated the interindividual variances for all four basic model PK parameters: total body clearance (CL_T), the apparent volumes of distribution of the central (V_{d1}) and peripheral (V_{d2}) compartments, and the intercompartmental clearance (Q). The plot of the predicted (PRED) vs. the observed ara-C plasma concentrations (dependent variable, *DV*) indicated "significant bias" in the fit of the data by this PK model analysis, suggesting the need to include covariates in the regression model that could account for the large interindividual variability of the data.

The regression model was subsequently expanded to include the evaluation of the effects of the covariates *AGE* and surface area (*SA*) of the pediatric patients on the PK model of ara-C. The next best-fit model, which resulted in a significant improvement of the data fit [PRED vs. DV – (1)] and a substantial decrease in the minimum value of the "objective function" was the one in which all four basic PK parameters (*see* above) were expressed as linear functions of the covariate product of estimates ($AGE \times SA$). The final PK parameter estimates were $CL_T = 2.59 \times (AGE \times SA)$ L/h; $Q = 2.01 \times (AGE \times SA)$ L/h; $V_{d1} = 0.48 \times (AGE \times SA)$ L; and $V_{d2} = 38.1 \times (AGE \times SA)$ L. It is imperative to note the distinction in that, routinely, the units of volume of distribution (V_d) are expressed in units of volume, milliliters or liters per square meter of patients. However, in these population PK analyses, the covariate term *SA* has already been considered in the regression model; hence, the units of these V_d estimates are expressed in units of volume only.

These analyses provided the following coefficients of variation (%CV): for CL = 83.8%; for Q = 12.1%; for V_{d1} = 40.0%; and for V_{d2} = 52.54%. The complete results from these PK population analyses are summarized in Table 1.

Table 1
PPK Analyses of ara-C Alone (Model I)

Model	*CL (L/h)*	*Q (L/h)*	V_{d1} *(L)*	V_{d2} *(L)*	ω^2_{CL}	ω^2_0	ω^2_{Vd1}	ω^2_{Vd2}	σ^2_1	σ^2_2	*OBF*
I-1	15.1	28.7	3.82	77.0	26.9	58.5	1.1E3	0.29	5E-3	613.0	3410.5
	(5.8)	(13.7)	(1.9)	(31.0)	(27.0)	(61.1)	(9.5E2)	(1.4)	(0.02)	(237.0)	
I-2	$2.6 \times P^a$	$1.82 \times P$	$0.46 \times P$	$34.6 \times P$	0.65	0.17	0.017	0.43	0.13	122.0	2837
	(0.62)	(0.86)	(0.22)	(22.6)	(0.24)	(0.3)	(0.32)	(0.7)	(0.07)	(39.9)	

The numbers in parentheses are the standard errors (SEs) of the estimated parameters. OBF, minimum values of the objective function.
[a]P, product of covariates *AGE* × *SA*.

6.2. NONMEM Population PK Analyses of ara-C/ara-U Data (Model II)

The ara-C and ara-U data have been analyzed simultaneously using the model depicted in Schema II. The same database was used for these analyses as for the previous ones. The PK characteristics of ara-U data were best described by the one-compartment open model as previously determined by the two-stage PK analyses (STS) for this metabolite. The first population PK analysis was done using the basic regression PK model, and it did not include covariates. The variance-covariance (OMEGA) matrix was constrained to be diagonal, as in the previous modeling described above.

The intraindividual variability of ara-C was best described by a combined Additive and Proportional (CCV) Error model, in which the two variance terms σ^2_1 and σ^2_2 were "fixed" to the final parameter estimates obtained via the *NONMEM* analyses of ara-C using model II. The intraindividual variability of ara-U was described by the Proportional (CCV) Error model.

The elimination of ara-C from the central compartment was described by two first-order rate constants: k_{13}, which described the metabolic conversion for ara-C to ara-U in the body, and by k_{10}, which described the renal elimination of ara-C from the central compartment. The simultaneous population analysis by the *NONMEM* program of ara-C and ara-U PK data using model II estimated a very low k_{10} value, approx equal to zero, and $k_{13} = 3.65\ h^{-1}$ or for a $t_{1/2,el} = 0.19$ h. Therefore, the best-fit population PK model was altered in a manner so that the clearance of ara-C from the central compartment was described primarily only by the k_{13} rate of metabolic elimination; that is, the clearance of ara-C from the central compartment of the pediatric patient population occurred via its metabolic conversion to ara-U rather than by renal elimination as unchanged parent drug.

The observed differences between the ratios of ara-U to ara-C in infants and older children suggested the possibility of an *AGE*-dependent metabolic conversion of ara-C to ara-U. The ratio of ara-U to ara-C was approx 1 in infant patients, but it averaged 7 in the older age pediatric patients. To examine the effect of the covariate *AGE* in the ara-U/ara-C ratio, the parameter k_{13} was expressed as a linear function of the *AGE* covariate as shown in Eqs. 12 and 13:

$$k_{13} = \theta(1) + \theta(2) \times AGE \tag{12}$$

$$k_{13} = \theta(2) \times AGE \tag{13}$$

where, $\theta(1)$ and $\theta(2)$ are parameters that express the metabolic rate constant k_{13} and are estimated by the final population analysis (*NONMEM*). Expression of k_{13} in terms of *AGE* in Eq. 12 was highly significant as determined by the Likelihood Ratio Test. However, the estimate of $\theta(1)$ in Eq.

12 is approx zero, suggesting that this parameter was not very important in the (best-fit) model, and perhaps a more simplified expression for k_{13} should be used. This was accomplished by utilizing the model with Eq. 13 (Table 2). Table 2 shows the parameter estimates obtained for the basic and expanded PK regression models.

The microconstant parameters of ara-C (k_{12}, k_{21}, k_{13}, and V_{d1}) estimated using model II-3 (Table 2) are very similar to the values for these parameters obtained when ara-C data were analyzed alone (Table 2, model II-4), by the two-compartment open model with first order of elimination from the central compartment utilizing the *ADVAN3/TRANS1* subroutines of the *NONMEM* program. Therefore, these results conclude that ara-C is eliminated from the central compartment with a first-order elimination, but this step is catalyzed by a remarkably large quantity of the Cyt DA enzyme; hence, no Michaelis-Menten (MM) saturable kinetics apply.

The final parameter estimates of both ara-C and ara-U for model II-3 (the best-fit one) were $k_{12} = 1.16 \pm 0.30$ h^{-1}, $k_{21} = 0.08 \pm 0.01$ h^{-1}, $k_{13} = 1.53 \times AGE$ h^{-1}, $V_{d1} = 1.37 \pm 0.51$ L, $k_{30} = 0.41 \pm 0.06$ h^{-1} ($t_{1/2,el} = 1.69$ h), and $V_{d3} = 4.98 \pm 1.50$ L (the volume of distribution of ara-U).

7. FLUDARABINE PHOSPHATE NONMEM POPULATION PK-PD ANALYSES IN PEDIATRIC PATIENTS WITH LEUKEMIAS

The combination of anthracycline and ara-C has been the basis for the therapy of AML since the 1970s. It was hypothesized that further progress in this disease therapy can be achieved by the use of newer, more effective drug combinations, such as Idarubicin followed by infusions of fludarabine monophosphate (F-araAMP) and ara-C regimen *(6)*.

The metabolism of nucleoside analog prodrugs (F-araAMP, ara-C) have been studied extensively in vitro and in vivo in both adult and pediatric leukemia patients *(4,7–9)*.

F-araAMP, the soluble analog of fludarabine (F-ara-A), once injected in the bloodstream, is dephosphorylated rapidly to F-ara-A by the alkaline phosphatases. When radiolabeled F-araAMP was administered, a half-life of 4–7 min was estimated in mice. Therefore, after 12–24 min of the F-araAMP injection, only F-ara-A circulates in plasma. Therefore, PK analyses of F-ara-A have been estimated in patients. F-ara-A is transported into the leukemia cells, where it is phosphorylated by cytosolic dCk to the monophosphate F-araAMP and by other kinases to the triphosphate F-araATP. F-araATP is considered to be the active anabolite of the drug *(7)*.

F-ara-A followed by the ara-C regimen has been the cornerstone of successful antileukemic regimens for leukemia patients in relapse as well as in front-line treatments in both adult and pediatric patients *(6,10,11)*.

Table 2
Simultaneous PPK Analyses of ara-C and ara-U Data (Model II)

Model	k_{12} (h^{-1})	k_{21} (h^{-1})	k_{10} (h^{-1})	k_{13} (h^{-1})	k_{30} (h^{-1})	V_{d1} (L)	V_{d3} (L)	σ^2_1	σ^2_2	σ^2_3	*OBF*
II-1	4.9	0.24	3E-9	3.65	3.78	1.5	0.47	0.13	107.0	0.06	7456.8
II-2	4.89	0.24	—	3.65	3.78	1.5	0.47	0.13	107.0	0.06	7456.8
II-3	1.16	0.08	—	1.53 3 × A[a]	0.4[b]	1.37	4.98	0.13	107.0	0.10	6764.14
	(0.30)	(0.08)		(0.25)	(0.06)	(0.51)	(1.51)			(0.40)	
II-4[c]	1.37	0.07	1.19 3 × A[a]	—	—	2.17	—	0.38	67.5	—	2810.17
	(0.48)	(0.04)	(0.13)			(0.39)		(0.19)	(29.7)		

The numbers in the parentheses represent the standard errors (SEs) of the estimated parameters. The variance terms σ^2_1 and σ^2_2 of the ara-C PK data were assigned fixed values. The variance term σ^2_3 of the ara-U PK data was estimated during the *NONMEM* analyses. OBF, minimum value of the objective function.

[a]A, *AGE* surrogate marker.

[b]The half-lives of elimination of ara-U were 0.18 h and 1.69 h for models II-2 and II-3, respectively. The estimates of half-life in model II-1 (similar to model II-2) and II-2 were highly biased; the half-life of ara-U in model II-3 was very similar to that estimated by STS analyses of the drug.

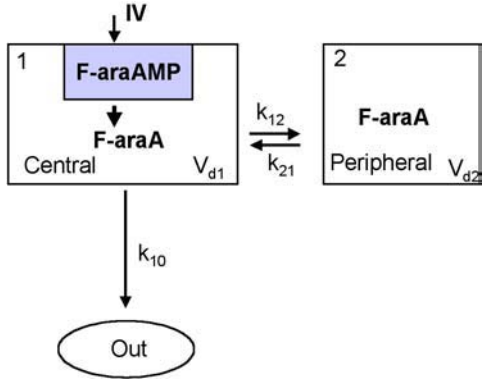
[c]This model estimated the PK parameters of ara-C along with ara-U using the *ADVAN3/TRANS1* subroutines of *NONMEM*.

The pharmacokinetic and pharmacodynamic studies of F-ara-A or ara-C were established long before the combination regimen of these drugs was designed for use in pediatric patients with leukemia. Specifically, the phase I–II of F-ara-A PK-PD data in pediatric leukemia patients were examined with the two-stage (STS) method and by the use of the *NONMEM* population analyses *(12)*. The data presented here are only a brief summary of these population PK-PD efforts.

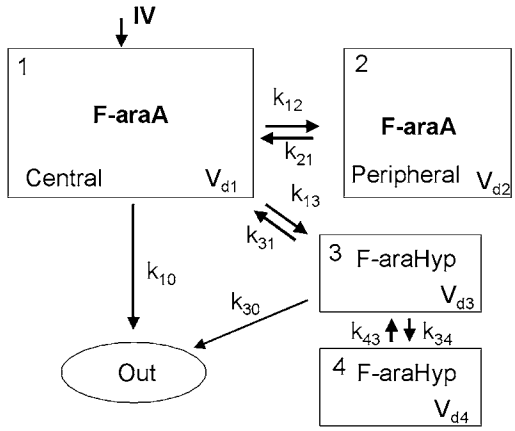
The unpredicted neurotoxicity after F-araAMP administration in adult patients was associated with the high peak plasma levels of F-ara-A, a highly lipophilic drug. To avoid this type of host toxicity in children with leukemias and because of the long half-life of elimination of the drug, F-araAMP was administered as an LB followed immediately by a CI 5-d regimen, which, as stated above, is rapidly dephosphorylated to F-ara-A *(12)*. The maximum tolerated dose of F-araAMP consisted of an LB of 10 mg/m^2 followed by an infusion of 30.5 mg/m^2/d for 5 d. The idea of LB plus CI was to achieve steady-state plasma concentrations (C_{ss}) of F-ara-A immediately after initiation of the regimen. Pharmacokinetic evaluations by the classical method (STS) of plasma 2 h after the initiation of the LB plus CI regimen demonstrated that the C_{ss} of F-ara-A was achieved and remained relatively constant for the duration of the 5-d infusion. The PK characteristics of the drug were then determined after the 5-d infusion. The terminal half-life of F-ara-A was similar to that reported in adult patients and after six dose-level escalations, suggesting the linear handling of the drug by pediatric patients over a wide range of dosing. The total body clearance of F-ara-A averaged 0.6 ± 0.1 L/m^2/h, and it was shorter in children than the reported value in adult patients. The drug obeys a two-compartment open model; the half-life of distribution ($t_{1/2,\alpha}$) averaged 1.25 ± 0.4 h and the terminal half-life ($t_{1/2,\beta}$) averaged 12.5 ± 3.1 h (mean of means ± SDEV of six dose levels). The intercept constants ratio (A/B) averaged 9.2, which indicated the high peak plasma levels achieved *(12)*.

8. *NONMEM* POPULATION PHARMACOKINETICS MODELING OF F-ara-A AND F-araATP

The *NONMEM* PK-PD population analyses of F-ara-A in plasma and F-araATP in leukemic blast cells were then performed. First, a two-compartment open PK model was devised to account for the fact that F-araAMP was the administered drug in patients (Schema III, model III). This *NONMEM* PK model was characterized by the *ADVAN3/TRANS1* subroutines. However, because of the vast amount and prevalence of alkaline phosphatases in plasma, liver, red blood cell surface, and so on, than phosphorylases, it was rejected. Then, and because F-ara-A is extensively catabolized to F-ara-hypoxanthine



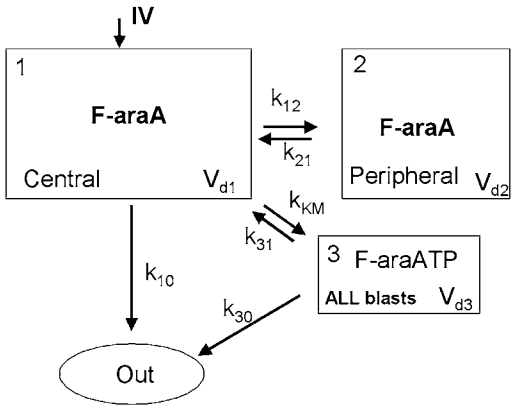
Schema III. PK model III.



Schema IV. PK model IV.

(F-araHyp), which itself obeys a two-compartment model, model in Schema IV, Model IV was devised. This PK model describes the plasma concentrations of F-ara-A and F-araHyp as each obeys a two-compartment open model; *the ADVAN6/TOL = 5* subroutines were used. Because the examination of F-araHyp did not alter the PK results of F-ara-A (no influence), this model was also rejected.

Finally, the Schema V, Model V was devised to describe simultaneously the PK distribution and elimination of F-ara-A from plasma and the concomitant formation/accumulation of its active anabolite F-araATP in leukemic cells. This PK-PD model was customized to insert the Michaelis-Menten (M-M) equation in the formation of F-araATP, even though the rate-limiting step in its formation is only at F-ara-A $\rightarrow$ F-araAMP;



Schema V. PK model V.

ADVAN6/TOL = 5 subroutines were used. The following equations were used to solve this PK-PD model V.

$$dA/dt(1) = -k_{12} \times A(1) + k_{21} \times A(2) - [A(1) \times V_1 \times VM] /[V_1 \times KM + A(1)] - k_{10} \times A(1) \quad (14)$$

$$dA/dt(2) = k_{12} \times A(1) - k_{21} \times A(2) \quad (15)$$

and

$$dA/dt(3) = [A(1) \times V_1 \times VM]/[V_1 \times KM + A(1)] - k_{30} \times A(3) \quad (16)$$

The concomitant *NONMEM* population pharmacokinetic and pharmacodynamic (PPK) model for F-ara-A and its cellular triphosphate anabolite F-araATP has been completed. The *A*/*B* intercept ratio for F-ara-A in plasma remained 9.2. The *NONMEM* population $t_{1/2,\alpha}$ of F-ara-A was 1.6 h, and the terminal $t_{1/2,\beta}$ of F-ara-A was 9.8 h (model V). These population values are similar to the ones obtained from the STS analyses. The V_{d1} of F-ara-A (central compartment) was 1.71 L/m^2, and the V_{d2} of F-ara-A was 3.35 L/m^2. However, the volume of distribution at steady state (V_{dss}) was higher, averaging 10.83 ± 3.5 L/m^2, strongly indicating that F-ara-A is accumulated in a slowly perfused peripheral compartment, most likely consisting of adipose and other lipophilic tissues.

The total body clearance (CL_T) of F-ara-A was 0.75 L/m^2/h, and the intercompartmental clearance (Q) from the central to the peripheral compartments of F-ara-A was 0.56 L/m^2/h. There is no MM kinetics in the distribution or elimination phases of F-ara-A in pediatric patients with leukemias.

The *NONMEM* PPK model for F-ara-A was tested for associations between *AGE* and *SA* covariates with the volumes of distribution (V_{d1}, V_{d2})

and *CL*. No associations were seen between *SA* and V_{d2} of F-ara-A. However, there was a good association (95% confidence interval, CI) between *SA* and V_{d1} of F-ara-A. In addition, a good association was seen between *SA* and *CL* of F-ara-A. There was a minor association (90% CI) between *CL* and *AGE* of patients from 5 to 19 yr of age.

The *NONMEM* PPK model for F-ara-A simultaneously modeled the F-ara-A plasma concentrations and the F-araATP cellular concentrations under MM conditions (model V). The K_m of F-ara-A for the dCk rate-limiting step was 142 μ*M*, and the V_{max} of F-ara-A phosphorylation was 30.6 nmol/h/m^2. The MM parameters (K_m and V_{max}) of F-ara-A from these *NONMEM* PPK analyses for dCk were similar to values determined under biochemical conditions of initial rates using a partially purified (2500-fold purification, (K_m = 200–400 μ*M*) dCK enzyme. Furthermore, the addition of the additive error for k_{12}, k_{KM}, the volume of the leukemic blast cell (V_M), and k_{10} produced significant differences, and it was an integral part of the final PK-PD model. In contrast, the Error, either Additive or Proportional, for V_{max}, k_{10}, k_{30} did not make a significant difference; therefore, the simpler model subroutines were accepted as the best fit of this PK-PD model of F-ara-A in plasma and F-araATP in leukemic cells. The ω-parameter values for ω^2_{12}, ω^2_{KM}, ω^2_{Vd1}, ω^2_{Vd3} ranged from 1.27 to 4.85E-8.

The volume of the leukemic cells averaged 114 mL, which represents the theoretical volume of the leukemic cell burden (packed leukemic blast volume). According to cell size estimation of the patients' leukemic cell volume, the 114 mL represents a tumor burden of approx 10 × E11 cells at the time of treatment. Under these MM conditions of F-ara-A metabolism by a significant number of leukemic cells, the $t_{1/2,\alpha}$ of F-ara-A increased to 3.4 h, and the terminal $t_{1/2,\beta}$ of F-ara-A was 11.2 h. These values are superimposable to the ones estimated by the STS method *(12)*.

On the secondary evaluations using the final model, it was found that there is no correlation, but a weak association between *CL* of F-ara-A and patients' *SA*. However, there was an association between *CL* and *AGE*, with 2σ of the paired data points falling within the 90% confidence interval (%CI) of the best-fit line. There was no association between the volume of distribution of the peripheral compartment of F-ara-A (V_{d2}) and *SA* or between the intercompartmental clearance *Q* and *SA*. However, there was an association between the volume of distribution of the central compartment of F-ara-A (V_{d1}) and *SA*.

Finally, the intercompartmental rates of transfer or the half-lives of transfer of F-ara-A between the central to the peripheral compartment were within physiological rates of transfer for most lipophilic drugs and were under physiological clearance values.

Table 3
Simultaneous Population PPK Analyses of F-ara-A in Plasma and F-araATP in Leukemic Blast Data (Model V)

Model	k_{12} (h^{-1})	k_{21} (h^{-1})	K_m (μ*M*)	V_{max}	$k_{10}{}^a$ (h^{-1})	k_{30} (h^{-1})	V_{d1} (*L*)	V_{d3} (*L*)	ω^2_{12}	ω^2_{KM}	ω^2_{Vd1}	ω^2_{Vd3}
V	0.203	0.125	142	30.6	0.0617	0.186	1.82	1.14E-1	1.27	0.39	4.8E-8	0.139

OBF (minimum value of the objective function) = 303.29; E, exponent.

[a]The population PK-PD model V final half-life of elimination of F-ara-A from the central compartment averaged 11.23 h. The previous models had an estimated half-life for F-ara-A of 19.85 h.

9. DISCUSSION

By utilizing the *NONMEM* PK modeling methodology, the analyses of ara-C in a pediatric patient population with leukemias have been described. The classical two-stage PK approach did not explain the significant variabilities in the elimination of this drug from the central compartment or the high peak plasma concentrations in younger patients *(3–6,11)*. For example, after the HDara-C regimen, there was greater than 100-fold difference in the peak plasma concentrations of ara-C and approx a 10-fold difference in the half-life of elimination of the drug in children than that reported in adult ALL patients. *NONMEM* population approaches resolved these variabilities and the mechanism of metabolic clearance of ara-C to ara-U systemically *(1)*. As stated, the simple PK model I showed considerable/ significant bias in the best fit of the ara-C PK data. However, the addition of covariates on the PK of the drug strongly indicated that substantial interindividual variability could be attributed to differences of age and surface area of the body (body size). As a result of the examination of these covariates, the total body clearance, the apparent volumes of distribution of the central and peripheral compartments, and the intercompartmental clearance of ara-C had statistically significant linear relationships with the covariate product [$AGE \times SA$]. The influence of [$AGE \times SA$] demonstrated an increase in the PK parameter values with increasing patient age (*AGE*) and surface area (*SA*). The results from these final analyses could therefore explain the lower drug plasma concentration in the older children as compared to that of the infants and younger patients with leukemias *(1)*.

In an effort to determine whether the increasing values of ara-C clearance with increasing *AGE* were caused by differences in the capacity of the body to deaminate the parent drug to ara-U, as suggested by the experimentally determined ara-U/ara-C ratios, the PK of ara-C and ara-U were modeled simultaneously in the pediatric patient population as a limited physiological model (model II). This was the most significant contribution of these population analyses to the medical sciences, the fact that ara-C is cleared almost 100% via metabolic deamination to ara-U *(1)*. This fact explained the high plasma concentrations of ara-C in infants and the low concentrations in teenaged patients *(1,3–5)*. By extrapolation of these findings and based on the reported PK concentrations of ara-C in adult leukemia patients, it is suggested that ara-C is cleared metabolically in these patients as well.

These *NONMEM* population analyses demonstrated that the clearance of ara-C from the central compartment occurred primarily via its deamination to ara-U. In addition, the rate of deamination increased with increasing *AGE* and *SA* as the expected enzymatic capacity of the body increased. Hence, patients' age was significant in explaining the interindividual variability

of this important antileukemic drug, strongly suggesting that infant patients, who have underdeveloped metabolic systems, do not eliminate ara-C from plasma as readily as older children via its deamination pathway to ara-U. This finding confirmed with a mathematical population model the previous observation that infant leukemia patients were accumulating very high plasma ara-C concentrations, had a very long half-life of elimination, and had a significant increase in central nervous system toxicity, facts that the STS PK method could explain *(1,4,5)*.

Many important findings were made by the population PK-PD analyses of F-ara-A and its active anabolite F-araATP in the leukemic blast cells. The similarity of the half-life of elimination between the final population model V (PPK-PD) analyses and those reported by the STS method in either pediatric or adult patients is remarkable, as is the estimation of the K_m of F-ara-A to F-araATP in blast cells in vivo and under steady-state plasma concentrations. In addition, the estimation of the secondary parameters contributes to the understanding of the accumulation of this drug to adipose or other lipophilic tissues, which may explain some of the drug's central nervous system toxicities. Unlike ara-C, the catabolic pathway to F-araHyp did not play an important role in the PK-PD handling of this drug in children.

Finally, these studies allow us to conclude that the *NONMEM* PPK analyses of F-ara-A and F-araATP under MM conditions produce important findings between the antileukemic drugs and the pediatric leukemia patients' surrogate markers. Overall, *NONMEM* population PK-PD analyses are complicated and time consuming and require a large amount of data or data from many patients. However, they produce the best possible PK-PD analyses for a drug and its metabolites, the parameters of which give better understanding of the biochemical pathways in plasma and in leukemic blasts simultaneously, which can then be applied to many other patients and future clinical trials.

REFERENCES

1. Periclou AP, Avramis VI. NONMEM population pharmacokinetic studies of cytosine arabinoside after high-dose and after loading bolus followed by continuous infusion of the drug in pediatric patients with leukemias. Cancer Chemother Pharmacol 1996;39:42–50.
2. Ellison RR, Holland JF, Weil M, et al. Arabinosyl cytosine: a useful agent in the treatment of acute leukemia in adults. Blood 1968;32:507–523.
3. Ochs J, Sinkule JA, Danks MK, Look AT, Bowman WP, Rivera G. Continuous infusion high-dose cytosine arabinoside in refractory childhood leukemia. J Clin Oncol 1984;2:1092–1097.
4. Avramis VI, Biener R, Krailo M, et al. Biochemical pharmacology of high dose 1-β-D-arabinofuranosylcytosine in childhood acute leukemia. Cancer Res 1987; 47:6786–6792.

5. Avramis VI, Weinberg KI, Sato JK, et al. Pharmacology studies of 1-β-D-arabinofuranosylcytosine in pediatric patients with leukemia and lymphoma after a biochemically optimal regimen of loading bolus plus continuous infusion of the drug. Cancer Res 1989;49:241–247.
6. Dinndorf PA, Avramis VI, Wiersma S, et al. Phase I/II study of idarubicin given with continuous infusion fludarabine followed by continuous infusion cytarabine in children with acute leukemia: a report from the Children's Cancer Group. J Clin Oncol 1997;15:2780–2785.
7. Avramis VI, Plunkett W. Metabolism and therapeutic efficacy of 9-β-D-arabinofuranosyl-2-fluoroadenine (F-araA) against murine leukemia P388. Cancer Res 1982;42:2587–2596.
8. Momparler RL. A model for the chemotherapy of acute leukemia with 1-β-D-arabinofuranosylcytosine. Cancer Res 1974;34:1775–1787.
9. Iacoboni SJ, Plunkett W, Kantarjian HM, et al. High-dose cytosine arabinoside: treatment and cellular pharmacology of chronic myelogenous leukemia blast crisis. J Clin Oncol 1986;4:1079–1088.
10. Gandhi V, Plunkett W. Modulation of arabinosylnucleoside metabolism by arabinosylnucleotides in human leukemia cells. Cancer Res 1988;48:329–334. 11.
11. Avramis VI, Wiersma S, Krailo MD, et al. Pharmacokinetic and pharmacodynamic studies of fludarabine and cytosine arabinoside administered as loading boluses followed by continuous infusions after a phase I/II study in pediatric patients with relapsed leukemias. The Children's Cancer Group. Clin Cancer Res 1998;1:45–52.
12. Avramis VI, Champange J, Sato J, et al. Pharmacology and phase I/II trial of fludarabine as a loading bolus and continuous infusion in pediatric patients. Cancer Res 1990;50:7226–7231.

APPENDIX: *NONMEM*: GUIDELINES TO GET STARTED

1. Enter the PK-PD data in a formatted table and label the file: Pt #; dose # (DOSE AMOUNT); TIME Dependent variable (Drug conc.) #1, #2, #3, etc.; Surrogate marker(s), like surface area (SA), body weight, AGE, creatinine clearance, outcome response vs. non-response, and so on.
2. Go to the appropriate computer compartment and select *NMIV* subroutine, for instance, :\NMIV\RUN.
3. Copy or transfer the data file to the NMIV\RUN.
4. Select a basic PK-PD subroutine, for instance, one-compartment open model, two-compartment open model, two-compartment Michaelis-Menten model, and so on, for example, ADVAN3TRLA.LNK.
5. Go to the INPUT file of the *NONMEM* program. Here is where all the subroutines of the PK selection and their customized modifications (if any), the number of compartment(s); the presence of metabolite data (e.g., ara-C/ara-U, ara-C/araCTP, F-ara-A/F-ara-Hyp, F-ara-A/F-araATP); the PK microconstants; the K_m; volume of distribution, volume of distribution of the peripheral compartment, or the *n*th compartment (i.e., the volume of leukemic blasts); the selection of type of Error(s), for instance, Additive, Proportional, Exponential, or Exponential plus

Additive, and their upper and lower limits wanted; the THETA (θ), OMEGA (ω), and SIGMA (σ) Error range fluctuations. Then, the differential equations, if any, and their relationship to the PK-PD equations (fusion). Then, the maximum evaluations, whether to print the outcome, and whether to abort on an Error or to continue and show the Error step(s). Last, print the scatterplot between PRED(icted) vs. DV (dependent variable) and the scatterplot between the (weighted error) WRES vs PRED. Give the INPUT file a name and insert the PK-PD run name, date, version, and so on; the data file; and the ADVAN.LNK file.

6. Select the *NM-TRAN* subroutine and click to "RUN".
7. If all files and subroutines have been entered correctly, then the *NM-TRAN* RUN will run without any Errors. If there are Errors, then it will define the Error, but not its location. Search and correct the Error. It usually is in the entry of the data (data file).
8. Go to NMIV\RUN and select a DOIT (command followed by identification of run) and ENTER (e.g., DOITVA.BAT).
9. If all subroutines and data files are well, then the program will come to a NO ERROR stop.
10. Then, GO to the appropriate compartment of the program and select: NONMEM.EXE. If all is well, then it will run the program(s) and print the data or print until the Error. If all is well, then rerun the data with variations of the ADVAN and PK subroutines until the MINIMUM VALUE OF OBJECTIVE FUNCTION is not changing (significantly). Then, one has arrived at the best-fit model for the data, and the FINAL PARAMETER ESTIMATE(s) can be used with the confidence of the ETA Error(s). Last, the predicted (PRED) vs. the DV values are compared at each and every time interval chosen, and the surrogate marker evaluations can be incorporated into the PK-PD or limited physiological model. For a completed mammalian physiological model one has to design a 13-compartment open model with a heart-lung shunt and the MM-controlled biliary excretion (liver) and reabsorption from the gastrointestinal mucosa.

15 The *cyclo*Sal-Nucleotide Delivery System

Development of Chemical Trojan Horses as Antiviral Agents

Chris Meier, PhD, Jan Balzarini, PhD, and Astrid Meerbach, PhD

CONTENTS

SUMMARY

Pronucleotides represent a promising alternative to improve the biological activity of nucleoside analogs against different viral diseases and cancer chemotherapy. Moreover, pronucleotides are valuable tools for studies concerning nucleoside/nucleotide metabolism. The basic idea is to achieve nucleotide delivery into cells, bypassing limitations with intracellular formation of nucleotides from their nucleoside precursors. The *cyclo*Saligenyl (*cyclo*Sal) concept is one of several pronucleotide systems reported so far but is the only approach in which a pronucleotide is cleaved successfully by simple but selective chemical hydrolysis. Beside others, for the nucleoside analog 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T), the application of the *cyclo*Sal approach improved antiviral potency. The basic concept, the

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

chemistry, different structural modifications, and their effects on the antiviral potency of the *cyclo*Sal-d4T 5′-monophosphate triesters are discussed. The application of the approach to different biologically active nucleoside analogs against different targets is discussed. First results of a conceptual extension of the original *cyclo*Sal approach are summarized. Once the pronucleotides have passed the membrane, the aim is to trap the *cyclo*Sal-phosphate triesters inside the cells. Therefore, enzyme-cleavable groups have been attached via a linker to the *cyclo*Sal moiety.

1. INTRODUCTION

Since the discovery of 3′-azido-3′-deoxythymidine (AZT) as the first nucleoside drug for the treatment of acquired immunodeficiency syndrome *(1)*, considerable efforts have been made to develop new nucleoside analogs that would be more-active and less-toxic inhibitors of human immunodeficiency virus (HIV) reverse transcriptase (RT) *(2,3)*. These analogs differ from the natural nucleosides in the modifications of the glycon or the aglycon residue *(4)*.

Today, synthetic nucleoside mimetics represent a highly valuable source of antiviral and anticancer compounds that contribute significantly to the arsenal of agents for the treatment of viral diseases (e.g., HIV, herpes and hepatitis virus infections) and cancer. The general mode of action of most of the nucleoside analogs is through the inhibition of DNA polymerases, including RT, by acting as competitive inhibitors or as DNA chain terminators. To act as DNA chain termination agents/polymerase inhibitors, intracellular conversion of the nucleoside analogs into their 5′-mono-, 5′-di-, and finally 5′-triphosphates is a prerequisite after cell penetration *(2,5)*. However, the efficient anabolism to the corresponding nucleoside analog triphosphates often is a major hurdle because of limited anabolic phosphorylation or catabolic processes as deamination of the aglycon or cleavage of the glycosidic bond. Therefore, their eventual therapeutic efficacy is compromised *(2,6)*. For example, the first phosphorylation step of the anti-HIV active dideoxynucleoside analog 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) **1** *(7–9)* (Fig. 1) into d4T 5′-monophosphate (d4TMP) catalyzed by thymidine kinase (TK) is the critical rate-limiting step in human cells *(10)*.

Despite the example given, the intracellular fate of the majority of nucleoside analogs has not been studied in detail. These compounds are often exclusively tested as nucleosides and discarded if found inactive. As they are rarely studied against the target polymerases as triphosphates, this ends in a black-box metabolism (Fig. 2).

However, a lack of uncovering where the metabolic blockade exists prevents further successful development. On the other hand, knowing the limitations on phosphorylation of a nucleoside offers a chance to develop derivatives with improved biological potential. In principle, the direct

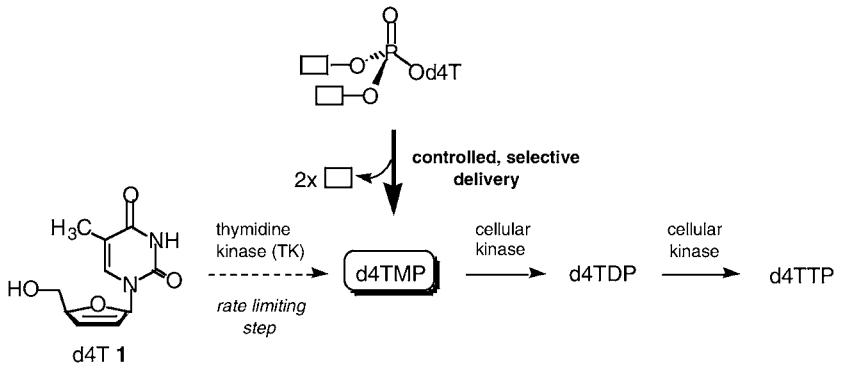


Fig. 1. Bioactivation of 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) **1** and the principle of the pronucleotide approach for the nucleotide d4TMP.

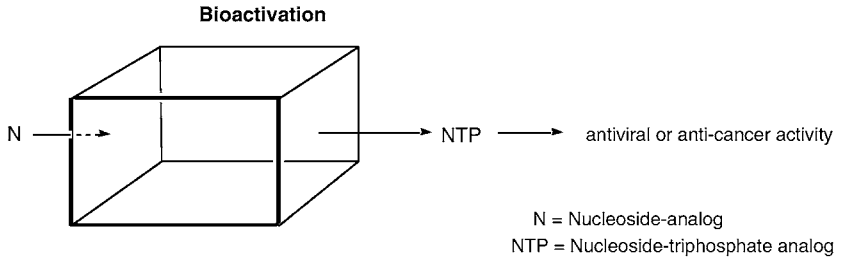


Fig. 2. Bioactivation of nucleosides into their triphosphates.

administration of nucleotides like d4TMP should bypass the limiting step in the TK-based anabolism of some nucleosides and thus improve their biological activity. Unfortunately, nucleotides are very polar molecules and do not easily pass cellular membranes. However, this difficulty can be surmounted by linking suitably degradable lipophilic carrier groups to the phosphate moiety, which leads to neutral, membrane-permeable nucleotide delivery systems (*pronucleotide approach*; Fig. 1) *(11–15)*.

It should be mentioned that strategies also have been studied to improve the uptake and therefore the efficiency of antiviral and anticancer nucleoside analogs. Examples are valacyclovir, famciclovir, and 5′-lipid-, cholesterol-, or simple alkyl esters of the nucleosides. However, these drugs work as prodrugs of the parent nucleoside and not of the nucleotides. Hence, they cannot help in the bypass of a metabolic limitation during phosphorylation. Therefore, these compounds are not further discussed in the review. Here, the focus is on nucleotide-releasing compounds.

Two masking groups are necessary to obtain a neutral, lipophilic phosphate ester because of the presence of at least one negatively charged phosphate oxygen under physiological conditions (phosphate monoester

pK_as are ~1.6 and ~6.6). Moreover, the efficient intracellular delivery of nucleotides from a pronucleotide requires the design of a specific delivery mechanism. Several strategies using different nucleotide delivery mechanisms have been developed to achieve this goal *(11–15)*. Among these, simple dialkyl-, diphenyl-, and dibenzylphosphate triesters based on pure chemical hydrolysis proved to be unsuccessful in vitro and in vivo *(11,12, 14,15)*. More recent pronucleotide approaches are based on selective enzymatic or chemical activation of the masking group, which leads to a second, spontaneous reaction (tripartate prodrug system; *16*). These approaches utilize and exploit carboxyesterase activity and pH. The concepts working via an enzymatic trigger mechanism are as follows: bis(pivaloyloxymethyl) (POM), bis(*iso*propyloxycarbonyloxymethyl) (POC), bis(*S*-glycosylthioethyl) (SGTE), bis(4′-acylbenzyl) (AB), aryloxyphosphoramidate (APA), phosphoramidate monoester, and (modified) bis(*S*-acylthioethyl) (SATE). The delivery mechanisms of these enzyme-cleavable compounds have been summarized *(12–14)*. All these enzyme-triggered approaches have demonstrated in vitro that the successful intracellular delivery of nucleotides is possible.

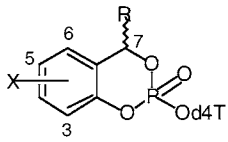
However, the only successful, pH-driven nucleotide delivery strategy is the *cyclo*Saligenyl (*cyclo*Sal) approach *(17)*. This approach, which also belongs to the group of tripartate prodrug delivery systems, has been developed in our laboratories and is the topic of this chapter. The effects of differently modified *cyclo*Sal triesters **2** and **3** of the nucleoside analog d4T are described. Three groups of derivatives are discussed. The so-called prototypes (**2**), a second series of compounds (**3**) bearing alkyl residues in the benzyl position (7-position), and finally a series of triesters having ester-functionalized X substituents but unsubstituted in the 7-position (**4**; Fig. 3). Moreover, several examples are summarized in which the prototype *cyclo*Sal structure has been attached to different antiviral and anticancer (5-fluoro-2′-deoxyuridine) nucleosides.

The first two compound series are discussed concerning their hydrolytic behavior. These first-generation compounds will offer valuable mechanistic insights into the designed cascade cleavage mechanism as well as their antiviral potential. The third series is discussed in the last part of this chapter. These compounds may act as second-generation *cyclo*Sal-pronucleotides.

2. THE *cyclo*SALIGENYL-PRONUCLEOTIDE APPROACH: THE FIRST GENERATION

2.1. Cyclo*Saligenyl-Nucleotides (*cyclo*Sal-Nucleoside Monophosphates): The Design of a Concept*

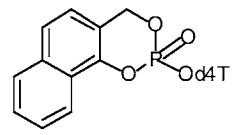
In contrast to the approaches mentioned in Section 1, the aim was the development of a selective delivery mechanism based on an exclusively



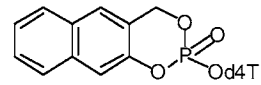
2a : X = 5-Cl; R = H
2b : X = 6-Cl; R = H
2c : X = R = H
2d : X = 5-C_6H_5 (Ph); R = H
2e : X = 3-C_6H_5 (Ph); R = H
2f : X = 3-CH_3 (Me); R = H
2g : X = 3,5-CH_3 (Me); R = H
2h : X = 3-*t*Bu; R = H
2i : X = 3,5-*t*Bu; R = H
2j : X = 3,5-*t*Bu, 6-F; R = H

3a : X = H; R = CH_3 (Me)
3b : X = H; R = CCl_3
3c : X = H; R = $CHCl_2$
3d : X = H; R = CH_2Cl

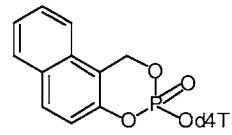
4 : X = CH_2CH_2-Ester; R = H



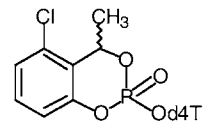
Benzo[a] -*cyclo*Sal-d4TMP **2k**



Benzo[b] -*cyclo*Sal-d4TMP **2l**



Benzo[c] -*cyclo*Sal-d4TMP **2m**



6-Chloro-7-methyl-*cyclo*Sal-d4TMP **3e**

Fig. 3. Various prototype *cyclo*Sal-d4TMP triesters **2** and **3**.

pH-dependent, chemically induced cascade mechanism *(18)*. However, the chemically driven release of the free nucleotide from a lipophilic precursor is not as easy as it seems *(19–21)*. By contrast to the enzymatically triggered pronucleotides *(12)*, the *cyclo*Sal strategy requires only one activation step to deliver the nucleotide, and because of the bifunctional character of the *cyclo*Sal group, the ratio of the masking unit per nucleotide molecule is 1:1. Other pronucleotide concepts employ ratios up to 4:1 *(22–24)*.

As summarized in Fig. 4, simple bis(phenyl)- (**5**) or bis(benzyl) nucleotide triesters (**6**) are unable to deliver the nucleotide *(25–27)*. Hydrolysis always stops at the phosphate diesters **7** and **8**, respectively, without formation of d4TMP (**9**). An exception to this are the bis acyclobenzyl (AB) pronucleotides developed by Freeman *(22)*. Although this concept should rely on enzymatic activation by carboxyesterases, work in our laboratory showed that these compounds are also chemically labile and deliver the nucleotide. However, the mechanism is the same as the enzymatic activation process and no S_NP-reaction is necessary for the cleavage of the intermediate AB-phosphate diester.

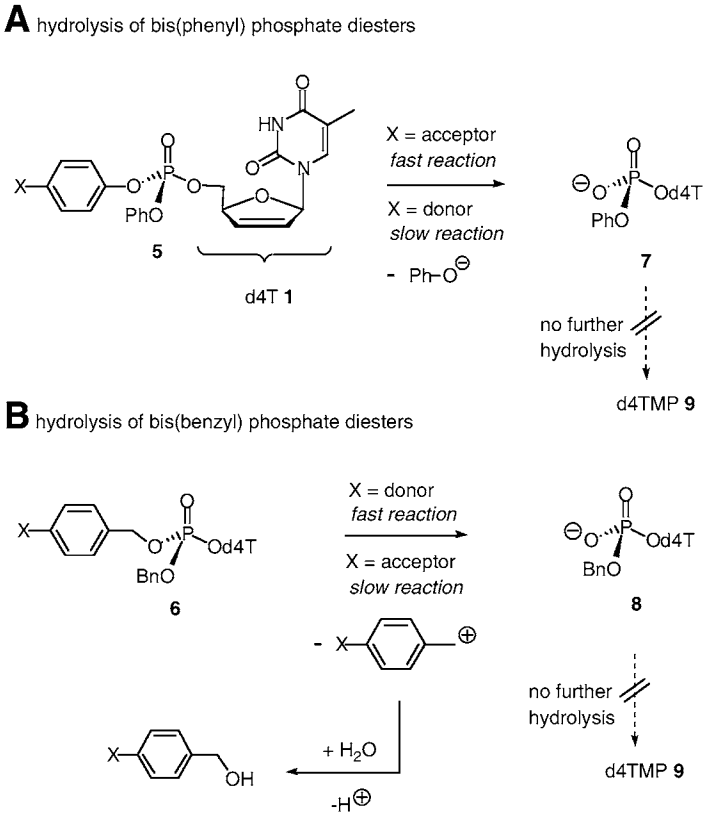


Fig. 4. Hydrolysis of *bis*(phenyl)- and *bis*(benzyl) phosphate triesters **5,6**.

Interestingly, the influence of substituents in the aromatic rings of **5** or **6** are just the opposite: Although acceptors in bis(phenyl) esters **5** cause fast hydrolysis, donors in bis(benzyl) esters **6** cause fast cleavage to yield the diester and finally benzyl alcohol (Fig. 4). However, a combination of both may form the basis of a suitable pronucleotide approach. Thus, the basis of the *cyclo*Sal concept consists of a combination of two ester bond types as part of a cyclic bifunctional group (masking unit).

Salicyl alcohols have been attached via a phenyl- and a benzylester bond. In addition, the nucleoside analog is attached through an alkyl ester bond. Only the introduction of these three ester bonds would allow sufficient discrimination between the different phosphate ester bonds. Thus, the designed chemically induced coupled process (tandem or cascade mechanism) is the following *(18,28,29)*: The phenyl ester bond in the *cyclo*Sal-triester structure should be the most labile one after nucleophilic attack

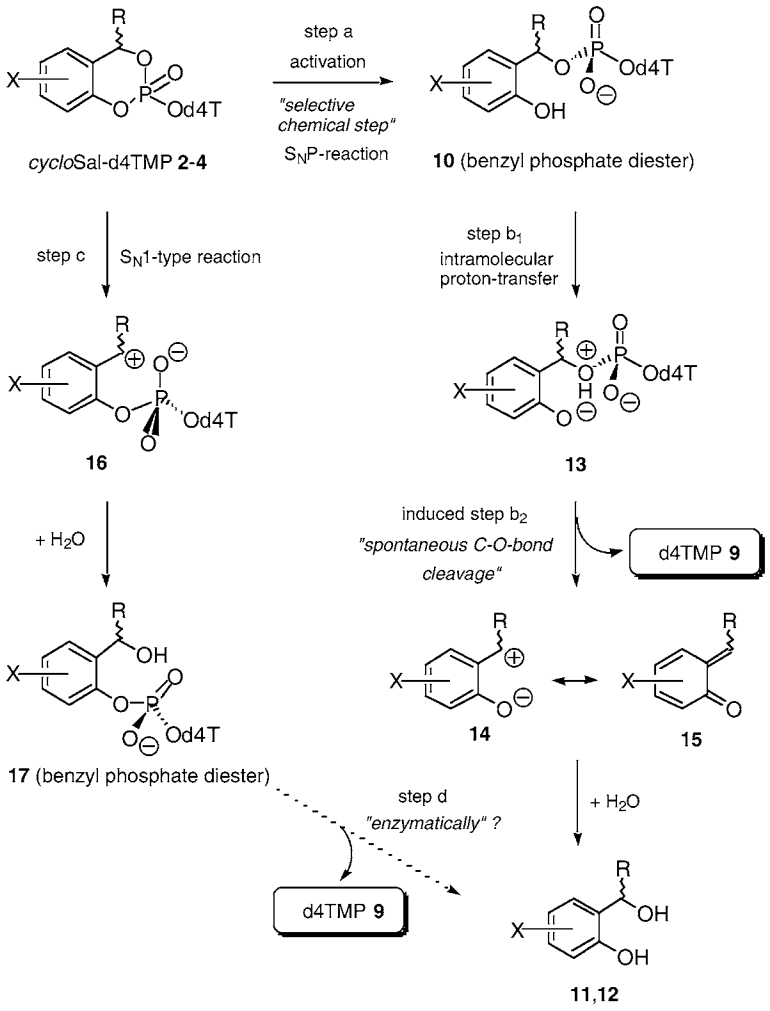


Fig. 5. Two possible hydrolysis pathways of the *cyclo*Sal-d4TMP triesters **2–4**.

of hydroxide to phosphorus (S_NP reaction). The developing negative charge can be delocalized by the aromatic system and thus make the phenolate the best leaving group in the triester. Cleavage yields a 2-hydroxy-*benzyl*phosphate diester (**10**; Fig. 5, step a). As a consequence of the initial step, the *ortho*-substituent to the benzyl ester is changed from a very weak electron-donating group (phosphate ester) to a strong electron-donating

group (hydroxyl). This effect of the 2-substituent intrinsically activates the remaining masking group, and this induces spontaneous rupture of diester **10** to yield the nucleotide and salicyl alcohols **11** (R = H) and **12** (R = alkyl) (cascade reaction; steps b_1 and b_2). This rupture proceeds presumably after intramolecular proton transfer (intermediate **13**) via zwitterion **14** or 2-quinone methide **15**. By this pathway, a cleavage mechanism is achieved that takes place within the masking group only and so prevents a pseudorotation process *(19)* that may partly lead to nucleoside liberation instead of the nucleotide. This is the preferred pathway via the benzyl phosphate diester intermediate **10**.

Although unfavored, a cleavage of the benzyl ester bond should also be taken into account (step c). Benzyl esters may be cleaved via S_N1-type C_{benzyl}–O bond rupture, which leads to the formation of a stabilized benzyl cation and an anionic phosphate ester group (intermediate **16**). The cation **16** is rapidly trapped by water to yield the phenyl phosphate diester of type **17** (Fig. 5). However, no further hydrolysis can be expected from this diester, thus hindering nucleotide release. The reason is the formation of a negative charge at the phosphorus atom. This prevents a second nucleophilic attack at the phosphorus atom in phenyl phosphate diesters. It also would decrease the leaving group properties of the 5′-nucleoside phosphate fragment in benzyl phosphate diesters *(21)*. Consequently, this C_{benzyl}–O bond rupture would lead to a dead end (Fig. 5) by formation of the phenyl phosphate diester **17**.

Salicyl alcohols **11** and **12** used as masking units were tested for their biological potency but showed neither antiviral activity nor cytotoxicity *(28,29)*. In addition, feeding of mice with 250 mg/kg of 3-methyl salicyl alcohol did not cause any visible toxic side effect. It should be added that salicyl alcohol (saligenin) is used as part of the antirheumatic and analgetic drug salicin (2-[hydroxymethyl]phenyl-β-D-glucopyranoside; Assalix®) *(30)*. β-Glucosidases hydrolyze salicin to D-glucose and saligenin, and the latter is then slowly oxidized by cytochrome P450 to salicylic acid in the blood and in the liver.

2.2. Chemistry

For the synthesis of the prototype *cyclo*Sal-d4TMP triesters **2**, the salicyl alcohols **11** had to be prepared first from the corresponding salicylic aldehydes, acids, or esters by reduction ($NaBH_4$ or $LiAlH_4$; Fig. 6) in high yields. In most cases, the aldehydes/acids/esters were not commercially available. Diols **11** then were synthesized starting with appropriately substituted phenols. Selective *ortho*-formulations have been achieved according to Casiraghi *(31)* or the Rieche formulation protocols *(32)*. Both methods led to salicyl aldehydes, which then could be reduced to the corresponding diols **11**. Alternatives are direct hydroxymethylation according

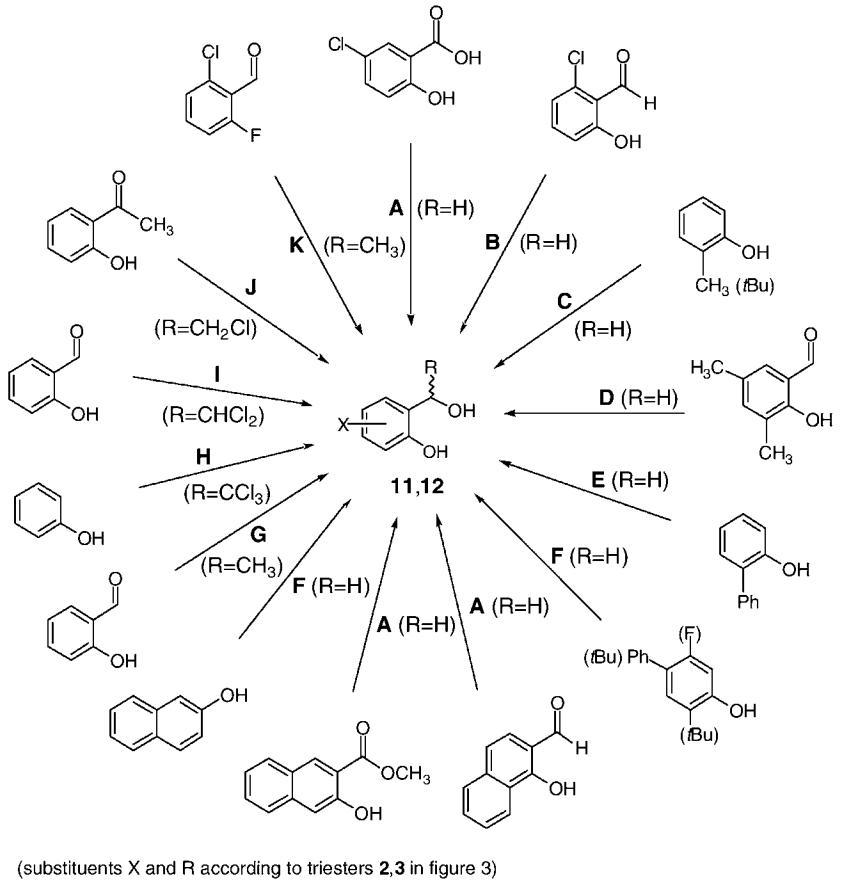


Fig. 6. Synthetic pathways to the salicyl alcohols **11,12**.

to Nagata et al. *(33)* or direct hydroxymethylation using formaldehyde in aqueous basic medium *(34)*. The last methods are the mildest of the procedures (Fig. 6).

These generally highly efficient methods are suitable for the synthesis of the prototype *cyclo*Sal derivatives without substituent in the benzyl position. For the second series of derivatives (triesters **3**), 7-methylated diols **12** were prepared by alkylation of salicyl aldehyde with methyllithium or dichloromethyllithium to give diols **12a** and **12c**. 7-Chloromethylsalicyl alcohol **12d** was prepared by chlorination of *ortho*-hydroxy acetophenone using 2,3,4,5,6,6-hexachloro-2,4-cyclohexadiene-1-one to give the ketone intermediate, which was reduced to give the alcohol. The 7-trichloromethyl

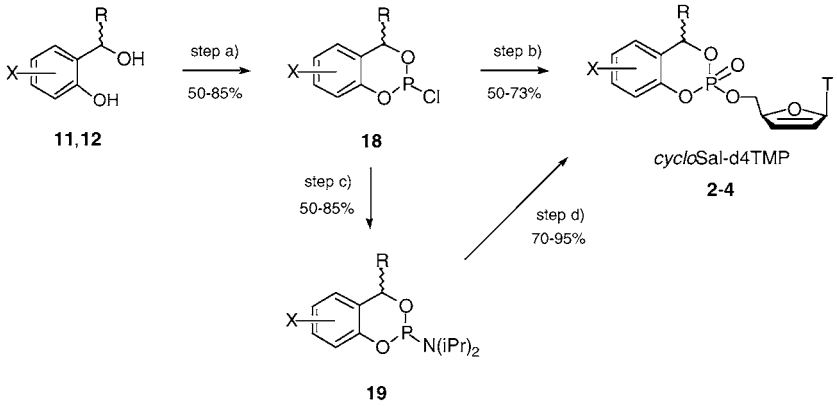


Fig. 7. Synthesis of the *cyclo*Sal-d4TMP triesters **2–4**.

derivative **12b** was synthesized using chloral instead of formaldehyde; double 6,7-modified salicyl alcohol **12e** was prepared by nucleophilic substitution of the fluorine in 2-chloro-6-fluoro-benzaldehyde by hydroxide and subsequent aldol-type addition *(35)*.

The synthesis of the *cyclo*Sal-pronucleotides was carried out using reactive phosphorus(III) reagents (Fig. 7). Therefore, diols **11** and **12** were reacted with phosphorus trichloride to give the cyclic chlorophosphites **18**. Phosphites **18** were reacted directly with the nucleoside analog (e.g., d4T) in the presence of diisopropylethylamine (Hünig's base) to yield the cyclic phosphite triesters, which were oxidized in a one-pot reaction using *t*-butylhydroperoxide or dimethyldioxirane. The phosphate triesters **2–4** were obtained in reasonable yields (50–73%) as mixtures of stereoisomers *(28,29)*.

Alternatively, chlorophosphites **18** were treated with diisopropylamine to yield the phosphoramidites **19**. The coupling with the nucleoside analog was carried out in acetonitrile in the presence of pyridinium chloride or imidazolium triflate as a coupling activator. In some cases, the last procedure resulted in yields greater than 90% using imidazolium triflate as the activator *(36)*.

2.3. Proof of Principle

2.3.1. d4TMP Release From *cyclo*Sal-d4TMP Pronucleotides

Extensive studies have been performed to investigate the designed delivery mechanism of d4TMP from the *cyclo*Sal-triesters *(28,37)*. Chemical hydrolysis studies in different buffer solutions at different pH values proved

Table 1
Half-Lives, Product Ratio and Antiviral Data of the Prototype *cyclo*Sal-d4TMP Triesters 2 and the 7-Modified *cyclo*Sal-d4TMPs 3

				EC_{50} (μM)[a]			
Compound	*Modification X and/or R*	$t_{1/2}$; *37°C*[b]; *pH 7.3*[c] *(h)*	*Product ratio d4TMP: 17*[d]	*CEM/O HIV-1*	*CEM/O HIV-2*	*CEM/ TK⁻ HIV-2*	CC_{50} (μM)[e]
2a	5-Cl	1.1	100:0	0.42	1.40	2.67	49
2b	6-Cl	0.9	100:0	0.087	0.15	0.8	36
2c	5-H	4.4	99:1	0.20	0.22	0.15	50
2d	5-C_6H_5	3.1	100:0	0.40	0.47	2	54
2e	3-C_6H_5	5.1	97:3	0.13	0.27	0.15	22
2f	3-CH_3	17.5	94:6	0.057	0.07	0.048	32
2g	3,5-CH_3	29	92:8	0.09	0.17	0.08	21
2h	3-*t*Bu	96	92:8	0.18	0.65	0.33	35
2i	3,5-*t*Bu	73	66:34	1.1	1.2	2.0	27
2j	3,5-*t*Bu; 6-F	6.2	100:0	1.23	0.73	0.6	26
2k	benzo[a]	2.8	91:9	0.14	0.12	0.6	53
2l	benzo[b]	1.4	97:3	0.41	0.50	4	132
2m	benzo[c]	2.8	88:12	0.09	0.17	0.8	>50
3a	7-CH_3	0.25	17:83	0.22	0.34	35	152
3b	7-CCl_3	0.9	85:15[f]	0.19	0.60	42	54
3c	7-$CHCl_2$	1.4	97:3	0.16	0.55	27	67
3d	7-CH_2Cl	2.8	100:0	0.19	0.35	22	56
3e	6-Cl,7-CH_3	2.2	100:0	0.19	0.25	7	42
d4T **1**	—	—	—	0.18	0.55	28	35

[a]Antiviral activity: 50% effective concentration.
[b]Hydrolysis half-lives in hours
[c]25 m*M* sodium phosphate buffer.
[d]Ratio of d4TMP: phenyl phosphate diester **17** determined by ^{31}P-NMR.
[e]Cytotoxic concentration: 50% cytostatic/toxic activity.
[f]Benzyl diester **4**: phenyl diester **17**.

that all prototype compounds released d4TMP selectively in a pH-dependent manner. As a second product, the salicyl alcohols **11** were also detected by high-performance liquid chromatographic (HPLC) analysis. The d4TMP was identified using ion-pairing eluents and the coelution technique. The half-lives of the triesters were determined in these studies (Table 1).

Within the prototype compound series, the following interesting effects were observed: As expected, the half-lives depend on the substitution pattern

of the aromatic ring. In general, acceptor substituents like the 5- or 6-chloro atom cause a decrease in hydrolytic stability, and donor substituents like 3-methyl-, 3,5-dimethyl-, and particularly 3-*t*-butyl or 3,5-di-*t*-butyl groups lead to an increased stability with respect to the unsubstituted prototype triester **2c**.

However, the phenyl ring substituent in the 3- (**2e**) and 5-positions (**2d**), respectively, showed a stabilizing effect for the first ($t_{1/2}$ = 5.1 h) and a destabilizing effect for the latter ($t_{1/2}$ = 3.1 h) compared to a $t_{1/2}$ of 4.4 h for the unsubstituted prototype triester **2c**. It should be mentioned that these differences are not within the experimental error (±0.2 h) and are relevant. Our interpretation is that the phenyl ring in the case of 5-phenyl-*cyclo*Sal-d4TMP **2d** is involved in the delocalization of the negative charge developed after the initial cleavage of the phenyl ester bond to give the benzyl phosphate diester **10** (Fig. 5). Thus, the accelerating effect dominates because of a (–)M-effect of the phenyl group, and the stability of the triesters decreases. In contrast, the rotation of the phenyl-aryl bond in 3-phenyl-*cyclo*Sal-d4TMP **2e** is hindered because of the bulky dioxaphosphorine-2-oxide ring, and the delocalization of the negative charge in diester **10** is less effective. Thus, a weak donor (+)I-effect dominates, and the stability increases. However, this electron-donating effect is considerably weaker compared to the strong (+)I-effect of a methyl group, as reflected by the longer half-life of 3-methyl-*cyclo*Sal-d4TMP **2f**. Another contribution of the 3-phenyl ring may be hindering the nucleophilic attack of water or hydroxide to the P-atom because of its lipophilicity by making the trajectory less available. By contrast, the presence of *t*-butyl groups has an unexpectedly big influence on the hydrolysis stability. Although the electron-donating property of a *t*-butyl group differs only slightly from the +I-effect of a methyl group, triesters **2h** (3-*t*Bu) and **2i** (3,5-*t*Bu) showed considerably higher half-lives compared to the methyl counterparts **2f** and **2g**. We attribute this to differences in the lipophilicity and therefore in the accessibility of the phosphate group.

It is interesting to note that the compounds bearing an extended aromatic system **2k**–2**m** *(37)* all showed decreased hydrolytic stability compared to the unsubstituted prototype triester **2c**. Half-lives ranged between 2.8 and 1.4 h for compound benzo[a]- (**2k**), benzo[b]- (**2l**), and benzo[c]-*cyclo*Sal-d4TMP **2m**. This may be explained by the increasing stabilization of the negative charge in the extended aromatic ring system, particularly in the case of the benzo[b]-derivative **2l** compared to the monocyclic prototype counterparts.

In addition to these HPLC-based studies, we were able to study the delivery mechanism by a ^{31}P nuclear magnetic resonance (NMR) experiment. The reason for using this technique was that the detection limit judged by integration of the resonance signals of possible reaction side

products in the NMR technique using the ^{31}P nucleus as a probe is much lower (1%) compared with the HPLC method (~5%). However, phosphate buffer is not an appropriate buffer system for this experiment. Compounds were dissolved, therefore, in an imidazole/HCl buffer adjusted to pH 7.3. A similar NMR experiment using only a dimethyl sulfoxide/water mixture was performed earlier in our lab for the hydrolysis of 5-nitro-*cyclo*Sal-d4TMP *(28)*. In that case, d4TMP was detected as the only hydrolysis product, pointing to an entirely selective delivery reaction.

The prototype *cyclo*Sal-d4TMP **2c** led to 99% formation of d4TMP in the imidazole/HCl buffer at pH 7.3. Moreover, 1% phenyl phosphate diester of type **17** (Fig. 5) was detected (Table 1). Diester **17** proved to be entirely stable for several weeks in the NMR tube at 37°C. This diester of the S_N1-type reaction has also been found in other cases in amounts of 3% (3-phenyl derivative **2e**) up to 8% for the 3,5-dimethyl triester **2g** and 3-*t*-butyl triester **2h** (Table 1). The formation of the phenyl phosphate diesters could not have been followed in the HPLC studies because of lower detection sensitivity. The benzo-annulated benzo[a]- and benzo[c]-*cyclo*Sal-triesters **2k** and **2m** formed 9% and 12% of the phenyl phosphate diester under the same reaction conditions, respectively.

The difference between the above benzo-annulated triesters **2k**, **2m**, and benzo[b]-*cyclo*Sal-d4TMP **2l** (3% diester **17**) results from better mesomeric stabilization of the cation intermediate of the first two compounds. This additional stabilization increases the rate of the S_N1-type bond rupture. By contrast, the worst case was found for the introduction of two *t*-butyl groups in the 3- and 5-position (compound **2i**), which led to the formation of 34% phenyl phosphate diester. Here, the concurrent S_N1-type reaction plays a considerable role in the degradation of triester **2i**. In contrast, 5-chloro- (**2a**), 6-chloro- (**2b**), 6-fluoro (data not shown), and 5-phenyl-*cyclo*Sal-d4TMPs **2d** turned out to deliver d4TMP exclusively. No side products were observed in the NMR experiment.

In conclusion, the S_NP-type hydrolysis of the prototype *cyclo*Sal-triesters showed strongly favored delivery of d4TMP following the designed cascade mechanism. However, a small amount of the phenyl phosphate diester **17** was also formed via an S_N1-type reaction. Hydrolysis half-lives may be controlled by introduction of appropriate substituents in the aryl moiety. Benzo-annulated compounds do not show favorable properties with respect to the half-life as well as delivery mechanism.

Interesting effects were observed for the second series of compounds. First, 7-methyl-*cyclo*Sal-d4TMP **3a** was investigated. Surprisingly, an enormous decrease in chemical stability was measured. The half-life dropped to 0.25 h (compared to 4.4 h for prototype triester **2c**), and even product distribution changed dramatically. NMR studies confirmed that the major product was the phenyl phosphate diester **17** (83%; Table 1). Thus, the S_N1-type

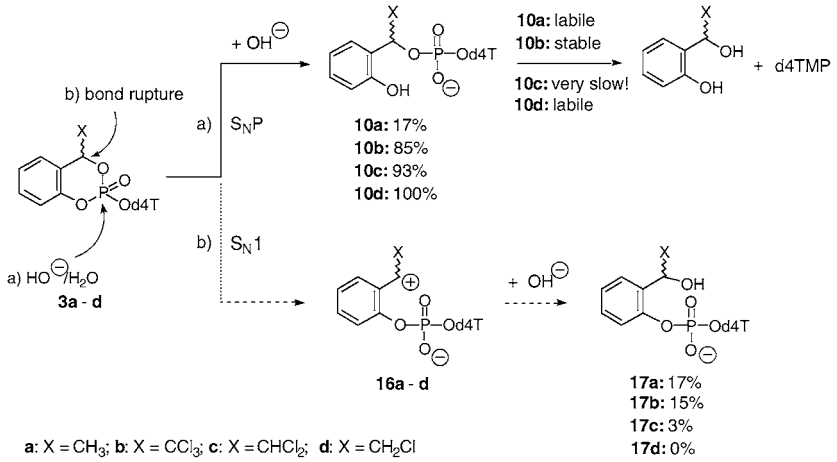


Fig. 8. Hydrolysis pathways of benzyl-substituted *cyclo*Sal-d4TMP triesters **3**.

reaction is now preferred. This may be because of the formation of a secondary benzyl cation intermediate as compared to a primary benzyl cation in the prototype cases. The additional alkyl residue stabilizes the cation by a (+)I-effect. This interpretation has been further confirmed by density functional theory calculations on the B3LYP/6–311G(d,p) level on model compounds of the prodrug systems *(38)*.

The activation energy barrier for the initial S_N1 reaction is considerably lower for 7-methyl-*cyclo*Sal-methylMP compared to the prototype triester analog. On the other hand, this predominant S_N1-type reaction should be avoided by reduction of the methyl (+)I-effect or by introduction of a strong electron-withdrawing substituent like a halogen atom in the 6-position of the aromatic ring. Both should decrease the stability of the intermediate benzyl cation and therefore should favor again the phenyl ester bond cleavage.

Therefore, the corresponding *cyclo*Sal-triesters **3b**–**3e** bearing a 7-trichloromethyl, a 7-dichloromethyl, a 7-chloromethyl, and a 6-chloro,7-methyl group instead of a 7-methyl group (**3a**) were prepared. The half-lives were determined as before (Table 1). NMR studies proved that, in all cases, the favored degradation pathway is the phenyl ester bond cleavage giving benzyl phosphate diesters **10a**–**d** (Fig. 8).

Unexpectedly, the intermediate benzyl diester bearing a 7-trichloromethyl group proved to be entirely stable in phosphate buffer at pH 7.3 as well as in imidazole/HCl at pH 7.3. No formation of d4TMP was observed. By introduction of three chloro atoms, the second, spontaneous step leading to the formation of d4TMP is prevented completely (Fig. 8). By introducing only two chloro atoms, 7-dichloromethyl-*cyclo*Sal-d4TMP **3c** gave the diester **10**

at 93%, which subsequently delivers d4TMP. However, the reaction proceeds very slowly. 7-Chloromethyl-*cyclo*Sal-d4TMP **3d** led to an entirely selective cleavage of the triester to give the benzyl phosphate diester **10**, which then delivered d4TMP as known for the prototype triesters **2**. Thus, a single chloro atom in the 7-methyl group completely turns the delivery mechanism from a predominant S_N1-type reaction into the desired, highly selective S_NP reaction (Fig. 8) *(35)*.

For 6-chloro-7-methyl-*cyclo*Sal-d4TMP **3e**, similar results were obtained: Again, highly selective d4TMP delivery was observed. Obviously, the introduction of the destabilizing 6-chloro atom compensates the stabilizing effect of the 7-methyl group efficiently. It should be mentioned that also the prototype 6-chloro-*cyclo*Sal-d4TMP **2b** (and the 6-fluoro derivative; data not shown) gave an exclusive d4TMP delivery as discussed above. The additional 7-methyl group did not interfere, although it resulted in an increase in chemical stability (0.9 h for **2b** vs 2.2 h for **3e**).

Finally, the tremendous effect of a halogen atom in the 6-position leading only to the formation of d4TMP **9** has been transferred to the case of 3,5-*t*-butyl *cyclo*Sal-triester **2i**. Our aim was to avoid or at least minimize the formation of the hydrolytically stable phenyl phosphate diester. With the introduction of a fluorine atom in the 6-position in addition to two *t*-butyl groups in the 3- and 5-positions in triester **2j**, the concurrent S_N1-type reaction should be limited; moreover, this fluorine atom should have an impact on the chemical stability at the same time. Triester **2j**, as expected, showed a pronounced decrease in chemical stability (6.2-h half-life instead of 73 h for **2i**); moreover, to our surprise, this compound delivers d4TMP **9** highly selectively (0% of the phenyl phosphate diester for **2j** instead of 34% for **2i**) *(39)*. Thus, again the concurrent S_N1-type cleavage of the triesters was entirely excluded.

In conclusion, it became apparent that besides the d4TMP formation, the prototype triesters also led to a small amount of the phenyl phosphate diesters **17**. However, this can be efficiently avoided by introduction of an electron-withdrawing 6-substituent or by a mono-acceptor-substituted methyl group in the 7-benzyl position.

No evidence of an enzymatic degradation in RPMI-1640 medium containing 10% fetal calf serum (pH 7.3) has been observed. Studies in CEM cell extracts showed that the hydrolysis half-lives slightly decreased only as compared to the buffer hydrolyses. Further studies of the prototype triesters in human serum (10% serum in phosphate buffer) exhibited no difference in stability compared to the buffer hydrolysis studies. Again, no enzymatic contribution could be detected, thus confirming the initial idea of a delivery mechanism independent of enzymatic activation.

All data obtained from hydrolysis and NMR studies are in perfect agreement with the designed degradation cascade reaction mechanism and

showed convincingly that the mechanism may be controlled efficiently by structural modification of the *cyclo*Sal moiety (Fig. 5).

2.3.2. Antiviral Activity

The in vitro antiviral potency of the *cyclo*Sal-nucleotides against HIV-1 and HIV-2 in CEM cells was assessed. It became apparent that the unsubstituted prototype (**2c**), 3-phenyl- (**2e),** 3-methyl- (**2f),** and 3,5-dimethyl-*cyclo*Sal-d4TMP (**2g**) showed comparable or even higher antiviral potency (0.087 μ*M*) in a wild-type T-lymphocytic cell line (CEM/O) compared to d4T (**1**) (0.18 μ*M*, Table 1). Moreover, particularly striking is the complete retention of the antiviral potency in mutant TK-deficient cells (CEM/TK$^-$) of the unsubstituted (**2c**), 3-phenyl- (**2e**), 3-methyl- (**2f**), 3,5-dimethyl- (**2g**), 3-*t*-butyl- (**2h**), and 3,5-*t*-butyl-6-fluoro- (**2j**) substituted triesters. The antiviral data and the hydrolysis half-lives clearly point to the fact that certain stability is needed, but beyond this point no further improvement of activity could be observed.

By contrast, the short half-life of the 5-chloro-triester **2a** ($t_{1/2}$ = 1.1 h) seems to be responsible for considerable loss of antiviral activity in the CEM/TK$^-$ cell assay, although the antiviral activity in the TK-competent cells (CEM/O cells) was comparable to that of d4T. The compound hydrolyzes extracellularly to yield d4TMP, which cannot be taken up by the cells. After extracellular dephosphorylation, d4T is taken up inside the cells and can be converted into the triphosphate. This is only possible in the TK-competent cells. Similar results were obtained for the 6-chloro derivative and all the triesters bearing the extended aromatic ring system. All of them are losing activity in the CEM/TK$^-$ cell assay.

An interesting observation has been made in the case of the unsubstituted, the 5-phenyl-, and the 3-phenyl prototype triesters **2c**, **2d**, **2e**, respectively. Although the 3-phenyl and the unsubstituted triester retained all antiviral potency in the CEM/TK$^-$ cells, 5-phenyl-*cyclo*Sal-d4TMP **2d** loses activity (fourfold). Half-lives were 5.1, 4.4, and 3.1 h, respectively. This may point to a threshold of about 4 h of hydrolytic stability needed to obtain a biologically active compound in the CEM/TK$^-$ cell assay.

Having this in mind, it is also reasonable that all compounds of the second series bearing the additional group in the 7-position and 3,5-*t*-butyl-*cyclo*Sal-triester **2i** lack antiviral activity in the CEM/TK$^-$ cells. Even the most stable derivative, 7-chloromethyl-*cyclo*Sal-d4TMP (**3d**), lost all antiviral potency in the TK-deficient cell line (Table 1). Moreover, a loss in activity also has been found for some of the compounds in the CEM/O cells. This can be attributed to the fact that predominantly the phenyl phosphate diester **17** or a stable benzyl phosphate diester **10** was formed that did not release d4TMP efficiently in the time-scale of the in vitro assay. It

should be added that, from experiments using an isolated recombinant RT/ribonucleic acid/DNA template primer, it became clear that the *cyclo*Sal-triesters themselves have no inhibitory effect on DNA synthesis, which is consistent with a mechanism of action for the *cyclo*Sal-triesters that relies on the formation of free d4TTP. Taken together, these results confirm (1) the cellular uptake of the compounds, (2) the highly selective intracellular delivery of d4TMP, and (3) the independence of the biological activity on cellular TK activation for some of the described *cyclo*Sal-phosphate triesters.

Nevertheless, the in vitro anti-HIV assays give only indirect proof of the intracellular delivery of d4TMP. Therefore, a series of incubation experiments with wild-type CEM/O and CEM/TK$^-$ cells and radiolabeled 3-methyl-*cyclo*Sal-d4TMP **2f** (tritium label in the methyl group of thymine) was conducted *(40)*. The amount of d4TMP in CEM/O cells was considerably higher (15-fold, 6-h incubations) compared to the amount of d4TMP resulting from the metabolism of d4T in the same cell line. In addition, an increase in the concentration of d4TTP was observed (16-fold, 6-h incubation), which may explain the higher activity of the prototype *cyclo*Sal-d4TMPs in wild-type CEM cells compared to d4T. These results are consistent with a mechanism of the *cyclo*Sal-d4TMPs that successfully bypasses TK and releases d4TMP inside the cells.

Furthermore, the *cyclo*Sal-d4TMP triesters demonstrated significant antiviral activity in AZT-resistant H9^{r}AZT^{250} cells *(41)*. The resistance is concomitant with a fivefold lower expression of the TK gene in comparison to parental H9 cells. The consequence is that d4T also showed reduced antiviral potency because of insufficient phosphorylation. In contrast, the prototype *cyclo*Sal-d4TMP **2c** proved to be equipotent in parental and in H9^{r}AZT^{250} cells (EC_{50} = 0.3 μ*M* and 0.5 μ*M*, respectively) proving again the entire independence of the expressed TK levels.

2.4. Cyclo*Sal-Pronucleotides of Different Nucleoside Analogs*

The *cyclo*Sal-approach has further been applied to different nucleoside analogues (Fig. 9). In addition to d4T (**1**), the *cyclo*Sal approach has been applied to 2′,3′-dideoxyadenosine (ddA) **20** and 2′,3′-dideoxy-2′,3′-didehydroadenosine (d4A; **21**). The anti-HIV activity has been increased 100-fold compared to the parent nucleoside analog ddA *(42)*, and the activity of d4A (**21**) has increased by 600-fold by the introduction of the *cyclo*Sal mask *(42)*. Thus, both *cyclo*Sal derivatives achieved the adenosine deaminase bypass. In the same project, two fluorinated ddA derivatives (**22**, **23**) were used *(43)*. The activity of 2′-*ara*-F-ddA **22** was improved (10-fold), but more interestingly, the entirely inactive nucleoside 2′-*ribo*-F-ddA **23** was converted into an anti-HIV active compound after

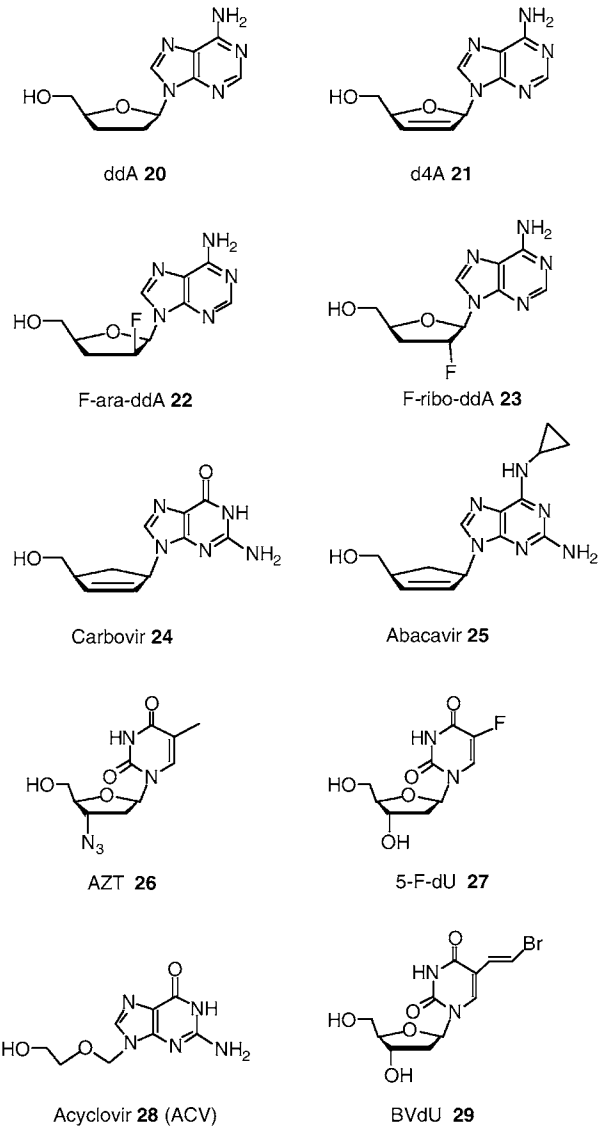


Fig. 9. Nucleoside analogs use with the *cyclo*Sal-approach.

modification with the 3-methyl-*cyclo*Sal moiety. The reason for the unexpected difference in the bioactivity of the two nucleosides may be related to a conformational difference caused by the introduction of the fluorine atom. This was the first example for a conversion of an inactive nucleoside into a bioactive derivative by the *cyclo*Sal approach.

The purine nucleoside analogs carbovir **24** and abacavir **25** have been used. For carbovir, no improvement against HIV and herpes simplex virus (HSV) 1/2 was observed compared to the parent nucleoside. However, the antiviral potency of abacavir was improved after *cyclo*Sal modification against HIV, and antiviral activity has been observed also against HSV-1/2 *(44)*.

2.4.1. The Failure of the *cyclo*Sal-3′-Azidothymidine Monophosphate Triesters

The approach has been applied to the delivery of the antiviral 3′-azidothymidine monophosphate (AZTMP), the monophosphate ester of the clinically used AZT (**26**; Fig. 9). In this case, the antiviral activity surprisingly observed in the TK-deficient cells clearly showed the failure of the concept *(45)*.

The preparation of these compounds was achieved by the chlorophosphite method. Chemical hydrolysis studies confirmed that these triesters behave as the corresponding *cyclo*Sal-d4TMP compounds: they release AZTMP selectively with half-lives slightly higher compared to the d4TMP derivatives. The antiviral test of these compounds showed pronounced anti-HIV activity in the wild-type CEM cells against both HIV-1 and HIV-2. However, the *cyclo*Sal-AZTMP derivatives **30** were losing most of their antiviral activity in mutant TK-deficient CEM/TK$^-$ cells. Nevertheless, a few of the compounds were still considerably more active compared to the parent nucleoside AZT **26** (triesters **30c** and **30d**; Table 2).

From the conceptual point of view, this was an unexpected result because the mutant cell line is unable to phosphorylate AZT into its monophosphate, and the retention of antiviral activity was expected again. However, it is known that the metabolic bottleneck in the activation of AZT into its triphosphate is the conversion of AZTMP into AZTDP *(2–5,46–48)*. Thus, a special metabolic limitation seems to act against the retention of activity in the TK$^-$ cells.

To investigate the reason for the failure of the *cyclo*Sal-AZTMP derivatives **30**, a study using radiolabeled 3-methyl-*cyclo*Sal-AZTMP R_p/S_p-**30c** was conducted *(49)*. From studies in CEM/O cells and CEM/TK$^-$ cells, it became apparent that the amount of AZTMP found in the wild-type CEM cells incubated with the *cyclo*Sal-AZTMP triester was considerably lower compared to the AZTMP concentration found in the same cell line incubated with AZT itself. However, after only 48 h of incubation the AZTMP levels formed from the triester reached the same level as those formed from the nucleoside AZT. In contrast, AZT formed extremely low levels of AZTMP in CEM/TK$^-$ cells (slightly over detection limit); the levels formed by hydrolysis of the *cyclo*Sal-AZTMP triester were considerably higher. This at least explains why the compounds kept some antiviral activity in

Table 2
Anti-HIV-Data of the *cyclo*Sal-AZTMP Triesters 30 in CEM Cells

			Antiviral activity EC_{50} (μM)[a]				
Compound	*Substance X*	*log P value*[b]	*CEM/O HIV-1*	*CEM/O HIV-2*	*CEM/TK⁻ HIV-2*	*Cytotoxicity* CC_{50} (μM)[c]	*SI value*[d]
30a	5-NO_2	0.81	0.008	0.02	>100	79	9800
30b	5-H	1.12	0.004	0.005	>20	68	17,000
30c	3,5-Me	1.81	0.007	0.017	7	40	5700
30d	3-Me	1.43	0.006	0.013	15	40	6600
AZT **26**	—	0.037	0.006	0.005	>100	>100	>16600

[a]50% effective concentration.
[b]log *P*: partition coefficient in 1-octanol/water.
[c]50% cytotoxic concentration.
[d]Selectivity index: ratio 50% cytotoxic concentration/50% effective concentration.

contrast to AZT. Nevertheless, the comparison of the AZTTP levels formed from the triester in CEM cells and in mutant CEM/TK⁻ cells after 6 h showed, as expected, a 100-fold decreased AZTTP level in the mutant cell line. This may explain the 100-fold decrease in antiviral activity in the mutant cell line compared to wild-type CEM cells.

Our interpretation of the failure of the AZTMP triesters is as follows: Because the bottleneck in the activation of AZT is the conversion of AZTMP into AZTDP, an increase of the AZTMP level by release from a pronucleotide would act counterproductively for the forward phosphorylation. Moreover, assuming efficient enzymatic dephosphorylation of AZTMP into AZT, the intracellular delivery of AZTMP cannot improve the activity. In wild-type cells, AZT formed by this catabolic reaction is readily rephosphorylated into AZTMP by the cellular TK; thus, no effect on the antiviral activity will be observed.

In contrast, in mutant CEM/TK-deficient cells, this process would have a severe consequence: If the released AZTMP from the pronucleotide is readily dephosphorylated, the intracellular pool of AZTMP is dramatically reduced because no TK phosphorylation restores the AZTMP amount. For AZT, this dephosphorylation/rephosphorylation metabolism plays a significant role because of rate-limiting phosphorylation into AZTDP and the nonphosphorylation to AZTMP in TK⁻ cells (Fig. 10). An identical process does not occur in the case of d4T because the bottleneck within the metabolism is the formation of d4TMP by the cellular TK, while d4TMP released from the pronucleotide is readily phosphorylated into d4TDP.

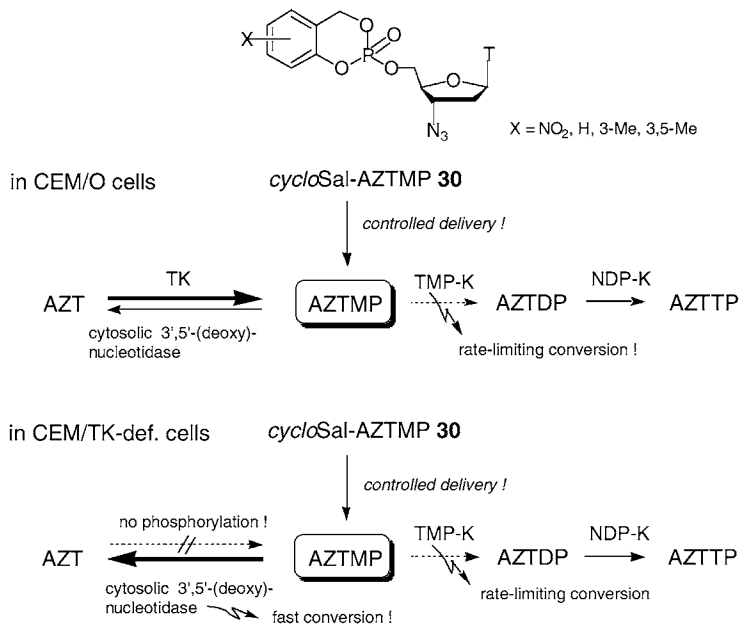


Fig. 10. Comparative metabolism of *cyclo*Sal-AZTMP in CEM/O- and CEM/TK$^-$ cells.

An enzyme that may be involved in the dephosphorylation of AZTMP is 3′,5′-(deoxy)nucleotidase *(49,50)*. From kinetic studies using this enzyme, it was shown that the relative efficacy for the dephosphorylation of dTMP, d4TMP, and AZTMP was 1, 0.08, and 2, respectively. This shows that AZTMP is even a better substrate for this enzyme than dTMP; d4TMP is about 20-fold less efficiently converted into d4T than AZTMP to AZT. This study may point to an important factor when pronucleotides are used to bypass metabolic limitations: Although a selective release of the nucleotide occurs, this alone may not be sufficient to overcome a limitation if unknown catabolic processes are involved.

Finally, it should be mentioned that other pronucleotides of AZTMP also lost most of their activity in the TK-deficient cell line *(13–15,24)*. This clearly points to an intrinsic problem during AZT metabolism and not to a general problem of the *cyclo*Sal-pronucleotide concept because the delivery concepts involved are entirely different from the *cyclo*Sal concept.

2.4.2. *Cyclo*Sal-Pronucleotides of 5-Fluoro-2′-Deoxyuridine

In antitumor chemotherapy, the 5-fluorouracil (5-FU) is used frequently. The mechanism of action of this compound includes the intracellular

formation of 5-fluoro-2′-deoxyuridine monophosphate (FdUMP). FdUMP does not need to be phosphorylated because it acts as an inhibitor of thymidylate synthase, which is responsible for the methylation of dUMP into dTMP. It appeared reasonable to apply the *cyclo*Sal concept (starting from 5-Fluoro-2′-deoxyuridine [FdU], **27**; Fig. 9) to the direct intracellular delivery of FdUMP *(51,52)*.

The chemical synthesis was first carried out using the chlorophosphite method *(52)*. However, the regioselectivity could be controlled only moderately: We obtained a 2:1 selectivity for the 5′-*O*-phosphorylated product and the 3′,5′-*O*-bisphosphorylated side product. Using *cyclo*Sal-phosphorchloridates instead of the phosphorchloridites, the regioselectivity increased to 5:1 in favor of the 5′-*O*-phosphorylated compound *(51)*. Interestingly, only a minor amount of the also-possible 3′-*O*-phosphorylated *cyclo*Sal-FdUMP triester could be isolated. It was interesting to note that *cyclo*Sal-FdUMP triesters were easily separable by semipreparative HPLC into the two diastereomers. Chemical hydrolysis studies proved again that FdUMP was released selectively.

Biological evaluation of the separated diastereomers of the *cyclo*Sal-FdUMP phosphate triesters was done in the following cell lines: L1210/O, L1210/TK$^-$, FM3A/O, FM3A/TK$^-$, Molt4/C8, CEM/O, and CEM/TK$^-$ (Table 3). In FM3A/O cells, the triesters showed even lower antitumor activity (0.057 μ*M*) compared to the nucleoside 5-FdU (0.009 μ*M*). Moreover, as for FdU itself, we observed a loss in antitumor activity in L1210/TK$^-$ and FM3A/TK$^-$ cells (IC_{50} 2.5–5 μ*M*). However, in wild-type L1210 (0.004 μ*M*), Molt4/C8 (16 μ*M*) and CEM cells (0.03 μ*M*) the biological activity of the triesters and the parent nucleoside **27** proved to be equipotent. A considerable loss (1000- to 200-fold, respectively) in activity was observed for compounds in L1210/TK$^-$ and CEM/TK$^-$ cells compared to the wild-type cell line (Table 3). In addition, the antithymidylate synthase activity was determined in a specific inhibition assay. As before, *cyclo*Sal-FdUMP triesters proved to be 10-fold less efficient than FdU independently if L1210/O cells or mutant L1210/TK$^-$ cells were used.

It should be mentioned that in the CEM/O assay, 5-FU showed only an IC_{50} of 0.7 μ*M*, and thus 5-FU is considerably less active (23-fold) than the *cyclo*Sal-compounds.

Nevertheless, taken together, from these data it is obvious that inefficient cellular nucleotide delivery took place. One possibility for the lack of activity was excluded. In contrast to the anti-HIV nucleoside analogs used so far, FdU possess a hydroxyl group at the 3′-position and therefore an internal nucleophile. This hydroxyl group may attack the phosphorus atom instead of the external nucleophile. This would still lead to the cleavage of the *cyclo*Sal-mask but with formation of 3′,5′-cyclic FdUMP instead of

Table 3
Antitumor Activity of the *cyclo*Sal-FdUMP Triesters in Different Cell Lines

		Antiviral activity IC_{50} (μM)[a]						
Compound	*Substance X*	*L1210/ 0*[b]	*L1210/ TK*$^{-}$[b]	*FM3A/ 0*[b]	*FM3A/ TK*$^{-}$[b]	*Molt4/ C8*[c]	*CEM/ 0*[c]	*CEM/ TK*$^{-}$[c]
a	5-Cl	0.0041	4.3	0.077	3.00	20.2	0.054	10.9
b	H	0.0057	4.2	0.012	3.07	20.3	0.070	≥5.7
c	3-Me	0.0077	4.9	0.12	3.25	20.9	0.029	4.85
d	3,5-DiMe	0.0081	3.8	0.18	4.63	17.7	0.072	11.5
FU	—	—	—	—	—	—	0.7	—
FdU **27**	—	0.0034	3.0	0.0096	1.98	15.5	0.058	4.22

[a]50% inhibitory effect.
[b]Murine leukemia cells.
[c]Human T-lymphocyte cells.

FdUMP. This cyclic diester cannot act as an inhibitor of thymidylate synthase. However, all data obtained from chemical hydrolysis studies exclude the formation of cyclic FdUMP.

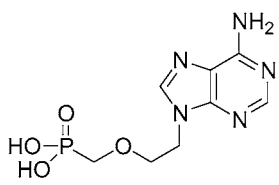
One limitation may be the cellular uptake of the triesters. Evidence for this assumption may be taken from the following experiment: The aforementioned thymidylate synthase assay was repeated in permeabilized L1210/O cells. As test compounds, FdU **27**, FdUMP, and 5-Cl-*cyclo*Sal-FdUMP were used. The IC_{50} values were 2.50, 0.15, and 0.30 μ*M*, respectively. Here, the triester clearly showed comparable biological activity to FdUMP and a higher potency compared to FdU **27**. This experiment proves that FdUMP and not FdU is released from the pronucleotide and may point indirectly to problems during uptake in nonpermeabilized cells for some unknown reasons.

However, as for the corresponding AZTMP compounds, a competing dephosphorylation cannot be excluded that led to fast intracellular clearance of FdUMP released from the pronucleotide. Indeed, it was shown that FdUMP is a very good substrate for 3′,5′-(deoxy)nucleotidase *(50)*.

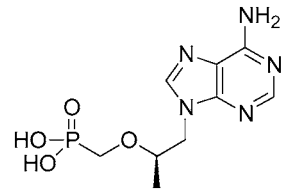
As in the case of AZTMP pronucleotides, other reported pronucleotides of FdUMP also lost their activity in the mutant TK-deficient cell lines. Again, this clearly points to an intrinsic problem during FdU metabolism.

2.4.3. Application of the *cyclo*Sal Approach to Antiviral Acyclic Nucleoside Phosphonates

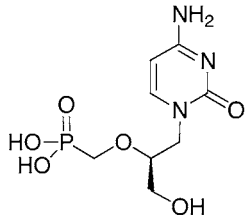
Nucleoside phosphonates represent a further class of interesting antiviral agents *(52)*. The most prominant compounds are 9-(2-phosphonylmethoxyethyl)adenine **31** (PMEA, adefovir), (*R*)-9-(2-phosphonyl- methoxypropyl)



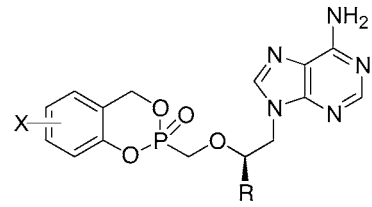
PMEA **31**



PMPA **32**



HPMPC **33**



34a: X = 3-*t*Bu, R = H
34b: X = 3.5-*t*Bu, R = H
35a: X = 3-*t*Bu, R = Me
35b: X = 3.5-*t*Bu, R = Me

Fig. 11. Structural formulae for acyclic nucleoside phosphonates PMEA, PMPA, and HPMC and the *cyclo*Sal-derivatives of PMEA and PMPA.

adenine **32** (PMPA, tenofovir) and (*S*)-9-(3-hydroxyphosphonylmethoxypropyl)cytosine **33** (HPMPC, cidofovir) (Fig. 11).

De Clercq and Holy reported in 1986 on the antiviral activity of such compounds against DNA viruses, which started a tremendous effort to find highly potent derivatives of this class of antiviral agents *(54)*. Mostly, these nucleoside phosphonates exhibited a broad antiviral activity spectrum; for instance, PMEA and PMPA were found to be active not only against HIV but also against DNA viruses like hepatitis B virus. HPMPC shows a broad spectrum of activity against DNA viruses such as the whole herpes family, pox virus, and papillomavirus. HPMPC is used clinically against cytomegalovirus (CMV) retinitis and is sold under the name Vistide®.

The mechanism of action of these nucleoside phosphonates is identical to that of the nucleoside analogs. They have to be converted into the corresponding nucleoside phosphonate diphosphates, the nucleoside triphosphate analog. However, because of the present phosphonate group, the parent compounds are analogs of the nucleoside monophosphates and thus do not need the first kinase-catalyzed conversion. On the other hand, the compounds are highly polar because of the charged phosphonate group, and

the bioavailability and cellular uptake are limited. Thus, the aim of prodrugs of these nucleoside phosphonates is to enhance membrane uptake and not an enzyme bypass as in the case of the nucleoside analogs.

A few prodrugs of nucleoside phosphonates are known: the bis(POM)-PMEA (adefovir dipivoxil, Hepsera®) *(55,56)*, bis(POC)-PMPA (tenofovir diisoproxil, Viread®) *(57,58)*, bis(SATE)-PMEA *(59)*, bis(AB)-PMEA *(60)*, and arylphosphoramidate prodrugs of PMEA and PMPA *(61)*. The first two lipophilic prodrugs are approved by the Food and Drug Administration for clinical use against hepatitis B virus and HIV, respectively. In all given examples, the parent drug is released by enzymatic activation.

The application of the *cyclo*Sal approach to this class of compounds deserves an entirely different chemical synthesis pathway. It was principally not possible to introduce the *cyclo*Sal moiety already attached to the phosphate group. Therefore, the *cyclo*Sal group was attached to the nucleoside phosphonates directly. However, it turned out that this approach was not as easy as it seemed. Numerous attempts failed, but finally the *in situ* conversion of the dialkyl esters of the parent phosphonates into the dichloride and subsequent one-pot esterification with the salicyl alcohol, leading to **34** and **35**, was successful. In the case of PMPA, direct esterification occurred with the salicyl alcohol using intermediate active esters.

Studies regarding the hydrolysis properties of the *cyclo*Sal-PMEA and PMPA derivatives showed a surprising effect: The half-lives of compounds **34** and **35** were 15-fold lower compared to the corresponding *cyclo*Sal-d4TMP triesters (e.g., 3,5-*t*butyl-*cyclo*Sal-PMPA: $t_{1/2}$ = 5.91 h; and 3,5-*t*butyl-*cyclo*Sal-d4TMP: $t_{1/2}$ = 91 h at pH 7.3 in phosphate buffer, 37°C). However, the hydrolysis in the case of the PMPA derivative was highly selective to PMPA without any trace of the formation of phenyl phosphonate monoester. In comparable hydrolysis studies of 3,5-*t*butyl-*cyclo*Sal-d4TMP, 35% of the corresponding phenyl phosphate diester was formed.

Further hydrolysis studies in CEM cell extracts proved that the compounds are not cleaved by enzymes present because the detected stability data were identical to those found in the chemical hydrolysis assays.

Moreover, it was interesting to note that the *cyclo*Sal-nucleoside phosphonates were entirely noninhibitory to human butyrylcholinesterase (BChE); for some, *cyclo*Sal-d4TMP triesters were inhibitory to that enzyme (3-*t*butyl-*cyclo*Sal-PMPA: $IC_{50} \geq 50$ μ*M*; and 3-*t*butyl-*cyclo*Sal-d4TMP: IC_{50} = 4.2 μ*M* after 5-min incubation). In addition, both compounds proved to be noninhibitory to human acetylcholinesterase (AChE) *(62)*.

Finally, the anti-HIV activity of the *cyclo*Sal-nucleoside phosphonates has been determined in wild-type CEM cells as well as in CEM/TK-deficient cells. The EC_{50} values are summarized in Table 4.

Table 4
Anti-HIV Data of the *cyclo*Sal-Nucleoside Phosphonates of PMEA and PMPA in CEM Cells

			Antiviral activity EC_{50} (μM)[a]				
			CEM/O		*CEM/TK⁻*	*Cytotoxicity*	
Compound	*Substance X*	*$t_{1/2\ at}$ at pH 7.3*	*HIV-1*	*HIV-2*	*HIV-2*	*CC_{50} (μM)*[b]	*IC_{50} (BChE)*[c]
34a	3-*t*-butyl	4.06	3.0 ± 1.4	4.5 ± 0.7	4.0 ± 1.4	29.3 ± 6.5	>50
34b	3,5-*t*-butyl	3.05	5.5 ± 0.7	5 - ≥ 10	4.5 ± 0.7	16.8 ± 0.6	>50
PMEA	—	—	4.0	3.5 ± 0.7	3.5 ± 0.7	≥ 250	—
35a	3-*t*-butyl	3.39	2.1 ± 1.3	1.6 ± 0.6	1.65 ± 0.49	116 ± 11.3	>50
35b	3,5-*t*-butyl	5.91	1.1 ± 0.4	1.0 ± 0.57	0.85 ± 0.21	97.9 ± 5.8	>50
PMPA	—	—	10.5 ± 6.4	10.0	10.0	50.0 ± 12.9	—

[a]50% effective concentration.
[b]50% cytotoxic concentration.
[c]50% inhibitory effect to human butyrylcholinesterase (BChE).

*Cyclo*Sal-nucleoside phosphonates showed considerable anti-HIV activity against HIV-1 and HIV-2 in wild-type CEM cells as well as in the TK-deficient cells. The EC_{50} values observed were two- to fourfold lower compared to those of the parent nucleoside phosphonates PMEA and PMPA. Two known prodrugs [bis(POM)-PMEA and bis(POC)-PMPA] were tested in the same series for comparison. Both compounds were more active than the *cyclo*Sal derivatives but also showed a considerable increase in the toxicity (CC_{50} value). A possible reason for the lower activity of the *cyclo*Sal derivatives might be the lower chemical stability of these derivatives. In comparative hydrolysis studies, bis(POM)-PMEA showed a half-life of 16 h, while the most stable *cyclo*Sal-PMEA derivative showed a half-life of only 4 h.

2.5. Application of the cyclo*Sal-Pronucleotides Against DNA Viruses*

DNA viruses are also an attractive target for the application of pronucleotides. In contrast to ribonucleic acid viruses like HIV, these viruses do not rely on reverse transcription of their genome prior to replication. Therefore, the target is not RT but a viral DNA polymerase. Moreover, some of the known antivirals against DNA viruses are not monophosphorylated by cellular TK but by a virus-encoded TK *(63)*. Often, drug-resistant virus strains are selected in vivo. One reason for this resistance seems to be associated with a downregulation of the expression of viral TK *(64)*. However, not all the virus types belonging to the herpesvirus family express viral TK activity (e.g., Epstein-Barr-virus [EBV] and CMV. Therefore, the *cyclo*Sal approach has been applied to the broad-spectrum acyclic purine nucleoside acyclovir (ACV; **28**) and to the pyrimidine-modified nucleoside 5-[(*E*)-2-bromovinyl]-2′-deoxyuridine (BVdU or brivudin) (**29**; Fig. 9).

2.5.1. *Cyclo*Sal-Pronucleotides of ACV

Among the most active and most broadly applicable nucleoside antivirals in this area is the purine-bearing acyclic nucleoside analog ACV (**28**) *(65–68)*. ACV is a potent inhibitor of HSV-1, HSV-2, but inhibits to a lesser extent varicella zoster virus (VZV), CMV, and EBV. ACV acts in its triphosphate form as a chain terminator (because its incorporation into DNA does not allow further chain elongation) or acts as inhibitor of the HSV DNA polymerase (K_i value for ACVTP vs the natural substrate deoxyguanosine 5′-triphosphate of 0.1 μ*M*). Although ACV is preferentially phosphorylated by a viral TK, it is not a particularly good substrate for HSV-TK, and the rate of phosphorylation is reckoned to be slow. Furthermore, ACVTP has a relatively short intracellular half-life of 0.7 h. More important, resistance to ACV seems to be associated with downregulation of the expression of viral TK.

The most common mutation is the selection of mutants deficient in TK activity or in mutants that express TKs with altered substrate specificity.

The chemical synthesis of 3-methyl-*cyclo*Sal-ACVMP triester may be carried out using the chlorophosphite method *(69,70)*. However, better results were obtained when the exocyclic amino group of the guanine residue was protected by dimethoxytritilation prior to the phosphitilation reaction using the phosphoramidite strategy with pyridinium chloride as activating agent. Reproducible yields of 80% were obtained. The protecting group has been cleaved by acid treatment of the N^2-blocked *cyclo*Sal-triester. The *cyclo*Sal-ACVMP triester was obtained as racemic mixtures. Chemical hydrolysis again showed selective delivery of ACVMP.

Antiviral evaluation showed an EC_{50} for the parent nucleoside ACV **28** of 0.62 μ*M* against HSV-1/TK^+ in Vero cells. As expected, ACV lost its activity in Vero cells infected with HSV-1/TK^- (EC_{50} = 58 μ*M*). Strikingly, 3-methyl-*cyclo*Sal-ACVMP showed identical antiviral activity values of 0.47 μ*M* and 0.51 μ*M* in the same HSV-1 TK^+ and TK^- systems, respectively, and an EC_{90} of 1.62 μ*M* against the mutant virus strain without increasing the toxicity. Again, the complete retention of activity clearly proves that ACVMP is delivered to the cells by the pronucleotide.

A comparable result was obtained for the antiviral activity against VZV. 3-Methyl-*cyclo*Sal-ACVMP showed an antiviral activity of EC_{50} of 4.1 μ*M* and 1.2 μ*M* against two wild-type virus strains (plaque reduction assay). This activity was completely retained in cells infected with VZV/TK^- (EC_{50} = 7.6 μ*M*) without changing the cell morphology (MCC > 200 μ*M*). As expected, ACV showed activity against the wild-type viruses but lost its antiviral potency in the mutant virus (YS/R) strain. Furthermore, 3-methyl-*cyclo*Sal-ACVMP exhibited antiviral activity against two VZV/TK^- strains (EC_{50} = 9–13 μ*M*); ACV was entirely inactive. As above, viral thymidylate kinase clearly is not involved in the activation into ACVDP. Here, cellular guanosine monophosphate kinase is involved in the phosphorylation.

The situation was somewhat different when 3-methyl-*cyclo*Sal-ACVMP was tested against CMV. Although ACV itself was completely inactive against two virus strains (EC_{50} > 200 μ*M* against the AD-169 and the Davis strain), the triester exhibited activity against both virus strain at EC_{50} of 13 μ*M* and 9 μ*M*, respectively, without changing the cell morphology (MCC > 200 μ*M*). This example clearly shows again the potential of the *cyclo*Sal-phosphate triester concept or—more generally—the potential of a pronucleotide: It converts a nucleoside analog that has no antiviral activity against a certain virus into a bioactive compound.

Finally, *cyclo*Sal-triester proved to be a potent and selective inhibitor of EBV DNA synthesis and EBV capsid antigen expression.

Further evidence for an effective intracellular delivery of ACVMP from the triester could be taken from the observed genotoxic effects caused by

3-methyl-*cyclo*Sal-ACVMP in wild-type Chinese hamster ovary (CHO) cells. The triester caused sister chromatid exchange and abberrations in this cell line in a concentration-dependent manner. Quantitatively comparable rates were found for ACV itself in CHO/TK^+ gene-transfected CHO cells. This clearly proves that ACVMP was liberated in the former case.

2.5.2. Application of the *cyclo*Sal Approach to the Antiherpes Drug Brivudine

As mentioned, the *cyclo*Sal concept has been applied to the nucleoside analog BVdU (brivudin, **29**), which is a very potent and highly selective inhibitor of the replication of HSV-1 and particularly VZV *(71)*. By contrast, BVdU is not markedly active against HSV-2 and EBV. Again, selectivity as inhibitor primarily depends on specific activation by HSV-encoded TK to the mono- and diphosphate and finally to the triphosphate by cellular enzymes *(72)*. BVdU triphosphate (BVdUTP) can act as either an inhibitor of the cellular DNA polymerase or an alternate substrate that would render the DNA more prone to degradation when incorporated in DNA *(73)*.

Some limitations for the use of BVdU are known: There is lack of activity during virus latency because of missing viral TK; drug-resistant virus strains are known; and BVdU will be enzymatically degraded to the nucleobase 5-[(*E*)-2-bromovinyl]uracil within 2–3 h from the bloodstream. Moreover, it has been shown that EBV does not express an HSV-like TK, and this may be the reason why BVdU is inactive to inhibit EBV replication *(74)*.

Obviously, cytosolic TKs are unable to activate BVdU. The aim was to prove if the *cyclo*Sal concept is able to broaden the application of BVdU against infections caused by EBV *(75)*. Such infections play a significant role as secondary infection (e.g., in patients with acquired immunodeficiency syndrome). It should be mentioned that two reports of pronucleotides of BVDU were published previously, but both were unsuccessful *(76,77)*.

Two different series of *cyclo*Sal-BVdUMP triesters were synthesized: *cyclo*Sal-BVDUMP (**36**) having different substituents in the aromatic residue and a series of 3′-*O*-esterified 3-methyl-*cyclo*Sal-derivatives (**37** and **38**) *(78,79)*. As 3′-*O*-modification, different lipophilic carboxylic acids (**37**) as well as α-amino acids (**38**) were used (Fig. 12).

The compounds were prepared using 3′-*O*-levulinylated BVdU or 3′-*O*-esterified BVdU derived from *N*-Boc-α-aminoacids like L-/D-alanine, L-phenylalanine, and carboxylic acids. These compounds were phosphorylated using the phosphoramidite/oxidation method. The levulinyl protection group was removed by treatment of the triester with hydrazine hydrate. The *N*-Boc protecting group was removed by treatment with trifluoroacetic acid.

First, chemical hydrolysis studies proved clearly the selective delivery of BVdUMP **39** as the sole product without formation of 3′,5′-cyclicBVdUMP

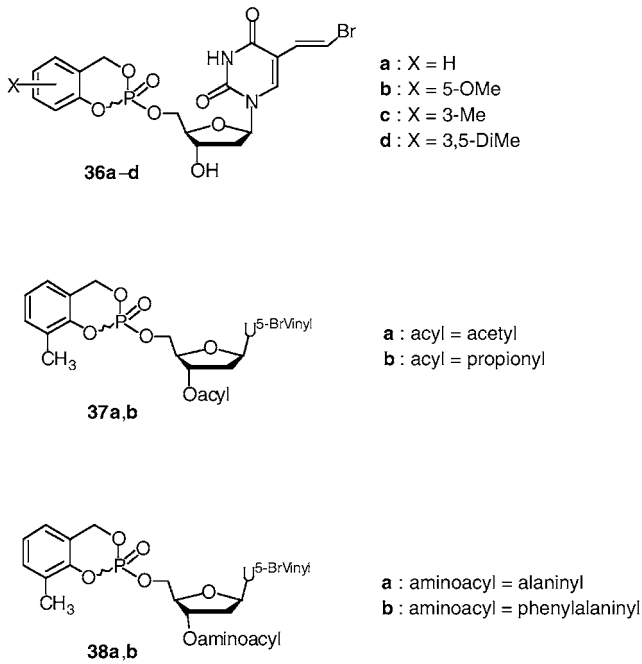


Fig. 12. Structural formulae of *cyclo*Sal-BVdUMP derivatives **36–38**.

40 (Fig. 13). By contrast, *cyclo*Sal-BVdUMP triesters modified by esterification with a carboxylic acid (**37**) led to the formation of the 3′-esterified BVdUMP **41**. However, the 3′-aminoacyl-esterified compounds **38** showed shorter half-lives compared to the others, but to our surprise the main product was 3-methyl-*cyclo*Sal-BVdUMP **36c** and not the corresponding 3′-aminoacyl-esterified BVdUMP **41**. 3-Methyl-*cyclo*Sal-BVdUMP **36c** then hydrolyzed as before to yield BVdUMP. Consequently, triesters **36** and **38** should act as sources for BVdUMP **39**.

First, 3-methyl-*cyclo*Sal-BVdUMP **36c** as well as the 3′-*O*-acetyl derivative **37a** were tested for inhibition of VZV replication. BVdU **29** proved to be highly active against VZV/TK$^+$, with EC_{50}s of 0.033 μ*M* and 0.010 μ*M* using the YS and the OKA strains, respectively. As expected, this activity was completely lost when VZV/TK$^-$ (YS/R and O7/1 strains; > 200 μ*M*) was used. Interestingly, both 3-methyl-*cyclo*Sal-BVdUMP triesters **36c** and **37a** showed comparable anti-VZV activity compared to the parent. However, both also lose all the antiviral activity against the VZV/TK$^-$ strains (EC_{50} > 50 μ*M*). This led to the conclusion that only the VZV/TK– associated viral thymidylate kinase activity is involved in the intracellular

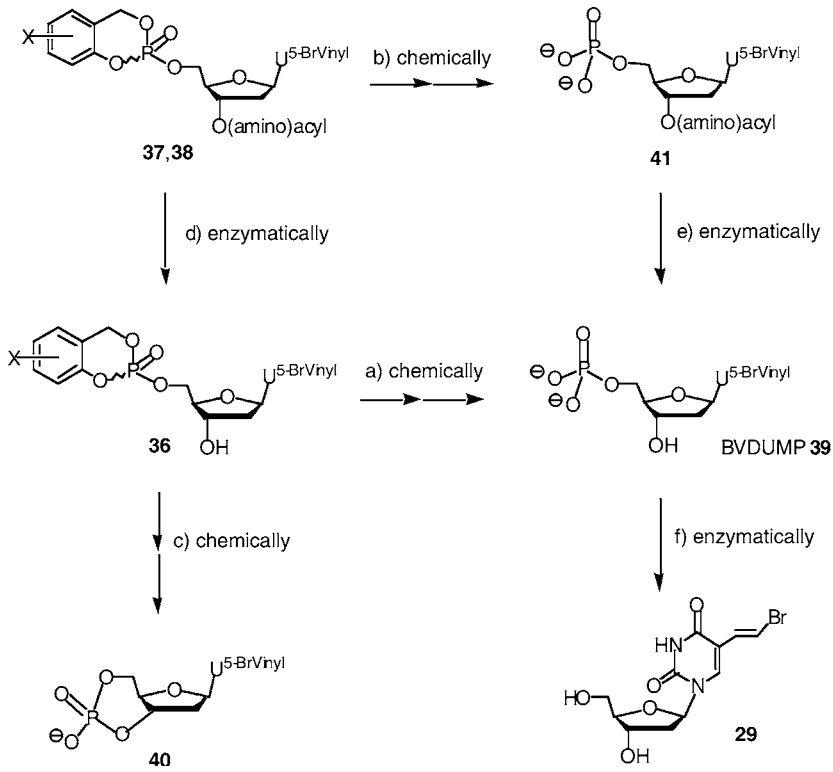


Fig. 13. Hydroysis pathways of *cyclo*Sal-BVdUMPs **36–38**.

formation of BVdUDP. Thus, cellular enzymes are unable to phosphorylate BVdUMP.

Much more interesting was the result obtained in the assays against inhibition of EBV replication in P3HR-1 cells *(50)*. BVdU itself was entirely inactive (EC_{50} > 100 μ*M* in the EBV DNA synthesis assay as well as the EB-VCA expression assay) *(74)*. It has been described that EBV does not possess an HSV-1-like TK. The lack of activity of BVdU clearly indicates that cellular kinases are unable to activate BVdU into its monophosphate BVdUMP. Strikingly, some of the *cyclo*Sal-BVdUMP triesters exhibited pronounced anti-EBV activity. The most active compound was the prototype 5-methoxy-*cyclo*Sal-BVDUMP **36b** (Table 5). As compared to the inactive BVdU **29**, triester **36b** was more than 166-fold more active and about 4-fold more active than the reference compound ACV.

The 3′-alanine *cyclo*Sal-triesters showed antiviral activity that was 5- (D-**38a**) and 12-fold (L-**38a**) lower compared to **36c**, but both were still

Table 5
Hydrolysis Data in Buffer, With Pig Liver Esterase (PLE) and Antiviral Activity of the *cyclo*Sal-BVdUMP Triesters 36–38 Compared to the Parent Nucleoside BVdU 29 and Acyclovir (ACV) 28

Compound	*Hydrolysis in aqueous $t_{1/2}$ (h) and product (%); PBS buffer; pH 7.3*	*Incubation with PLE $t_{1/2}$ (h) and product (%); PBS buffer; pH 7.3*	*Antiviral activity EC_{50} [μM][a]; DNA synthesis*	*Cytotoxicity CC_{50} [μM][b]*
36a	1.5; BVdUMP (100)	n.a.[c]	6.0	92
36b	2.3; BVdUMP (100)	n.a.[c]	1.8	137
36c	6.7, BVdUMP (100)	n.a.[c]	4.1	122
36d	8.6; BVdUMP (100)	n.a.[c]	11	143
37a	5.8, **41a** (100)	3.6; **36c** (100)	>85	110
37b	6.3, **41b** (100)	2.8; **36c** (100)	>150	>300
D-**38a**	1.39, BVdUMP (100)	0.3; **36c** (100)	9.5	83
L-**38a**	1.40; BVdUMP (100)	0.6; **36c** (100)	22	140
D-**38b**	1.68, BVdUMP (100)	n.a.[c]; **36c** (100)	7.6	66
L-**38b**	1.18, BVdUMP (100)	1.2; **36c** (100)	>100	78
ACV **28**	—	—	7.2	422
BVdU 29	—	—	>300	225

[a]EC_{50}: concentration required to reduce EBV DNA synthesis by 50%.
[b]CC_{50}: concentration required to reduce the growth of exponentially growing P3HR-1 cells by 50%.
[c]Not available.

significantly more potent than BVdU. It was interesting to realize that, in both cases, the attachment of L-amino acids led to lower antiviral activity compared to the unnatural D-amino acids. Surprisingly, both derivatives **37** esterified with carboxylic acids devoid of any antiviral activity (Table 5). Obviously, BVdUMP **39** released from the *cyclo*Sal-pronucleotide led to antiviral activity, and thus the phosphorylation of BVdUMP into the ultimate metabolite BVdUTP seems to be achieved by cellular enzymes. Such insights of biosynthetic pathways are only possible because *cyclo*Sal-pronucleotides led to intracellular delivery of the corresponding nucleotides. Thus, this is a good example that *cyclo*Sal-triesters may also be used as biochemical tools to study nucleoside metabolism.

To study the different behaviors of the mentioned 3-methyl-*cyclo*Sal-BVdUMP triesters, studies using isolated carboxyesterase *(80)* and cell extracts from CEM/O cells as well as P3HR-1 cells (the same cell line used for the antiviral evaluation) were performed.

In studies concerning possible enzymatic cleavage caused by intracellular (carboxy)esterases, the triesters **37a**,**b**, D/L-**38a**, and L-**38b** were treated with 50 units of pig liver esterase (PLE) in phosphate buffer at pH 7.3 as a model for enzymatic cleavage. The half-lives were markedly lower compared to previous studies in phosphate buffer at the same pH (Table 5). More important, the product was in all cases the 3′-*O*-deesterified 3-methyl-*cyclo*Sal-BVdUMP **36c**, which clearly proves efficient enzymatic cleavage and explains the shorter half-lives found in the study (given half-lives represent only the disappearance of the *cyclo*Sal-triester). After enzymatic deesterification, 3-methyl-*cyclo*Sal-BVdUMP released BVdUMP as in the chemical hydrolysis studies. In these incubation studies, 3′-*O*-esterified BVdUMP **41** was not detected. It is worth mentioning that the 3′-*O*-α-amino acid containing triesters **38** were cleaved to the same extent as the 3′-*O*-carboxylic acid bearing derivatives **37**. Hence, the reason for the significant differences in antiviral activity remains unclear (Table 5).

A few striking differences have been observed in P3HR-1 cell extracts. Triester **36c** was hydrolyzed to BVdUMP with a half-life comparable to that observed in chemical hydrolysis studies ($t_{1/2}$ = 8.9 h; Table 6). Thus, the degradation is chemically driven, not enzymatically. Again, no cBVdUMP **40** was detected *(81)*. In contrast to the chemical hydrolyses, BVdU **29** was observed also to a minor extent after 4 h (5%) and 8 h (22%), which was caused by an enzymatic dephosphorylation of BVdUMP by phosphatases/nucleotidases (path f, Fig. 13). In separate studies, BVdUMP was 13% converted to BVdU within 4 h.

The hydrolyses of the 3′-*O*-acyl derivatives **37** exhibited a clear difference with respect to the attached acid. For the 3′-*O*-Ac-derivative **37a**, enzymatic deesterification by carboxyesterases yielded the prototype **36c** as the major product (32%), but only 9% of BVdUMP was found. By contrast, the 3′-*O*-Prop derivative **37b** yielded the 3′-*O*-esterified BVdUMP derivatives **41c** as the major hydrolysis products because of the chemically driven cleavage of the *cyclo*Sal-mask (path b, Fig. 13) (Table 6).

The situation was significantly different for the α-amino acid-modified 3-methyl-*cyclo*Sal-BVdUMP triesters **38a**,**b**. All four compounds were rapidly deesterified to yield the prototype triester **36c** as the major product (40–44%). This result also differs considerably from the PLE studies described. The half-lives were dependent on the stereochemistry and the type of the α-amino acid. As for 3-methyl-*cyclo*Sal-BVdUMP **36c**, 33% BVdUMP was formed, starting from amino acid esters D/L-**38a**,**b** after incubation for 8 h (Table 6).

Comparable data were obtained in the CEM/O cell extract incubation. The major difference was that the triesters yielded higher amounts of BVdUMP after 8 h as in the P3HR-1 extracts. Again, the triesters modified

Table 6
Incubation Studies of the *cyclo*Sal-BVdUMP Triesters 36–38 in CEM and P3HR-1 Cell Extracts

	Hydrolysis in cell extracts[a], $t_{1/2}$ *(h) and (products [%])*	
Compound	*CEM cell extract*	*P3HR1 cell extract*
36a	2.3 [BVdUMP 82, **29** 18]	2.3 [BVdUMP 51, **29** 49]
36b	3.0 [BVdUMP 58, **29** 10]	3.2 [BVdUMP 42, **29** 24]
36c	4.8 [BVdUMP 50, **29** 8]	8.9 [BVdUMP 31, **29** 18]
36d	9.8 [BVdUMP 38, **29** 10]	10.9 [BVdUMP 22, **29** 15]
37a	4.1 [BVdUMP 22, **36c** 24, **41a** 17, **29** 4]	2.2 [BVdUMP 16, **36c** 29, **41a** 16, **29** 10]
37b	4.7 [BVdUMP 3, **36c** 3, **41b** 18, **29** <1]	4.5 [BVdUMP 3, **36c** 4, **41b** 13, **29** 2]
D-**38a**	0.8 [BVdUMP 52, **36c** 38, **29** 10]	0.5 [BVdUMP 33, **36c** 43, **29** 24]
L-**38a**	0.08 [BVdUMP 53, **36c** 37, **29** 10]	0.12 [BVdUMP 30, **36c** 44, **29** 26]
D-**38b**	n.d.[b] [BVdUMP 53, **36c** 35, **29** 12]	n.d.[b] [BVdUMP 35, **36c** 40, **29** 25]
L-**38b**	0.2 [BVdUMP 47, **36c** 39, **29** 14]	1.1 [BVDU BVdUMP MP 29, **36c** 46, **29** 25]

[a]Results shown in the table are at the end of an 8-h incubation; missing percentage to 100% is remaining *cyclo*Sal-phosphate triester.
[b]Not determined.

at C3′ with a carboxylic acid exhibited considerably lower amounts of BVdUMP (1–22%, Table 6). Moreover, dephosphorylation in CEM/O cell extracts BVdUMP proceeded to a lower extent compared to P3HR-1 cell extracts (13% vs 26%, respectively).

Taking these data together, the experiments with both extracts resulted in much higher formation of BVdUMP for triesters **36** and amino acid-modified triesters **38** compared to the carboxylic acid-modified derivatives. Extrapolated to a cellular situation, this would lead to higher BVdUMP concentrations and a higher degree of forward phosphorylation to the ultimate metabolite BVdUTP.

The promising anti-EBV data of some *cyclo*Sal-BVdUMP triesters (e.g., **36b**) proved that, by applying the *cyclo*Sal approach, the inactive BVdU can be converted into an anti-EBV active agent that is even more active than the reference ACV.

Moreover, it may be proven whether the *cyclo*Sal-BVDUMP triesters show also some anticancer activity. The US company New Biotics reported that aryl phosphoramidates of BVdU may act as potential anticancer drugs *(82)*.

2.6. Interaction of cyclo*Sal-Phosphate Triesters With Human AChE and BChE*

Because of their nature to be reactive organophosphate esters, *cyclo*Sal-phosphate triesters were studied concerning their potential inhibitory effect against human AChE. A large number of *cyclo*Sal-nucleotide triesters bearing different nucleoside analogs and substitution patterns in the aromatic ring have been studied concerning their ability to inhibit cholinesterases of different origins. It was shown that none of the tested triesters showed any inhibitory effect against the physiologically relevant human AChE (isolated enzyme) as well as against AChE from beef erythrocytes, calf serum, and electric eel (*Electrophorus electricus*) *(83)*. The assay was a modified Rappaport assay measuring the hydrolysis of acetylcholine catalyzed by these enzymes to yield acetic acid at pH 7.8. As indicator, *m*-nitrophenol is used. The progression of the reaction was therefore followed photometrically (absorption at $\lambda = 420$ nm) because of the decreasing amounts of *m*-nitrophenolate.

In contrast, inhibition of BChE has been observed for some triester derivatives in human and mouse serum. However, the physiological role of this enzyme is not known, and it is not related to nerve signal transfer. The *cyclo*Sal pronucleotides showed strong competitive inhibition with respect to the substrate acetylcholine chloride ($K_i/K_m \sim 2 \times 10^{-5}$) and acted by time-dependent irreversible inhibition of the human serum BChE. Detailed studies demonstrated that the inhibitory effect against BChE is highly dependent on the nature of the nucleoside analog, the substitution pattern

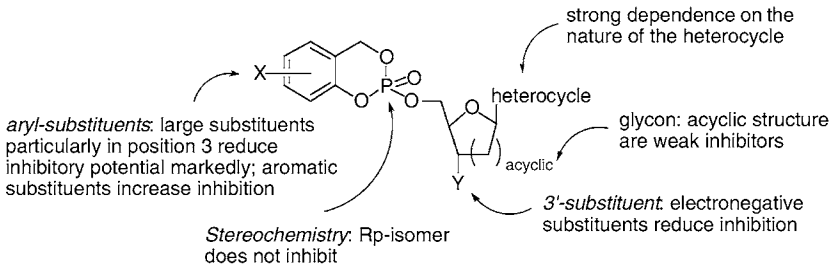


Fig. 14. Structure activity relationship of the interaction of *cyclo*Sal-triesters and butyrylcholinesterase.

in the *cyclo*Sal moiety, and particularly the stereochemistry at the phosphorus atom (Fig. 14) *(84)*.

A few *cyclo*Sal-NMP triesters have been separated into their diastereoisomers by semipreparative HPLC. These separated diastereomers were subjected to the assays with AChE from electric eel and BChE from human serum. Again, no inhibitory activity was found against AChE for both diastereoisomers. Much more interesting was the behavior of the triesters against human BChE. As expected, a marked difference in the inhibition was observed. In all cases, the *S*p-diastereoisomer was inhibitory toward the enzyme, while the *R*p-isomers were entirely noninhibiting. This behavior was independent of the nature of the nucleoside. To some extent, such stereoselectivity has also been reported for *Torpedo californica* AChE against E-2020 ((*R*,*S*)-1-benzyl-4-[(5,6-dimethoxy-1-indanon-2-yl)methyl]piperidine) (donepezil hydrochloride, Aricept®); the enzyme showed a fivefold higher affinity for the *R*-enantiomer (K_i = 3.3 n*M*) than for the *S*-enantiomer (K_i = 17.5 n*M*). In contrast, among the *cyclo*Sal-nucleotide prodrugs, the difference in IC_{50} between the two diastereomers was up to more than 200-fold.

Moreover, an interesting inverse correlation between the inhibitory potency and the antiviral activity was observed. As a rule, the most antivirally active diastereomers corresponded to the least BChE-inhibitory diastereomer. Thus, the *R*p diastereomer of 3-Me-*cyclo*Sal-d4TMP **2f**, although not inhibitory to human BChE at 50 µ*M*, was fivefold more anti-HIV active (0.087 µ*M*) than the *S*p diastereomer. In contrast, the *S*p diastereomer was markedly more inhibitory against BChE (0.24 µ*M*). In deciding which *cyclo*Sal anti-HIV prodrug should be chosen as a clinical candidate, the optimal configuration at phosphorus is *R*p, but it would be advantageous to choose *cyclo*Sal substituents with reduced anti-BChE activity of the *S*p configuration to avoid the need for separating the diastereomers.

In addition, the inhibitory potential of the *cyclo*Sal-triesters can be modulated by the substitution pattern of the aromatic ring. Particularly, bulky alkyl substituents in the 3-position led to considerable reduction of the

inhibitory potential. This effect was even more pronounced when two substituents were introduced in the 3- and 5-positions. Both diastereomers of 3,5-dimethyl-*cyclo*Sal-d4TMP **2g** and 3,5-*t*-butyl-*cyclo*Sal-d4TMP **2i** proved to be noninhibitory to BCHE. However, two properties of these two triesters were undesirable: (1) The chemical stability was very high (29 and 73 h, respectively; Table 1), and (2) there was formation of the phenyl phosphate diesters **17** instead of the benzyl phosphate diesters (8% and 34%, respectively; Table 1). However, by combination of different properties of various *cyclo*Sal-phosphate triesters reported in this chapter, a new *cyclo*Sal group attached to d4T **1** has been developed having four desirable properties: 3,5-*t*-butyl-6-fluoro-*cyclo*Sal-d4TMP **2j**.

As mentioned, the presence of two *t*-butyl groups is responsible for high stability of the triester but avoids the interaction with BChE. The fluorine atom in position 6 was introduced to achieve two goals: First, from previous studies using 6-chloro-*cyclo*Sal-d4TMP **2b**, it was concluded that an acceptor group in this position prevents the formation of the unwanted phenyl phosphate diester **17**. Thus, although two *t*-butyl groups are present, hydrolysis of **2j** should lead to highly selective delivery of d4TMP. Second, in the same study, the halogen atom in position 6 reduced the chemical stability of the *cyclo*Sal-triester (Table 1). Because of this effect, considerable decrease in the hydrolysis half-life for the triester **2j** compared to *t*-butyl- (**2h**) and di-*t*-butyl triester (**2i**) was expected.

The target triester **2j** was prepared starting from commercially available 3-fluorophenol. First, an acid-catalyzed alkylation with isobutene was carried out to give 4,6-*t*-butyl-3-fluorophenol **7** at a 70% yield. Reaction of this material with a basic formaldehyde solution led to the formation of the corresponding salicyl alcohol **11j** at a 71% yield. As shown in Fig. 7, the diol was reacted with PCl_3 and subsequently with d4T to give the target *cyclo*Sal-triester **2j** at a 47% yield.

As expected, the chemical hydrolysis of triester **2j** at pH 7.3 showed a significant reduction in stability (half-life 6.2 h; Table 1). In ^{31}P-NMR studies in imidazole/HCl buffer at pH 7.3 after 6 wk, only the formation of d4TMP was observed from triester **2j**, which proves that the introduced fluorine atom completely avoided the formation of the phenyl phosphate diesters **17** (Fig. 5). Incubation studies with human AChE showed that compound **2j** was not inhibitory to the enzyme. Moreover, incubations with human serum confirmed that the triester **2j** has almost no inhibitory potency toward BChE ($IC_{50(BChE)} = 48\ \mu M$). Finally, in antiviral tests using HIV-1- and HIV-2-infected wild-type CEM/O cells, triester **2j** exhibited pronounced antiviral potency that was even better than the activity of d4T **1**. Most important, the antiviral activity was fully retained in HIV-2-infected CEM/TK$^-$ cells, and thus triester **2j** achieved the TK bypass. Thus, so far this masking group is optimal *(39)*.

3. SECOND-GENERATION *cyclo*SAL-PHOSPHATE TRIESTERS

*3.1. "Lock-in" cyclo*Sal-d4TMP Triesters: A Conceptional Extension of the Trojan Horse Concept

The compounds described so far belong to the first-generation compounds of the *cyclo*Sal concept. Although these first-generation *cyclo*Sal-triesters led to convincing antiviral results, the use of a chemical hydrolysis mechanism may also have some limitations. We have clearly shown for several *cyclo*Sal-triesters that an enzymatic contribution to the hydrolysis could not be found in cell extracts or human serum. Moreover, we have clearly proven that the lipophilic *cyclo*Sal-triesters are able to penetrate the cell membranes and deliver nucleotides inside the cell. However, it cannot be excluded that the chemical hydrolysis also takes place outside the cells. In addition, although the compounds are lipophilic enough to migrate inside the cells, we cannot exclude that they also can diffuse in the opposite direction through the membrane. This would lead to the formation of an equilibrium. To avoid such a backdiffusion, we planned to convert the triesters inside the cell into a much more polar compound by an enzymatic reaction, thus preventing the efflux ("lock-in" mechanism; Fig. 15) *(85–87)*.

Therefore, we used a (carboxy)esterase reaction on a carboxylic ester attached to the *cyclo*Sal-aromatic ring via a linker. As a linker, a C2-alkyl chain was introduced. The ethylene spacer should separate the ester group efficiently from the aromatic ring to avoid an electronic effect on the hydrolysis of the phosphate triester moiety. Because of results obtained from the first-generation *cyclo*Sal-phosphate triesters, two positions in the *cyclo*Sal-moiety were selected for the introduction of the ester-spacer residue: the 3- and the 5-positions. As an ester group, two possibilities were used: (1) esterification of a *cyclo*Sal-acid (**42, 43**) with an alcohol and (2) esterification of a *cyclo*Sal-alcohol (**44, 45**) with a carboxylic acid, leading to compound series **46**, **47** and **48**, **49**, respectively (Fig. 16).

After enzymatic cleavage, the former triesters **46**, **47** should lead to the formation of a free carboxylic acid residue that should be deprotonated under physiological pH conditions; the triesters **48**, **49** would lead to a free alcohol group. Different esters bearing linear or branched alkyl groups were introduced, and the new concept first was applied to the nucleoside analog d4T.

The synthesis of the *cyclo*Sal-pronucleotides was done as for the prototype compounds **2** and **3**, respectively, using the above-mentioned reactive phosphorus(III) reagents. Again, phosphate triesters **46–49** were obtained in reasonable yields as diastereomeric mixtures *(85,86)*.

Originally, salicyl alcohols **11** were prepared from the corresponding salicylic aldehydes or acids by standard reduction protocols. However, the

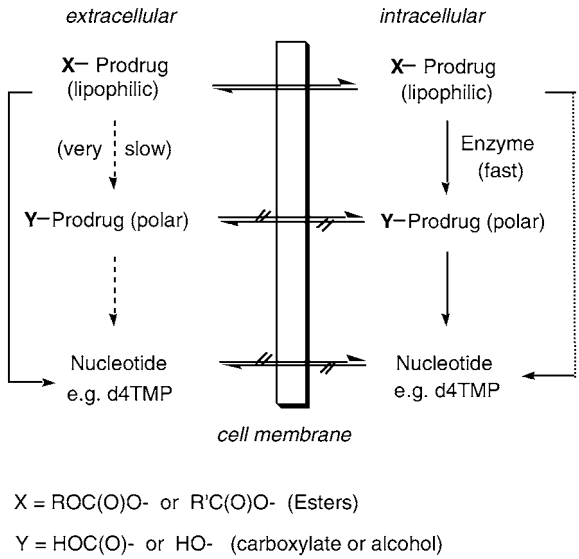
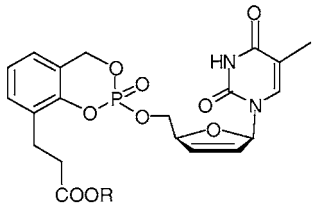


Fig. 15. "Lock-in" principle with the *cyclo*Sal-approach.

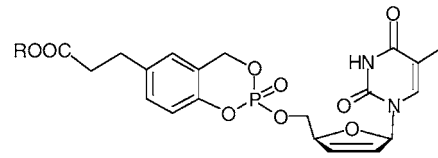
aldehydes/acids that were used here were not commercially available. Then, diols **11** were synthesized from the phenols by the mentioned selective *ortho*-hydroxymethylation according to Nagata. Unfortunately, there is no generally applicable method for the preparation of the substituted phenols bearing the ester-spacer residue, and thus each one was prepared separately. All triesters were studied concerning their properties to liberate the polar group by the enzymatic reaction as well as their chemical hydrolysis to yield d4TMP.

3.2. Proof of the Lock-In Concept

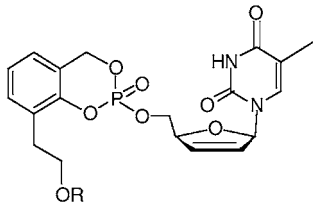
First, chemical hydrolysis studies were conducted. The results are summarized in Table 7. As expected, all new triesters were cleaved to yield d4TMP at pH 7.3 in 25 m*M* phosphate buffer. Half-lives were between 7.3 and 13.5 h in the case of the 3-modified triesters **43** and **45** and between 5.4 and 7.3 h for their 5-modified counterparts **47** and **49**. A comparison with the 3- (**2f**; $t_{1/2}$ = 17.5 h) and 5-methyl-*cyclo*Sal-d4TMP triesters ($t_{1/2}$ = 8.1 h) proved that the ethylene-spacer separates the electron-withdrawing ester group and the *cyclo*Sal aromatic ring sufficiently. Interestingly, both free acid-*cyclo*Sal-d4TMP triesters **42** and **43** showed up to twofold higher half-lives compared to the neutral ester-modified *cyclo*Sal-triesters. A possible explanation for this effect may be the presence of an overall negative charge on the molecule caused by the carboxylate formed at pH 7.3, which slows the nucleophilic reaction necessary for the initial cleavage step.



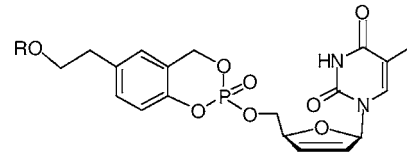
*cyclo*Sal-d4TMP acid **42**: R = H
*cyclo*Sal-d4TMP esters: **46a**: R = Me
46b: R = *i*Pr
46c: R = *t*Bu



*cyclo*Sal-d4TMP acid **43**: R = H
*cyclo*Sal-d4TMP esters: **47a**: R = Me
47b: R = *i*Pr
47c: R = *t*Bu



*cyclo*Sal-d4TMP alcohol **44**: R = H
*cyclo*Sal-d4TMP esters: **48a**: R = Ac
48b: R = Lev
48c: R = Piv



*cyclo*Sal-d4TMP alcohol: **45**: R = H
*cyclo*Sal-d4TMP esters: **49a**: R = Ac
49b: R = Piv

Fig. 16. Structural formulae of the "lock-in" *cyclo*Sal-derivatives **42–49**.

As expected, 3-*cyclo*Sal-triester **44**, having the hydroxyl group in the side chain, did not show such an increase in the half-lives ($t_{1/2}$ = 12.6 h; Table 7). This value is very close to the esters **48a**,**c** and the levulinyl-ester **48b** ($t_{1/2}$ = 12.5–13.6 h). In addition, the hydrolysis of 3-MePr-*cyclo*Sal-d4TMP **46a** followed by ^{31}P-NMR showed that also the phenyl phosphate diester was formed to a minor extent (~2%). However, this amount is considerably lower compared to the situation found for 3-methyl-*cyclo*Sal-d4TMP **2f** (5.5%). In both experimental setups for chemical hydrolysis, no cleavage of the carboxylic ester group was observed.

Next, studies in 25 m*M* phosphate buffer (pH 7.3) containing 50 units of PLE were carried out as a model for the enzymatic cleavage of the carboxylic esters (Table 7). It was observed that the half-lives of the 3-modified *cyclo*Sal-triesters **46** (methyl-, *i*-propyl, and *t*-butyl-esters) were slightly lower compared to the situation in pure phosphate buffer. However, no trace of the expected *cyclo*Sal-triester acid **42** could be detected. Thus,

Table 7
Antiviral Data of *cyclo*Sal-d4TMP Triesters 42–44 and 46–49

		Hydrolysis ($t_{1/2}$) at 37°C			*EC_{50} (μM)*[a]			
Compound	*Substance X*	*pH 7.3*[b] *[h]*[c]	*PLE*[d] *[h]*[c]	*CE*[e] *[h]*[c]	*CEM/O HIV-1*	*CEM/O HIV-2*	*CEM/ TK- HIV-2*	*CC_{50} (μM)*[f]
46a	3-MePr[g]	7.3	7.6	7.2	0.09	0.25	0.40	57
46b	3-*i*PrPr	12.5	9.3	10.1	0.14	0.80	1.50	54
46c	3-*t*BuPr	13.5	10.9	9.0	0.33	0.50	1.14	43
42	3-HPr	22.9	22.6	20.4	0.19	1.4	20	100
48a	3-AcEt[h]	13.6	8.3	1.9	0.16	0.33	0.15	40
48b	3-LevEt	12.5	n.d.[i]	1.9	0.13	0.15	0.33	58
48c	3-PivEt	13.1	8.2	6.6	0.16	0.70	0.40	55
44	3-HOEt	12.6	n.d.[i]	14.9	0.24	0.25	0.49	96
47a	5-MePr	7.0	4.0	5.7	0.33	1.05	1.20	58
47b	5-*i*PrPr	7.3	4.7	5.7	0.17	0.90	3.00	59
47c	5-*t*BuPr	7.1	5.5	5.3	0.18	2.40	4.00	42
43	5-HPr	12.5	9.1	11.4	0.14	0.80	50	76
49a	5-AcEt	6.3	5.8	2.6	0.15	0.80	0.55	44
49b	5-PivEt	5.4	4.8	5.5	0.23	0.90	0.60	22
d4T **1**	—	n.a.[j]	n.a.[j]	n.a.[j]	0.25	0.15	50	56

[a]Antiviral activity: 50% effective concentration.
[b]25 m*M* sodium phosphate buffer.
[c]Half-lives in hours.
[d]25 m*M* phosphate buffer + 50 units pig liver esterase (PLE).
[e]CEM cell extracts.
[f]Cytotoxicity: 50% cytostatic concentration.
[g]X = 3-MeOC(O)CH_2CH_2.
[h]X = 3-MeC(O)OCH_2CH_2.
[i]Not determined.
[j]Not available.

no enzymatic cleavage took place. In contrast, the acetyl and the pivaloyl triester **48a**,**c** showed a twofold decrease in the half-lives, and alcohol **44** was observed in the HPL chromatograms. Interestingly, the situation was different for the 5-modified *cyclo*Sal-d4TMPs. Here, none of the studied esters of the *cyclo*Sal-d4TMP acid **43** and the *cyclo*Sal-d4TMP alcohol **45** were hydrolyzed.

In conclusion, the outcome of these cleavage studies using PLE was disappointing. It should be added that first experiments using the methyl ester of the 3-propionate-*cyclo*Sal-mask showed extremely fast deesterification

under the same conditions. Therefore, we also expected fast ester hydrolysis in most of the cases of the *cyclo*Sal-triesters **46**. Nevertheless, d4TMP **9** was formed in all cases as a result of a chemical hydrolysis of the phosphate triester entity.

Further studies were done in CEM/O cell extracts. Triesters were incubated for 10 h at 37°C (Table 7). The acetyl- (**48a**) and the levulinyl ester of the *cyclo*Sal-d4TMP alcohol **48b** showed the most impressive result. These triesters were degraded six- to sevenfold faster compared to the buffer incubations, and the intermediate alcohol **44** was clearly detected in the chromatogram. So, the cleavage capacity of the extracts was markedly higher compared to the isolated enzyme PLE. However, the enzyme responsible for the ester hydrolysis in the extracts is not known. The pivaloyl ester **48c** was also cleaved (half-life dropped twofold); all the other esters (**46**) were again not cleaved. In the 5-ester-modified *cyclo*Sal-d4TMP series **47**, **49**, only the acetyl ester **49a** showed a two-fold decrease in stability. In conclusion, the acetyl esters were proven to be good substrates for the human esterases; all alkyl esters were not cleaved by the extracts. However, again the final products of the complete chemical hydrolysis of all triesters were d4TMP and the salicyl alcohols.

The reason for the insusceptibility of the alkyl esters is surprising because alkyl esters are often used in prodrug strategies, and at least the phosphoramidate approach developed by the McGuigan group is based on initial cleavage of such an ester group *(88)*. However, esters of natural α-amino acids were used in their case.

It was interesting to note that, in studies of the corresponding BVdUMP triesters, also the benzyl esters were cleaved in P3HR-1 cell extracts in addition to the acetyl ester (data not shown). So, obviously the cleavage is also dependent on the nature of the nucleoside analog. However, we cannot exclude that the extracts from P3HR-1 cells contain different esterases or different concentrations of esterases as CEM cell extracts.

3.3. Antiviral Activity

The triesters were tested for their antiviral potency in CEM/O cells infected with HIV-1 and HIV-2 as well as in HIV-2-infected CEM/TK$^-$ cells. The results are summarized in Table 7.

All *cyclo*Sal-triesters proved to be active in the wild-type cell line against both virus types. Only the 5-*t*Bu-ester **47c** was fivefold less active against HIV-2 compared to the reference compound d4T. More interesting are the results obtained in the TK-deficient CEM cells (CEM/TK$^-$). First, both *cyclo*Sal-triesters **42** and **43**, having the unesterified acid functionality in the side chain, lost all their antiviral activity in the mutant cell line. The reason is the charge at the carboxylate, which prevents efficient membrane

penetration. However, this result shows that, in the case of liberation of the carboxylate inside the cell by an enzymatic cleavage, the resulting polar product would stay trapped inside the cell. This is the first hint that the planned lock-in mechanism should work. Moreover, and in contrast to the parent d4T, all *cyclo*Sal-triesters bearing alkyl esters in the 5- or 3-position of the *cyclo*Sal aromatic ring retained their antiviral activity in the CEM/TK$^-$ cells (EC_{50} = 1–2 μ*M*), thus proving at least the TK bypass envisaged by these pronucleotides. Taking into account the results of the cell extract studies, no additional effect of the lock-in could be expected. However, *cyclo*Sal-triesters **48a** and **49a**, which were enzymatically cleaved in the extracts, showed lower EC_{50} values in the CEM/TK$^-$ cells. Although these triesters do not liberate a charged carboxylate but a more polar neutral alcohol group (**44** and **45**, respectively), it appears that these compounds show first evidence for successful trapping inside the cells.

4. CONCLUSION

To summarize, the *cyclo*Sal approach convincingly demonstrated the intracellular delivery of active nucleotides by a nonenzymatically induced cascade reaction. Compared to other pronucleotide systems, one advantage of *cyclo*Sal prodrugs is their easy synthesis and their reasonable solubility in aqueous media. Moreover, the drug/masking group ratio for *cyclo*Sal prodrugs is 1:1 only, whereas almost all enzymatically triggered nucleotide delivery systems show a ratio of 1:2 or 1:4. The 1:1 ratio may be favorable in terms of reducing potential toxicity. A further key feature of the *cyclo*Sal approach is the chemically driven release of the nucleotide. Thus, in contrast to the enzymatically driven systems, we are entirely independent to changing concentrations of the activating enzymes.

The *cyclo*Sal-pronucleotide system is further an ideal tool to study biochemical pathways in nucleoside metabolism. Modification of the *cyclo*Sal moiety and particularly of the benzyl position led to considerable differences in the hydrolysis pathway. This allowed further insights into the mechanism of degradation and at the same time gave clues to improve d4TMP delivery. It has considerably improved the antiviral activity of certain nucleoside analogs using the first-generation *cyclo*Sal-triesters. First attempts have been made to influence the equilibrium formed by a lipophilic phosphate triester through the membrane, resulting in the development of second-generation *cyclo*Sal-triesters having an ester-bearing side chain in the *cyclo*Sal aromatic ring. So far, only acetyl- and (partly) pivaloyl-esters were found to be substrates for cellular esterases. Nevertheless, the liberation of a negatively charged carboxylate would be advantageous to achieve an efficient intracellular trapping (lock-in) of the triester. Work to achieve this goal is currently in progress in our laboratories.

ACKNOWLEDGMENT

We would like to acknowledge the tremendous efforts of the following doctoral students for reported work: Martina Lorey, Andreas Lomp, Tina Knispel, Florence Munier, Thomas Hom, Jürgen Renze, Christian Ducho, Christian Müller, Manuel Ruppel, and Dalibor Vukadinovic-Tenter. Financial support by the Deutsche Forschungsgemeinschaft, Bonn, Germany, is gratefully acknowledged.

REFERENCES AND NOTE

1. Mitsuya H, Weinhold KJ, Furman PA, et al. 3′-Azidothymidine (BWA509U); an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in-vitro. Proc Natl Acad Sci USA 1985;82:7096–7100.
2. Balzarini J. Metabolism and mechanism of antiretroviral action of purine and pyrimidine derivates. Pharm World Sci 1994;16:113–126.
3. De Clercq E. Strategies in the design of antiviral drugs. Nature Rev Drug Discov 2002;1:13–25.
4. Herdewijn P, Balzarini J, De Clercq E. 2′,3′-Dideoxynucleoside analogues as anti-HIV agents. In: De Clercq E, ed. Advances in Antiviral Drug Design. Vol. 1. Greenwich, CT: JAI Press; 1993;233–318.
5. Furman PA, Fyfe JA St, Clair MH, et al. Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the triphosphate with human immunodeficiency virus reverse transcriptase. Proc Natl Acad Sci USA 1986;83:8333–8337.
6. Hao Z, Cooney DA, Hartman NR, et al. Factors determining the activity of 2′,3′-dideoxynucleosides in suppressing human immunodeficiency virus in vitro. Mol Pharmacol 1988;34:431–435.
7. Riddler SA, Anderson RE, Mellors JW. Antiretroviral activity of stavudine (2′,3′-didehydro-2′,3′-dideoxythymidine d4T). Antiviral Res 1995;27:189–203.
8. Hitchcock MJM. 2′,3′-Didehydro-2′,3′-dideoxythymidine (d4T), an antiviral agent. Antiviral Chem Chemother 1991;2:125–132.
9. Sommadossi JP. Comparison of metabolism and in vitro antiviral activity of stavudine vs other 2′,3′-dideoxynucleoside analogues. Infect Dis 1995;171(suppl. 2):88–92.
10. Balzarini J, Herdewijn P, De Clercq E. Differential patterns of intracellular metabolism of 2′,3′-didehydro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine. Two potent anti-human immunodeficiency virus compounds. J Biol Chem 1989;264:6127–6133.
11. Jones RJ, Bischofberger N. Nucleotide prodrugs. Antiviral Res 1995;27:1–17.
12. Freeman S, Ross KC. Prodrug design for phosphates and phosphonates. In: Ellis GP, Luscombe DK, eds. Progress in Medicinal Chemistry. Amsterdam: Elsevier Science B. V.; 1997; 34:111–147.
13. Meier C. Pronucleotides—recent advances in the design of efficient tools for the delivery of biologically active nucleoside monophosphates. Synlett 1998:233–242.
14. Wagner CR, Iyer VV, McIntee EJ. Pronucleotides: toward the in vivo delivery of antiviral and anticancer nucleotides. Med Res Rev 2000;20:417–451.
15. Périgaud C, Gosselin G, Imbach JL. Anti-HIV phosphotriester nucleotides. Basis for the rational design of biolabile phosphate protection. In: Torrence PF, ed.

Biomedical Chemistry/Applying Chemical Principles to the Understanding and Treatment of Disease. New York: John Wiley and Sons; 2000;55:115–141.
16. Carl PL, Chakravarty PK, Katzenellenbogen JA. A novel connector linkage applicable in prodrug design. J Med Chem 1981;24:479, 480.
17. Meier C. *Cyclo*Sal-pronucleotides—design of chemical trojan horses. Minirev Med Chem 2002;2:219–234.
18. Meier C. 2-Nucleos-5′-*O*-yl-4H-1,3,2-benzodioxaphosphinin-2-oxide—A new concept for lipophilic, potential prodrugs of biologically active nucleoside monophosphates. Angew Chem 1996;108:77–79; Angew Chem Int Ed Engl 1996;35:70–72.
19. Westheimer FH. Pseudorotation in the hydrolysis of phosphate esters. Acc Chem Res 1968;1:70–78.
20. Buchwald SL, Pliura DH, Knowles JR. Stereochemical evidence for pseudorotation in the reaction of a phosphoric monoester. J Am Chem Soc 1984;106:4916–4922.
21. Bunton CA, Mhala MM, Oldham KG, Vernon CA. The reaction of organic phosphates. Part III. The hydrolysis of dimethylphosphate. J Chem Soc 1960:3293–3301.
22. Routledge A, Walker I, Freeman S, Hay A, Mahmood N. Synthesis, bioactivation and anti-HIV activity of 4-acyloxybenzyl bis(nucleosid-5′-yl) phosphates. Nucleosides, Nucleotides 1995;14:1545–1558.
23. Shafiee M, Deferme SM, Villard AL, et al. New bis (SATE) prodrug of AZT 5′-monophosphate: in vitro anti-HIV activity, stability and potential oral absorption. J Pharm Sci 2001;90:448–463.
24. Saboulard D, Naesens L, Cahard D, et al. Characterization of the activation pathway of phosphoramidate triester prodrugs of stavudine and zidovudine. Mol Pharmacol 1999;56:693–704.
25. Meier C, Muus U, Renze J, Naesens L, De Clercq E, Balzarini J. Comparative study of bis(benzyl)phosphate triesters of 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T) and *cyclo*Sal-d4TMP—hydrolysis, mechanistic insights and anti-HIV activity. Antiviral Chem Chemother 2002;13:101–114.
26. Farrow SN, Jones AS, Kumar A, Walker RT, Balzarini J, De Clercq E. Synthesis and biological properties of novel phosphotriesters: a new approach to the introduction of biologically active nucleotides into cells. J Med Chem 1990;33:1400–1406.
27. Shimizu SI, Balzarini J, De Clercq E, Walker RT. The synthesis and biological properties of some aryl bis(nucleotid-5′-yl) phosphates using nucleosides with proven anti-HIV activity. Nucleosides Nucleotides 1992;11:583–594.
28. Meier C, Lorey M, De Clercq E, Balzarini J. *Cyclo*Sal-2′,3′-dideoxy-2′,3′-didehydrothymidine monophosphate (*cyclo*Sal-d4TMP): synthesis and antiviral evaluation of a new d4TMP delivery system. J Med Chem 1998;41:1417–1427.
29. Meier C, Lorey M, De Clercq E, Balzarini J. Cyclic saligenyl phosphotriesters of 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T)—a new approach. Bioorg Med Chem Lett 1997;7:99–104.
30. Glaser R. Aspirin. An ab *initio* quantum-mechanical study of conformational preferences and of neighboring group interactions. J Org Chem 2001;66:771–779.
31. Casiraghi G, Casnati G, Puglia G, Satori G, Terenghi G. Selective reactions between phenols and formaldehyde. A novel route to salicylaldehydes. J Chem Soc Perkin Trans I 1980:1862–1865.
32. Gross H, Rieche H, Matthey G. Neue Verfahren zur Darstellung von Phenolaldehyden. Chem Ber 1963;96:308–313.
33. Nagata W, Okada K, Aoki T. *ortho*-Specific α-hydroxyalkylation of phenols with aldehydes. An efficient synthesis of saligenol derivatives. Synthesis 1979:365–368.

34. No KH, Gutsche CD. Calixarenes. 8. Short, stepwise synthesis of *p*-phenylcalix[4]arene, *p*-phenyl-*p-tert*-butylcalix[4]arene, and derived products. J Org Chem 1982;47:2713–2719.
35. Meier C, Renze JT, Ducho C, Balzarini J. *Cyclo*Sal-d4TMP pronucleotides—structural variations, mechanistic insights and antiviral activity. Current Topic Med Chem 2002;2:1111–1121.
36. Mugnier F, Meier C. Phosphoramidite chemistry for the synthesis of *Cyclo*Sal-pro-nucleotides. Nucleosides Nucleotides 1999;18:941, 942.
37. Ducho C, Balzarini J, Meier C. Aryl-substituted and benzo-annelated *cyclo*Sal-derivatives of 2′,3′-dideoxy-2′,3′-didehydrothymidine monophosphate (d4TMP)—correlation of structure, hydrolysis properties and anti-HIV activity. Antiviral Chem Chemother 2002;13:129–141.
38. Meier C, Renze J, Balzarini J, De Clercq E. D4TMP delivery from 7-substituted *cyclo*Sal-d4TMPs. Nucleosides, Nucleotides Nucleic Acids 2003;22*:* 825–827.
39. Ducho C, Wendicke S, Görbig U, Balzarini J, Meier C. 3,5-t-Butyl-6-fluoro-*cyclo*Sal-d4TMP—a pronucleotide with a highly optimized masking group. Eur J Org Chem 2003:4786–4791.
40. Balzarini J, Aquaro S, Knispel T, et al. Cyclosaligenyl-2′,3′-didehydro-2′,3′-dideoxythymidine monophosphate: efficient intracellular delivery of d4TMP. Mol Pharmacol 2000;58:928–935.
41. Gröschel B, Meier C, Zehner R, Cinatl J, Doerr HW, Cinatl Jr. J. Effects of *Cyclo*Sal-d4TMP derivatives in H9 cells with induced AZT resistance phenotype. Nucleosides Nucleotides 1999;18:933–936.
42. Meier C, Knispel T, De Clercq E, Balzarini J. *Cyclo*Sal-Pro-nucleotides (*cyclo*Sal-NMP) of 2′,3′-dideoxyadenosine (ddA) and 2′,3′-dideoxy-2′,3′-didehydroadenosine (d4A): synthesis and antiviral evaluation of a highly efficient nucleotide delivery system. J Med Chem 1999;42:1604–1614.
43. Meier C, Knispel T, Marquez VE, Siddiqui MA, De Clercq E, Balzarini J. *Cyclo*Sal-pro-nucleotides of 2′-fluoro-ara- and 2′-fluoro-ribo-2′,3′-dideoxyadenosine (F-ara- and F-ribo-ddA) as a strategy to bypass a metabolic blockade. J Med Chem 1999;42:1615–1624.
44. Balzarini J, Haller-Meier F, De Clercq E, Meier C. Antiviral activity of cyclosaligenyl prodrugs of acyclovir, carbovir and abacavir. Antiviral Chem Chemother 2002;12:301–306.
45. Meier C, De Clercq E, Balzarini J. *Cyclo*Sal-3′-azido-2′,3′-dideoxythymidine monophosphate (*cyclo*Sal-AZTMP)—an unexpected failure of nucleotide delivery from a proven pronucleotide system. Eur J Org Chem 1998:837–846.
46. Balzarini, J, Herdewijn P, De Clercq E. Differential patterns of intracellular metabolism of 2′,3′-didehydro-2′,3′-dideoxythymidine and 3′-azido-2′,3′-dideoxythymidine, two potent anti-human immunodeficiency virus compounds. J Biol Chem 1989;264:6127–6133.
47. Lavie A, Schlichting I, Vetter IR, Konrad M, Reinstein J, Goody R.S. The bottle neck in AZT activation. Nature Med 1997;3:922–924.
48. Lavie A, Vetter IR, Konrad M, Goody RS, Reinstein J, Schlichting I. Structure of thymidine kinase reveals the cause behind the limiting step in AZT activation. Nature Struct Biol 1997;4:601–604.
49. Balzarini J, Naesens L, Aquaro S, et al. Intracellular metabolism of cyclosaligenyl-3′-azido-2′,3′-dideoxythymidine monophosphate, a prodrug of

3′-azido-2′,3′-dideoxythymidine (zidovudine). Mol Pharmacol 1999;56: 1354–1361.
50. Mazzon C, Rampazzo C, Scaini MC, et al. Cytosolic and mitochondrial deoxyribonucleotidases: activity wit substrate analogs, inhibitors and implications for therapy. Biochem Pharmacol 2003;66:471–479.
51. Lorey M, Meier C, De Clercq E, Balzarini J. New synthesis and antitumor activity of *cyclo*Sal-derivatives of 5-fluoro-2′-deoxyuridine monophosphate. Nucleosides Nucleotides 1997;16:789–792.
52. Lorey M, Meier C, De Clercq E, Balzarini J. *Cyclo*Saligenyl-5-fluoro-2′-deoxyuridine monophosphate (*cyclo*Sal-FdUMP)—a new prodrug approach for FdUMP. Nucleosides Nucleotides 1997;16:1307–1310.
53. Balzarini J, De Clercq E. Acyclic purine nucleoside phosphonates as retrovirus inhibitors. In: Jeffries DJ, De Clercq E, ed. Antiviral Chemotherapy, Chichester, UK: John Wiley and Son; 1995:41–63.
54. De Clercq E, Holy A, Rosenberg I, Sakuma T, Balzarini J, Maudgal PC. A novel selective broad-spectrum anti-DNA virus agent. Nature 1986;323:464–467.
55. Starrett JE Jr, Tortolani DR, Hitchcock MJM, Martin JC, Mansuri MM. Synthesis and in vitro evaluation of a phosphonate prodrug: bis(pivaloylosymethyl) 9-(2-phosphonylmethoxyethyl)adenine. Antiviral Res 1992;19:267–273.
56. Srinivas RV, Robbins BL, Connelly MC, Gong YF, Bischofberger N, Fridland A. Metabolism an in vitro antiretroviral activities of bis(pivaloyloxymethyl)-prodrugs of acyclic nucleoside phosphonates. Antimicrob Agents Chemother 1993;37:2247–2250.
57. Arimilli MN, Dougherty JP, Cundy KC, Bischofberger N. Orally bioavailable acyclic nucleoside phosphonate prodrugs: adefovir dipivoxil and bis(POC)PMPA. In: De Clercq E, ed. Advances in Antiviral Drug Design. Stamford, CT: JAI Press; 1999:69–91.
58. Robbins BL, Srinivas RV, Kim C, Bischofberger N, Fridland A. Anti-human immunodeficiency virus activity and cellular metabolism of a potential prodrug of the acyclic nucleoside phosphonate 9-*R*-(2-phosphonomethoxypropyl)adenine (PMPA), bis(isopropyloxymethylcarbonyl)PMPA. Antimicrob Agents Chemother 1998; 42:612–617.
59. Benzaria S, Pélicano H, Johnson R, et al. Synthesis, in vitro antiviral evaluation, and stability studies of bis(S-acyl-2-thioethyl) ester derivatives of 9-(2-phosphonometoxy)ethyladenine (PMEA) as potential PMEA prodrugs with improved oral bioavailability. J Med Chem 1996;39:4958–4965.
60. Glazier A, Yanachkova M, Yanachkov I, et al. Potent topical anti-herpes activity of a lipophilic phosphorus prodrug for the antiviral agent PMEA. Antiviral Res 1995;26:A306.
61. Ballatore C, McGuigan C, De Clercq E, Balzarini J. Synthesis and evaluation of novel amidate prodrugs of PMEA and PMPA. Bioorg Med Chem Lett 2001;11:1053–1056.
62. Meier C, Görbig U, Müller C, Balzarini J. *Cyclo*Sal-PMEA and *cyclo*Amb-PMEA—potentially new phosphonate prodrugs on the basis of the *cyclo*Sal-pronucleotide approach. J Med Chem 2005;48:8079–8086.
63. De Clercq E. Biochemical aspects of the selective antiherpes activity of nucleoside analogues. Biochem Pharmacol 1984;33:2159–2169.
64. Fields BN. Virology. Vol. 1. 2nd ed. New York: Raven Press; 1990:450, 451.
65. Elion GB. Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. Proc Natl Acad Sci USA 1977;74:5716–5720.

66. Wagstaff AJ, Faulds D, Goa KL. Acyclovir: a reappraisal of its antiviral activity, pharmacokinetic properties and therapeutic efficacy. Drugs 1994;47:153–205.
67. Vere Hodge RA, Sutton D, Boyd MR, Harden MR, Jarvest RL. Selection of oral prodrug (BRL 42810;famciclovir) for the antiherpesvirus agent BRL 39123 [9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine;penciclovir]. Antimicrobial Agents Chemother 1989;33:1765–1773.
68. Field HJ, Goldthorpe SE. Chemotherapy of herpes simplex virus infections: a laboratory perspective. In: Jeffries DJ, De Clercq E, eds. Antiviral Chemotherapy. New York: John Wiley and Sons; 1995:127–153.
69. Meier C, Habel L, Haller-Meier F, et al. Chemistry and anti-herpes simplex virus type 1 evaluation of *cyclo*Sal-nucleotides of acyclic nucleoside analogues. Antiviral Chem Chemother 1998;9:389–402.
70. Meerbach A, Klöcking R, Meier C, Lomp A, Helbig B, Wutzler P. Inhibitory effect of *cyclo*Saligenyl-nucleoside monophosphates (*cyclo*Sal-NMP) of acyclic nucleoside analogues on HSV-1 and EBV. Antiviral Res 2000;45:69–77.
71. De Clercq E, Descamps J, De Somer P, Barr PJ, Jones AS, Walker RT. (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine: a potent and selective anti-herpes agent. Proc Natl Acad Sci USA 1979;76:2947–2951.
72. Wutzler P. Antiviral therapy of herpes simplex and varicella-zoster virus infections. Intervirology 1997;40:343–356.
73. De Clercq E. In search of selective antiviral chemotherapy. Clinical Microbiol Rev 1997;10:674–693.
74. BVDU has been reported to inhibit EBV infections: Lin JC, Smith MC, Pagano JS. Effect of 12-*O*-tetra-decanoylphorbol-13-acetate on the replication of Epstein-Barr virus. 1. Characterisation of viral DNA. Antimicrob Agents Chemother 1985;27: 971–973, but these results could not be reproduced in our laboratories (*see* Table 2).
75. Anagnostopoulos I, Hummel M. Epstein-Barr virus in tumours. Histo-Pathol 1996;29:297–315.
76. Farrow SN, Jones AS, Kumar A, Walker RT, Balzarini J, De Clercq E. Synthesis and biological properties of novel phosphotriester: a new approach to the introduction of biologically active nucleotides into cells. J Med Chem 1990;33:1400–1406.
77. Herdewijn P, Charubala R, De Clercq E, Pfleiderer W. Synthesis of 2′-5′ connected oligonucleotides. Prodrugs for antiviral and antitumoral nucleosides. Helv Chim Acta 1989;72:1739–1748.
78. Meier C, Lomp A, Meerbach A, Wutzler P. *cyclo*Saligenyl-5-[(E)-2-bromovinyl]-2′-deoxyuridine monophosphate (*cyclo*Sal-BVDUMP) pronucleotides active against Epstein-Barr virus. ChemBioChem 2001;4:283–285.
79. Meier C, Lomp A, Meerbach A, Wutzler P. *Cyclo*Sal-BVdUMP pronucleotides: how to convert an antiviral-inactive nucleoside analogue into a bioactive compound against EBV. J Med Chem 2002;45:5157–5172.
80. Meier C, Lomp A, Meerbach A, Wutzler P. Synthesis, hydrolysis and anti-EBV activity of a series of 3′′-modified *cyclo*Sal-BVdUMP pronucleotides. Nucleosides Nucleotides Nucleic Acids 2001;20:307–314.
81. Lomp A, Meier C, Herderich M, Wutzler P. Evidence for cyclophosphate formation during hydrolysis of 3-methyl-*cyclo*Sal-PCVMP. Nucleosides Nucleotides 1999;18:943–944.
82. Larckey DB, Groziak MP, Sergeeva M, et al. Enzyme-catalyzed therapeutic agents (ECTA) design: activation of the antitumor ECTA compound NB1011 by thymidylate synthase. Biochem Pharmacol 2001;61:179–189.

83. Ducho C, Balzarini J, Meier C. Non-inhibition of acetylcholineesterase by *cyclo*Sal-nucleotides. Nucleosides Nucleotides Nucleic Acids 2003;22:841–843.
84. Meier C, Ducho C, Görbig U, Esnouf R, Balzarini J. Interaction of *cyclo*Sal-pronucleotides with cholinesterases from different origins—a structure-activity relationship. J Med Chem 2004;47:2839–2852.
85. Meier C, Ruppel MFH, Vukadinovíc D, Balzarini J. Second generation of *cyclo*Sal-pronucleotides with esterase-cleavable sites—the "lock-in"-concept. Nucleosides Nucleotides Nucleic Acids 2004;23:89–115.
86. Meier C, Ducho C, Jessen H, Balzarini J. Esterase-cleavable *cyclo*Sal-pronucleotides—the trapping concept. Coll Cech Chem Commun (Symp Ser.) 2005: 105–114.
87. Meier C, Ducho C, Jessen HJ, Vukadinovic-Tenter D, Balzarini J. Second generation *cyclo*Sal-d4TMP pronucleotides bearing esterase-cleavable sites—the trapping-concept. Eur J Org Chem 2006:197–206.
88. Saboulard D, Naesens L, Cahard D, et al. Characterisation of the activation pathway of phosphoramidate triester prodrugs of stavudine and zidovudine. Mol Pharmacol 1999;56:693–704.

16 Purine and Pyrimidine-Based Analogs and Suicide Gene Therapy

Zoran Gojkovic, PhD
and Anna Karlsson, MD, PhD

CONTENTS

SUMMARY

Nucleobase and nucleoside analogs are widely used chemotherapeutic agents in the treatment of cancer and viral diseases. These compounds inhibit or disrupt DNA synthesis, and as tumor cells usually divide more rapidly than normal cells, there is a narrow therapeutic window to be exploited. Suicide gene therapy delivers genes to the cancer cells, enabling them to convert relatively nontoxic prodrugs into active chemotherapeutic agents. With this strategy, drug activation occurs primarily in the cancer cells, thereby maximizing damage to the cancer cells while keeping the systemic toxicity low. A number of suicide gene systems utilizing nucleobase and nucleoside analogs have been described. The best-known and most studied examples are the herpes simplex virus thymidine kinase gene in combination with ganciclovir and the *Escherichia coli* cytosine deaminase gene, which activates 5-fluorocytosine. Additional promising genes for use in suicide systems include the bacterial purine nucleoside phosphorylase gene; different deoxyribonucleoside kinases, such as the multisubstrate insect nucleoside kinase; and several other genes acting on a variety of

From: *Cancer Drug Discovery and Development:*
Deoxynucleoside Analogs in Cancer Therapy
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

different analogs. The efficiency of the currently used suicide systems depends on various biological parameters, such as the potential of activation, degree of activation, and bystander effect. In addition to these parameters, in-depth understanding of the biopharmaceutical properties of the prodrugs and suicide system kinetics should be known before selecting a specific system. Each suicide system offers not only specific advantages but also some limitations, and full understanding of the system can help overcome a number of problems associated with this type of gene therapy. Effective tumor destruction also depends on the delivery systems. Various vectors, including liposomes, retroviruses, and different adenoviruses, have been used to transfer suicide genes to tumor cells. Several of these approaches have been successful in many early clinical trials. Advances in new suicide systems, improved modulation of existing systems, and cell-specific delivery will definitely improve the clinical efficacy of suicide gene therapy and hopefully lead to better cancer treatments.

Key Words: Acyclovir; cytosine deaminase; deoxynucleoside kinase; drug delivery system; fluorocytosine; ganciclovir; gene therapy; herpes thymidine kinase; suicide genes; thymidine kinase.

1. INTRODUCTION

Nucleoside and nucleobase analogs were among the first chemotherapeutic agents to be used for the medical treatment of cancer and viral diseases. Today, these compounds include a range of purine and pyrimidine derivatives with therapeutic activity in a variety of different tumors. Although used as first-line therapy for many cancer types, several potential disadvantages still remain. The most specific problem is insufficient therapeutic index, either because of the limited bioavailability in tumors with poor, unequal vascularization in the areas with necrosis or as a result of low selectivity in heterogenetic tumor cell populations. The lack of specificity often leads to emergence of drug-resistant subpopulations of cells, which ultimately results in an inadequate therapeutic effect. Therefore, it is not surprising that alternative strategies using gene therapy methods are under development to improve the therapeutic properties of these drugs. Combination therapy, with different subsets of analogs or other cytotoxic drugs, has been used to increase the cytotoxicity and activity of these drugs *(1,2)*. In general, tumors show greater susceptibility to multiple agents that simultaneously attack tumor cells by different mechanisms. Combinations of analogs and inhibitors of their degradative enzymes are also used to combat cancers *(3,4)*. Nevertheless, all these improvements showed quite limited success.

An increasing number of attempts are in motion to target therapeutic compounds to tumors selectively. These technologies include targeting cells with monoclonal antibodies *(5)* or the use of liposome formulation for

encapsulation of analogs *(6–8)*. In either case, the existing intravenous drugs are concentrated into the specific area of the body where the treatment is required. This in return maximizes the efficacy of the drug, lowers the dose requirement, and thus reduces the potential for side effects while still delivering the desired therapeutic effect. Rather than increasing the drug concentration at the tumor site, an alternative strategy is to kill cancer cells by the selective expression of a "suicide" gene. The suicide genes can be designed to cause either a direct or an indirect antitumor effect. *Toxin gene therapy* is the method by which the gene expresses toxic proteins that directly kill tumor cells. Examples of toxin suicide gene approaches include, for example, engineering cells with a diphtheria toxin A-chain (*DT-A*) gene *(9,10)*.

The therapy by which enzymes need to activate specific prodrugs to create the toxic effect is known as gene-directed enzyme prodrug therapy (GDEPT), gene prodrug activation therapy, or suicide gene therapy. Here, new functions that sensitize cells to drugs, at concentrations that would otherwise be harmless, are introduced. The expression of a suicide enzyme converts a relatively nontoxic prodrug into a potent cytotoxic metabolite that inhibits or disrupts DNA synthesis. The tumor cells subsequently die via necrotic or apoptotic pathways.

2. SUICIDE GENE SYSTEMS

There are many barriers to overcome in developing clinically useful suicide gene therapy. Effective tumor destruction depends on the chemistry of the prodrugs, the interaction between produced enzyme and administrated prodrug, the choice of the gene therapy vector, and finally specific targeting to tumor cells. Several factors critical to enzyme–substrate kinetics should be considered.

The "perfect" prodrug for suicide gene therapy should be rapidly activated by the enzyme. Then, issues like prodrug half-life, intracellular degradation, or clearance mechanisms will not be limiting factors, plus high concentrations of prodrug are unnecessary. Enzymes that are highly efficient in activating the prodrug should prove beneficial because gene transfer and gene expression are usually not high in in vivo setups. Because current delivery methods cannot transfer the gene into all tumor cells, the bystander effect, by which activated prodrug can kill neighboring tumor cells that are not expressing the suicide gene, is also required. The vectors, which deliver the transgenes to the tumor cells, must be carefully adapted to each specific suicide system and to the specific cancer type. The success of suicide cancer therapy also depends on how specifically cancer cells can be targeted and how high transfection rates can be achieved. All these key issues are described and discussed in the following sections.

2.1. Prodrugs

Nucleobases, nucleosides, and nucleotides are essential compounds for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis. These compounds serve as a general energy source (adenosine triphosphate [ATP] and guanosine 5′-triphosphate [GTP]), they are involved in phospholipid and polysaccharide biosynthesis and are components of many cofactors, vitamins, and allosteric activators. Therefore, it is not surprising that a variety of purine and pyrimidine derivatives has been synthesized with activity in both solid tumors and malignant disorders of the blood. Many biologically potent nucleobase and nucleoside analogs have limited clinical use because of their undesirable biopharmaceutical properties. Low bioavailability, caused by low permeability through biological barriers, such as the blood-brain barrier and the intestinal barrier, is often encountered. Undesirable physicochemical properties (e.g., charge, lipophilicity, hydrogen bonding potential, size) hinder the permeation of these drugs through biological barriers.

One of the most successful strategies to optimize all these characteristics is through the use of prodrugs *(11)*. The term *prodrug* is used to describe an agent that must undergo chemical or enzymatic transformation to the active or parent drug after administration. The metabolic product or parent drug can subsequently exert the desired pharmacological response. Nucleobase analogs, such as 5-fluorouracil (5-FU), enter human cells via both nonfacilitated diffusion and nonspecific carriers *(12)*. Analogs of the naturally occurring nucleosides are usually administered as prodrugs (e.g., in their unphosphorylated form) as the omission of the negative charges from the phosphate groups allows effective transport of the analog into the cell.

Several mammalian nucleoside transporter proteins have been identified so far *(13,14)*. They belong to two families: the equilibrative nucleoside transporters (ENTs) and the concentrative nucleoside transporters (CNTs). Once the nucleosides are inside the cell, they must be phosphorylated to exert a toxic effect. Phosphorylated analogs cannot freely diffuse across cell membranes, which greatly limits spreading of these drugs in surrounding tissues. Only a few of the nucleosides have been reported to accumulate in their mono- or diphosphorylated forms, and it is therefore generally assumed that the initial phosphorylation by salvage nucleoside kinases is the rate-limiting step in the activation process *(15)*. Accordingly, the initial phosphorylation by nucleoside kinases is well studied, and it is utilized in several suicide gene systems.

Nucleoside analogs and nucleobases are a pharmacologically diverse family, which includes cytotoxic compounds, antiviral agents, and immunosuppressive molecules (Fig. 1).

These drugs belong to the so-called antimetabolite family because they compete with naturally occurring compounds and interact with a large

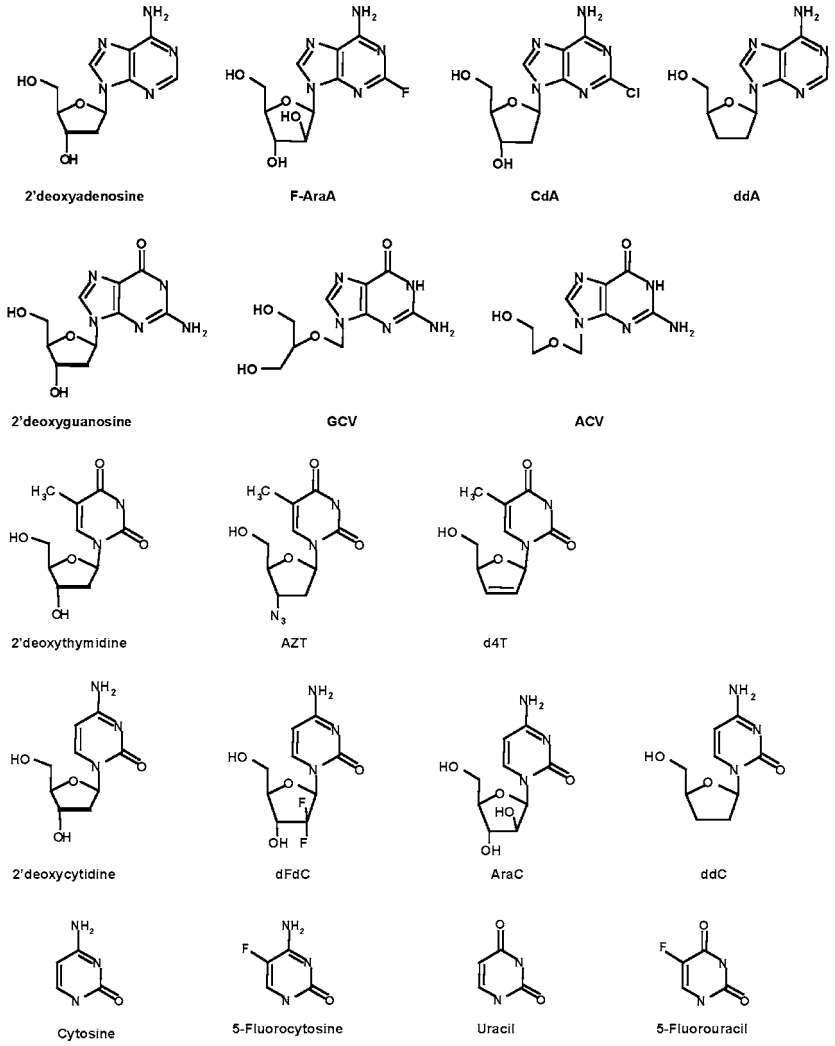


Fig. 1. Examples of naturally occurring nucleosides and nucleobases and some of the clinically used analogs (*see* text and Table 2 for chemical full names).

number of metabolic pathways and intracellular targets through which they exert their cytotoxic effect. The anticancer nucleosides include several analogs of physiological pyrimidine and purine nucleosides and nucleobases. The two primary purine analogs are cladribine (CdA, 2-chloro-2′-deoxyadenine) and fludarabine (F-ara-A, 2-fluoro-9-β-D-arabinofuranosyl-adenine). These drugs have mostly been used in the treatment of low-grade malignant

disorders of the blood. Among the currently available pyrimidine analogs, cytarabine (ara-C, 1-β-D-arabinofuranosylcytosine) is extensively used in the treatment of acute leukemia; gemcitabine (dFdC, 2′.2′-difluorodeoxycytidine) has activity in various solid tumors and some malignant hematological diseases; the fluoropyrimidines 5-FU and 5′-deoxy-5-fluorouridine (5-DFUR) in the form of its prodrug N^4-pentoxycarbonyl-5′-deoxy-5-fluorocytidine (capecitabine, Xeloda®) have shown activity in colorectal and breast cancers.

Antiviral drugs are extensively used and studied for suicide gene therapy applications. Acyclic nucleosides, such as ganciclovir (GCV) and acyclovir (ACV) are well known antiherpetic drugs activated by viral thymidine kinases. Anti-human immunodeficiency virus (HIV) compounds, like 2′,3′-didehydro-3′-deoxythymidine (d4T), and azidothymidine (AZT), which are phosphorylated by the same enzymes, might also find use in inhibiting DNA synthesis. Other approved HIV drugs, such as dideoxycytidine (ddC) or 2′-deoxy-3′-thiacytidine (3TC), have yet to be applied in combination with suicide therapy.

2.2. Suicide Genes

The most important and limiting factor in suicide gene therapy is the choice of the suicide gene. Numerous suicide gene/prodrug systems using a variety of drugs have been described so far. However, only suicide gene systems that activate nucleoside and nucleobase analogs are described here (Table 1).

The enzymes used for suicide gene therapy fall into two categories. The first group is comprised of enzymes of nonmammalian origin, with or without human homologs such as viral thymidine kinases (TKs) *(16–18)*, bacterial cytosine deaminase (CD) *(19)*, bacterial purine nucleoside phosphorylase (PNP) *(20)*, insect multisubstrate deoxynucleoside kinase (dNK) *(21)*, and prokaryotic xanthine-guanine phosphoribosyl transferase (XGPRT) *(22)* gene. The second category includes enzymes of human origin, such as deoxycytidine kinase (dCK) *(23)* and thymidine phosphorylase (TP) *(24)*.

The main advantage of the enzymes from the first category is their rather different substrate requirements compared to the human homologs. Disadvantages may be the potential of provoking an immune response in humans, although this may actually improve the therapeutic effect *(25)*. Enzymes from the second group will not induce an immune response, but the substrate kinetics of the transfected enzymes should first be modified, or the enzyme must be selectively delivered to the tumor cells to avoid unwanted prodrug activation in normal cells.

The suicide enzymes should activate their prodrugs to achieve at least a 100-fold increase in cytotoxic effect compared to the preactivated prodrug form *(26)*. Before choosing between various suicide systems, their efficiency should be known. However, the kinetic parameters (K_m, V_{max},

Table 1
Enzyme-Prodrug Systems Based on Nucleobase and Nucleoside Prodrug Analogs

Enzyme-prodrug system	*Origin*	*Prodrugs*	*Activated drug*
Cytosine deaminase (CD)	*E. coli* Yeast	5-Fluorocytosine (5-FC)	5-Fluorouracil (5-FU)
Deoxycytidine kinase (dCK)	Human	Cytosine arabinoside (ara-C)	Cytosine arabinoside monophosphate (ara-CMP)
Deoxyribonucleoside kinase (dNK)	*D. melanogaster*	Pyrimidine and purine 2-deoxynucleoside analogs	Pyrimidine and purine dNMP analogs
Purine nucleoside phosphorylase (PNP)	*E. coli*	Purine nucleosides	2-Fluoroadenine 6-Methylpurine
Thymidine kinase (TK)	Herpes simplex virus	Ganciclovir (GCV) Acyclovir (ACV)	GCV and ACV monophosphates
Thymidine kinase (TK)	Varicella zoster virus	Purine nucleosides FIAU, ara-M	Monophosphate nucleotide analogs
Thymidine phosphorylase (TP)	Human *E. coli*	Pyrimidine analogs 5-DFUR	5-Fluorouracil
Xanthine-guanine phosphorybosyl transferase (XGPT)	*E. coli*	6-Thiopurines	6-Thiopurine nucleoside

FIAU, 1-(2′-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil; araM, 6-methoxy-9-β-D-arabinofuranosylpurine.

and k_{cat}), which often are used to compare prodrug activation processes, are usually made under different experimental conditions. Therefore, direct comparison among different systems is quite difficult, although a low K_m and high V_{max} suggest a good suicide gene candidate. An alternative approach is to use an *Escherichia coli* system for testing and screening of genes that sensitize bacteria to specific prodrugs. DNA family

shuffling and a high-throughput robotic screening have been used to identify optimized suicide genes with an enhanced ability to phosphorylate AZT *(27)*. Kinetic measurements showed that these novel chimeric enzymes had acquired reduced K_m for AZT as well as decreased specificity for thymidine (Thd).

Taking into account the pharmacokinetics and activation steps of different prodrugs, it may be unwise to choose systems based solely on strong reaction rates. However, two biological parameters, the activation potential and the degree of activation, can be used to compare suicide systems *(28)*. The potential of activation is expressed as the ratio of the 50% inhibitory concentration (IC_{50}) of the prodrug to the IC_{50} of the active drug in a nontransfected cell system. It represents the maximum possible efficiency of a given enzyme-prodrug system toward a specific cell line. The degree of activation is defined as the ratio of the IC_{50} of the prodrug in the nontransfected cell line to the IC_{50} of the prodrug in the transfected cell line and demonstrates the system's efficiency in a given cell line. These parameters allow fair selection of the desired suicide system and might help in comparing and evaluating novel suicide systems. Unfortunately, not all systems can be compared in this way. The potential of activation is impossible to determine for nucleoside prodrug-based systems as mono-, di-, and triphosphate forms of activated analogs cannot cross cell membranes. Therefore, their IC_{50} values cannot be precisely determined.

Some authors have tried to determine cytotoxicity based on transfer of phosphorylated analogs between different cells *(29,30)*. However, many of the analogs do not accumulate and exert toxic effects not only by DNA incorporation but also by interference with other metabolic pathways. The interaction between different nucleoside drugs and metabolic pathways is not well established; therefore, it is difficult to predict fully their cytotoxic effect. The activation process is very complex and involves numerous parameters, which directly or indirectly influence this process. The degree of activation, which might be lower or equal to the potential of activation, reflects the efficiency of the system and can be a useful parameter to predict the in vivo effect.

The best-known and most-studied genes used for suicide gene therapy are the bacterial CD gene and the herpes simplex virus (HSV) TK gene. Both systems display several advantages over other suicide systems and have been tested in numerous clinical trials. CD mediates cell death through the conversion of 5-fluorocytosine (5-FC) to the potent cytotoxic agent 5-FU. Expression of the HSV-TK gene leads to cell death when growing cells are exposed to antiherpetic nucleoside analogs such as GCV as this prodrug is metabolized by HSV-TK to a toxic agent. Several other suicide systems based on nucleobase and nucleoside analogs have been described and tested. The majority of them used prodrugs that are

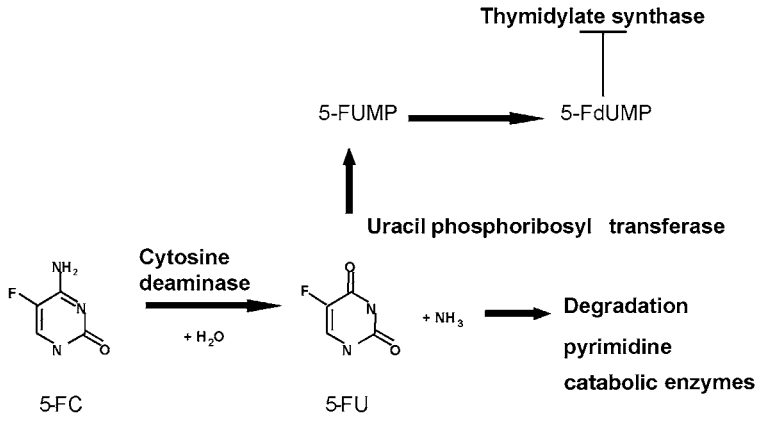


Fig. 2. The conversion of 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU) by cytosine deaminase.

approved anticancer or antiviral agents, and their side effects, pharmacokinetics, and dosage are well documented. This might facilitate their introduction into clinical trials and speed development of these suicide systems.

2.2.1. Cytosine Deaminase

One of the best-known genes used for suicide gene therapy is the bacterial CD gene (Enzyme Commission [EC] 3.5.4.1) *(31,32)*. CD catalyzes the hydrolytic deamination of cytosine and 5-methylcytosine to uracil and thymine, respectively. The enzyme also deaminates the nontoxic prodrug 5-FC to 5-FU, which is further metabolized to several 5-fluoronucleotides that inhibit both RNA and DNA synthesis *(33)* (Fig. 2). The CD gene is present in different prokaryotes and some fungi, but a mammalian counterpart does not exist. Therefore, 5-FC has relatively little toxicity, and it seems that most of the toxicity observed with oral use of 5-FC in humans is caused by deamination by intestinal bacteria *(34)*. 5-FC is an approved antifungal agent with a well-established clinical profile. Taking into account its biopharmaceutical properties, such as good oral absorption and crossing of the blood-brain barrier, 5-FC offers several advantages over other prodrugs used in cancer gene therapy.

Two different CD genes are used for suicide gene therapy. *Escherichia coli* CD is a hexamer of approx 300 kDa; the yeast *Saccharomyces cerevisiae* CD is a homodimer with a molecular mass of 35 kDa *(35)*. The significant differences between the bacterial and yeast enzymes include not only size and quaternary structure but also relative substrate specificities and affinities. The bacterial enzyme is capable of deaminating a wide range of cytosine derivatives, including 2-thiocytosine, 6-aza-cytosine,

4-azacytosine, and, of course, 5-FC *(36)*. The high-resolution crystal structure of *E. coli* CD is available *(37)*, which may help with specific protein design for gene therapy applications through the selection of modified enzyme forms with improved catalytic properties for 5-FC. The yeast CD has a different fold, active site architecture and binds other metal ions than the bacterial enzyme *(38)*. Although bacterial CD is more thermostable than its yeast counterpart, the yeast enzyme may be the better candidate for suicide gene therapy because of its lower K_m toward 5-FC *(39)*.

Many cancer cell lines are sensitive to 5-FC when transfected with the CD gene *(19,40–42)*. Mouse 3T3 cells transduced with the CD gene were sensitive to 5-FC, but no bystander effect was observed *(19)*. A similar result, again with minimal bystander effect, was observed in transduced mouse fibrosarcomas and adenocarcinomas *(43)*. In contrast, human colorectal cancer cells showed a very strong bystander effect *(40,44)*. With only 4% implanted tumor cells expressing the CD gene, 60% of the mice were tumor free after 5-FC treatment *(45)*. This discrepancy between mouse and human cells might be explained by a much higher CD expression in human cells. However, a higher level of CD expression does not ultimately result in higher effect. The effect may actually depend on the inherent sensitivity of the particular cell line to 5-FU. In sensitive cell lines, such as colon or lung carcinomas, the cytotoxicity of 5-FU directly correlates with the level of CD expression *(46)*. In resistant cells, the CD dose response is not as obvious, which might explain the absence of bystander effect in murine cells. On the other hand, the prodrug dose response is more obvious because higher 5-FC concentrations are more effective in killing CD-expressing cells than lower concentrations *(47)*. It is clear that the CD/5-FC system displays very strong bystander effect mediated by direct diffusion of 5-FU molecules across cell membranes. This can result in strong in vitro and in vivo tumor response, which is advantageous, especially when taking into account the current low rates of in vivo gene transfer.

The CD/5-FC combination has been used in many experimental studies *(48)*. However, its clinical use to date is rather limited *(49–51)*, although recent clinical trials have proven the safety and efficacy of the CD/5-FC system *(52)*. Use of a replication-competent, oncolytic adenovirus containing CD/HSV-TK genes together with radiation therapy showed that all these treatments can be safely combined and suggests a possible interaction between the oncolytic adenovirus or double-suicide gene therapies and radiation *(52)*.

CD is the second-most-used suicide system under clinical investigation. However, certain restrictions may have an effect on the use of this system. One disadvantage is actually that the strong 5-FU bystander effect as free diffusion is not selective and can also damage surrounding normal tissues.

Another problem associated with this suicide system is its quite low sensitivity for 5-FU as many tumor cells lack the uracil phosphoribosyl transferase enzyme that catalyzes the conversion of 5-FU to 5-fluorouridine monophosphate (Fig. 2), and the mammalian counterpart orotate phosphoribosyltransferase has a variable expression in tumor cells. The antitumor effect of 5-FU is enhanced by augmenting 5-fluorodeoxyuridine monophosphate converted from 5-fluorouridine monophosphate, which inhibits thymidylate synthase. To overcome this drawback, CD and uracil phosphoribosyl transferase were coexpressed, which led to 6000-fold activation *(53)*. The toxicity of 5-FU is cell proliferation specific; that is, it affects only highly proliferating tumor cells. Tumors contain highly variable cell populations in which only a few percent of the cells are actively dividing. To compensate for lower therapeutic effect, higher doses of both 5-FC and CD are needed, which may be difficult to achieve.

Finally, the active product, 5-FU, is rapidly degraded by pyrimidine catabolic enzymes, such as dihydrouracil dehydrogenase *(54)*, which has a high systemic (predominantly liver) expression; tumoral expression is related to the antitumor activity of 5-FU. The metabolic pathway of 5-FC activation is quite complex, which provides cancer cells with many opportunities to develop resistance to the therapy. Further research and advanced trials are necessary to develop this suicide system to its full potential.

2.2.2. Deoxycytidine Kinase

Once inside the cell, the nucleosides are monophosphorylated by the salvage kinases. Mammals have four deoxyribonucleoside kinases with overlapping specificities *(15,55)* (*see* Chapters 2 and 3). dCK (EC 2.7.1.74) phosphorylates deoxyadenosine (dAdo), deoxyguanosine (dGuo), and deoxycytidine (dCyd). Deoxyguanosine kinase (dGK; EC 2.7.1.113) only phosphorylates the purine nucleosides dAdo and dGuo; thymidine kinase 1 (TK1; EC 2.7.1.21) only phosphorylates Thd and deoxyuridine (dUrd). The pyrimidine-specific thymidine kinase 2 (TK2; EC 2.7.1.21) phosphorylates not only both Thd and dUrd, but also dCyd *(56)*.

dCK is also a crucial enzyme in the phosphorylation of a variety of antineoplastic and antiviral nucleoside analogs, including F-ara-A *(57)*, CdA *(58)*, dFdC (gemcitabine) *(59)*, and ddC (2′,3′-dideoxycytidine) *(60)* (Fig. 1). Deficiency of dCK actively mediates resistance to these drugs *(61,62)*. Conversely, increased dCK activity is associated with increased sensitivity to these compounds, which is utilized in suicide gene therapy *(23,63,64)*. A noticeable sensitization of glioma cells lines to ara-C was achieved by both retroviral and adenoviral transduction of the dCK gene *(23)*. Ara-C is a potent analog used for treatment of acute myeloid leukemia, but its activity against solid tumors is quite limited. Solid tumors have high expression of cytidine deaminase, an enzyme that degrades ara-C,

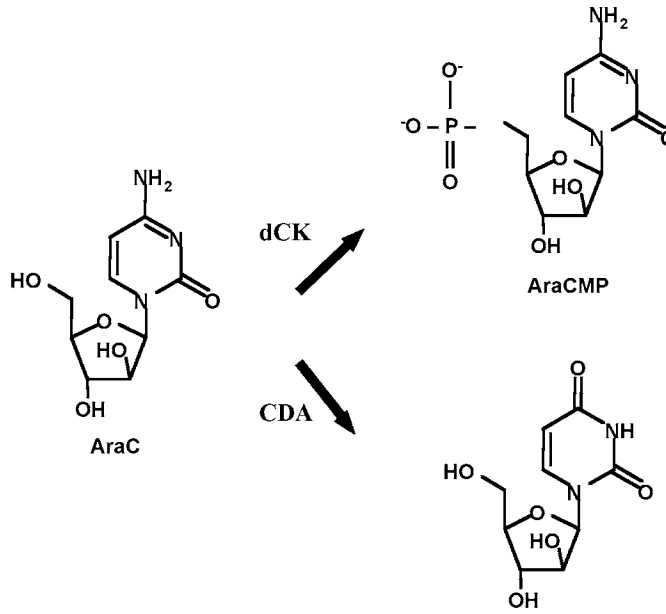


Fig. 3. Metabolism of pyrimidine nucleoside analog ara-C. This analog is either phosphorylated by dCK into ara-C monophosphate or degraded by cytidine deaminase (CDA) into chemotherapeutically inactive uracil nucleoside.

whereas the expression of dCK, which phosphorylates ara-C, is quite low (Fig. 3). Therefore, overexpression of dCK in solid tumors activates ara-C to ara-C monophosphate, which after further phosphorylation and incorporation into DNA results in significant antitumor effects *(65)*.

The dCK/ara-C system may be a useful approach for gene therapy. Several other prodrugs can be developed for successful suicide gene therapy. The recently solved crystal structure of human dCK *(66)* led to the construction of a mutant of dCK with improved kinetic parameters for dFdC, which might facilitate activation of this drug in tumor tissues. Other kinases, such as TK2 *(67)* and dGK *(68)*, are also able to phosphorylate substrate analogs of dCK. Human cancer cells transfected with the dGK gene show increased sensitivity toward CdA *(69)*. Nevertheless, none of these genes have as yet been used in suicide gene therapy.

A number of potential problems are associated with this suicide system. Solubility of some analogs used by dCK, such as F-ara-A and ara-A (vidarabine), is quite low *(70)*, and many of these drugs are rapidly inactivated *(59,71)*, which limits their applications. Cell specificity is another restriction; F-ara-A and CdA are used against hematological malignancies *(72,73)*, are specific for blood cells, and do not penetrate other tissues well *(58,74)*.

On the other hand, dFdC appears to be useful in the treatment of a wide variety of cancers, including both hematological malignancies *(75)* and solid tumors *(76)*. The major advantage of using dCK as a suicide gene is that activated metabolites may help trigger apoptosis *(77–79)*. The active species of nucleoside analogs are predominantly toxic to cells that are replicating their DNA. Even with fast-growing solid tumors, the proportion of actively dividing cells may be as low as a few percent *(80,81)*. Therefore, a large proportion of resting cells may be responsible for failure of some suicide therapies. By triggering apoptosis, slow-growing and quiescent cells can be eliminated, which is especially important for eradication of large solid tumors. So far, clinical trials using the dCK system have not been carried out.

2.2.3. Multisubstrate Deoxyribonucleoside Kinases

The fruitfly *Drosophila melanogaster* has a single enzyme that phosphorylates all the natural pyrimidine and purine deoxyribonucleosides *(82)*. The sequence of the *Drosophila melanogaster* nucleoside kinase (*Dm*-dNK; EC 2.7.1.113) is closely related to the human deoxyribonucleoside kinases (Fig. 4).

The enzyme also phosphorylates several nucleoside analogs, and therefore the *Dm*-dNK gene has been suggested as a candidate suicide gene in combined gene/chemotherapy of cancer *(83)*. In addition to its broad substrate specificity, the enzyme demonstrates other interesting properties that may be beneficial in a gene therapy application. Most important, the catalytic activity is 10- to 100-fold higher, depending on the substrate, than what has been reported for the mammalian enzymes, which is especially interesting because the initial activation step is rate limiting for the phosphorylation of the majority of nucleoside analogs. The further phosphorylation, to the corresponding di- and triphosphates, is performed by cellular mono- and diphosphate kinases with high capacity. Another important property is that the *Dm*-dNK protein can be expressed at high levels in mammalian cancer cell lines retrovirally transduced with the complementary DNA encoding *Dm*-dNK *(21)*. *Dm*-dNK prefers pyrimidine substrates to purines, and the K_m values for Thd and dCyd are more than 100-fold lower than the K_m for dAdo and more than 1000-fold lower than the K_m for dGuo. The efficiency to phosphorylate nucleoside analogs follows a similar pattern, with high efficiency for several pyrimidine and some dAdo analogs, whereas analogs of dGuo, like GCV and ACV, are inefficient substrates. The inhibitory activity (IC_{50}) of some nucleoside analogs on the phosphorylation of 1 μ*M* Thd and dCyd are shown in Table 2.

The pyrimidine nucleoside analog (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU) has been identified as an efficient substrate for *Dm*-dNK with low toxicity toward several human cell lines. Cancer cell lines

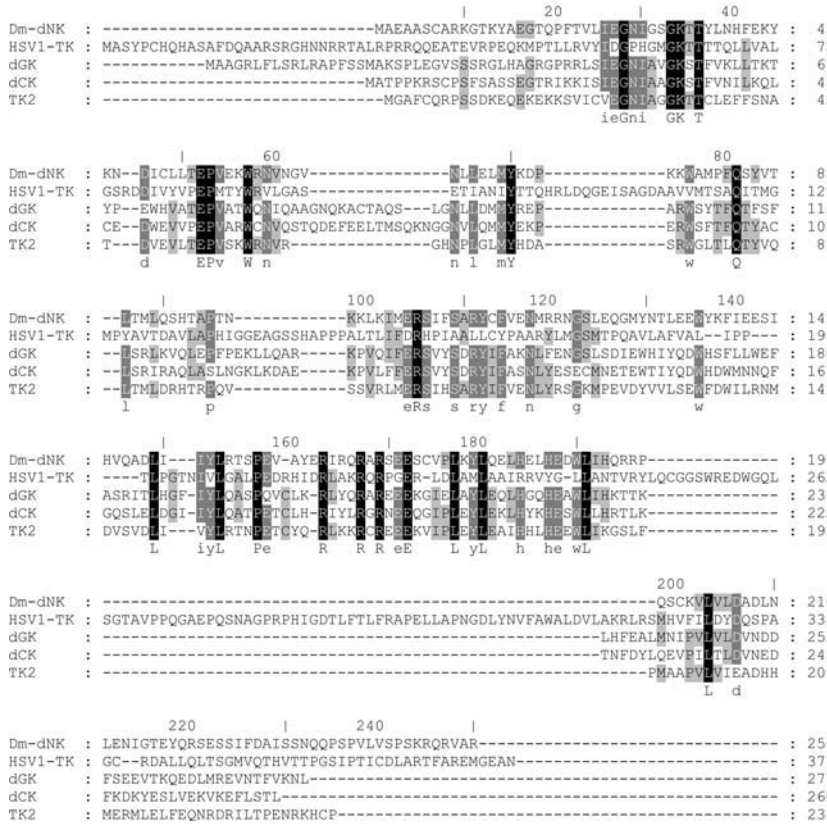


Fig. 4. Alignment of *Dm*-dNK with HSV-1 TK, dGK, dCK, and TK2. Black boxes indicate completely conserved amino acid residues (uppercase) and gray boxes indicate semiconserved residues (lowercase). Arrows indicate the location of the studied mutants. Numbering is based on the amino acid sequence of *Dm*-dNK.

expressing *Dm*-dNK exhibit increased sensitivity toward several cytotoxic nucleoside analogs, among which BVDU is the most efficient prodrug candidate *(21)*. On this ground, BVDU has been selected as a prodrug in combination with *Dm*-dNK for further preclinical studies for gene therapy applications. The cytotoxicity of BVDU can be enhanced in cell lines when the compound is used in combination with subtoxic concentrations of hydroxyurea. The inhibition of ribonucleotide reductase by hydroxyurea has been shown to increase the phosphorylation of Thd analogs, but the mechanism for this is not clear. Hydroxyurea also enhances the bystander effect in certain cells when combined with BVDU. BVDU is an efficient substrate for both HSV-TK and *Dm*-dNK, and the compound has previously been investigated as a prodrug in HSV-TK-transduced cells *(84)*.

Table 2
Inhibitory Activity (IC_{50}) (μ*M*) of Nucleoside Analogs on the Phosphorylation of 1 μ*M* Radiolabeled Thd or dCyd by *Dm*-dNK

		Dm-dNK	
Base	*Analog*	*Thd*	*dCyd*
A	CdA	123 ± 14	120 ± 6
	ara-A	>1000	>1000
G	GCV	>1000	>1000
	ara-G	>1000	>1000
C	ara-C	53.4 ± 18.9	89.0 ± 24.0
	ddC	760 ± 8	838 ± 53
	dFdC	145 ± 1	102 ± 30
	3TC	740 ± 31	868 ± 95
T	ara-T	64.8 ± 27.7	34.8 ± 5.6
	AZT	41.9 ± 12.9	29.6 ± 0.8
	d4T	>1000	805 ± 275
U	ara-U	570 ± 66	580 ± 49
	BVDU	27.7 ± 10.0	15.8 ± 2.8
	FdUrd	25.8 ± 0.8	23.5 ± 5.0
	FIAU	16.3 ± 0.1	15.4 ± 2.6

Source: Adapted from ref. *83*.

CdA, 2-chloro-2′-deoxyadenosine; araA, 9-β-D-arabinofuranosyladenine; GCV, ganciclovir; ara-G, 9-β-D-arabinofuranosylguanine; ara-C, 1-β-D-arabinofuranosylcytosine; ddC, 2′, 3′-dideoxycytidine; dFdC, 2′,2′- difluorodeoxycytidine; 3TC, 2′-deoxy-3′-thiacytidine; ara-T, 1-β-D-arabinofuranosylthymine; AZT, 3′-azido-2′,3′-dideoxythymidine; d4T, 2′,3′-didehydro-3′-deoxythymidine; ara-U, 1-β-D-arabinofuranosyluracil; BVDU, (*E*)-5(2-bromovinyl)-2′-deoxyuridine; FdUrd, 5-fluoro-2′-deoxyuridine; FIAU, 1-(2′-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil.

Although BVDU did not show any bystander effect in HSV-TK-transduced osteosarcoma cells, a clear bystander effect was detected in *Dm*-dNK-transduced osteosarcoma cells *(85)*.

An advantage with the *Dm*-dNK-based suicide system is the overlapping substrate specificity with dCK in addition to its activation of unique substrates. dCK activates several clinically important nucleoside analogs like ara-C, CdA, and dFdC, which are also substrates for *Dm*-dNK. The possibility to combine two or more prodrugs with different cytotoxic mechanisms should be interesting in a future clinical trial using the *Dm*-dNK suicide gene. The sequence and substrate specificity of *Dm*-dNK indicate that the enzyme has evolved close to mammalian TK2 *(86)*, but it has also similarities and overlapping substrate specificity with dCK, dGK, and

HSV-TK (Fig. 4). The three-dimensional structure of *Dm*-dNK and dGK indicate that only a few amino acids differ in the substrate-binding region of the enzymes *(87)*. Mutagenesis of three key residues in *Dm*-dNK converts the *Dm*-dNK substrate specificity from predominantly pyrimidine specific into purine specific *(88)*. Such alterations of *Dm*-dNK may be useful to obtain *Dm*-dNK mutants with specific properties for suicide gene therapy.

One suggested advantage of purine analogs is that the bystander effect is more efficient than for pyrimidine analogs *(84)*. Several mutant enzymes of *Dm*-dNK have been engineered to find an enzyme with higher efficiency in GCV phosphorylation because GCV is the nucleoside analog that has shown the most efficient bystander cell killing *(89)*. It is still not known if any of these mutants will demonstrate superior properties to the wild-type *Dm*-dNK for gene therapy applications. Clinical trials using *Dm*-dNK, or any of its mutants, have not yet been initiated.

Other insect multisubstrate nucleoside kinases that have been identified and characterized are the *Bombyx mori* deoxynucleoside kinase and the *Anopheles gambiae* nucleoside kinase *(90)*. Both these enzymes, similar to *Dm*-dNK, have the ability to phosphorylate all four natural deoxyribonucleoside substrates, although that of *B. mori* has a preference for pyrimidines, whereas the *A. gambiae* dNK shows higher efficiency for purine substrates *(91)*. Whether activation of immune responses toward the insect enzymes will affect their efficiency when used in humans is still unknown.

2.2.4. Purine Nucleoside Phosphorylase

PNP (EC 2.4.2.1) catalyzes the reversible phosphorolysis of purine (2′-deoxy)ribonucleosides to generate the corresponding purine base and (2-deoxy)ribose 1-phosphate. The enzyme has been isolated from variety of eukaryotic and prokaryotic organisms. There are two major classes of this enzyme: Mammalian PNP are trimers with 31-kDa monomer size; bacterial PNPs are hexameric proteins with 26-kDa monomers. The hexameric PNP is specific for adenosine and dAdo *(92)* and accepts purine arabinosides *(93)*. This property is utilized in suicide gene therapy as *E. coli* PNP cleaves substrates as 2-fluorodeoxyadenosine, fludarabine monophosphate, and 6-methyl purine-deoxyribose into toxic compounds 2-fluoroadenine (2-F-Ade) and methyl purine (6-MeP), respectively (Fig. 5).

These compounds are further incorporated into a variety of metabolites that inhibit RNA and protein synthesis. The PNP system exhibits several characteristics that make it a very attractive suicide method. Generated active compounds are very potent; 2-F-Ade is 1000 times more toxic then 5-FU. Because the molecules are as small as 5-FU, the bystander effect of these drugs is not dependent on gap junctions and does not require cell-to-cell contact. An entire cell population can be killed when as few as 0.1–1%

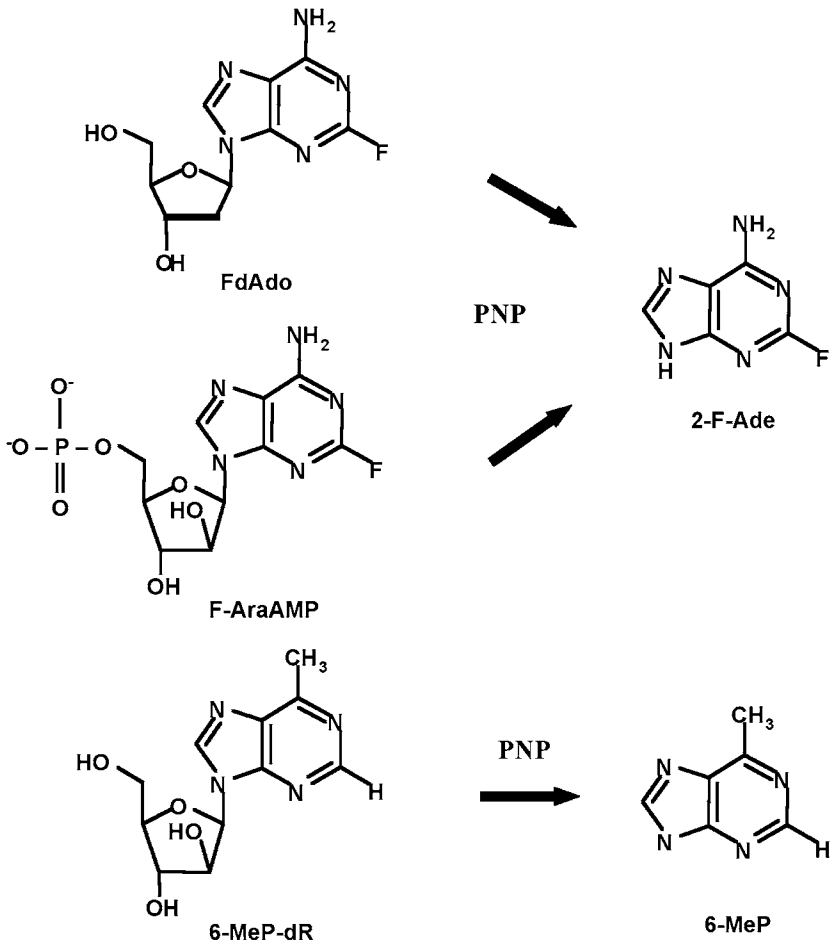


Fig. 5. Conversion of nucleoside analogs by *E. coli* PNP. Analogs are cleaved into cytotoxic purines and corresponding sugar molecules.

of the cells express the PNP gene *(94)*. Apart from being extremely potent toxins, these compounds appear to have very long half-life (greater than 24 h) with slow release from solid tumors *(95)*, and they are active against both dividing and nonproliferating cells. Both 6-MeP and 2-F-Ade are converted to ATP analogs and block reactions involving ATP, which ultimately leads to cell death. Therefore, these two compounds are quite different from, for example, GCV and 5-FU in that they block enzymes unrelated to DNA synthesis and can kill cells that are not actively dividing.

Several reports showed that expression of PNP led to superior killing of human prostate cancer (PC-3) cells both in vitro and in vivo *(96)* when compared to the HSV-TK/GCV system using identical adenovirus as a delivery. Strong in vivo antitumor effects in mouse models of liver carcinoma have also been shown *(97,98)*. The *E. coli* PNP system has several advantages over other suicide gene therapy strategies. However, rigorous clinical tests are required to fully prove usefulness of this system for cancer treatment.

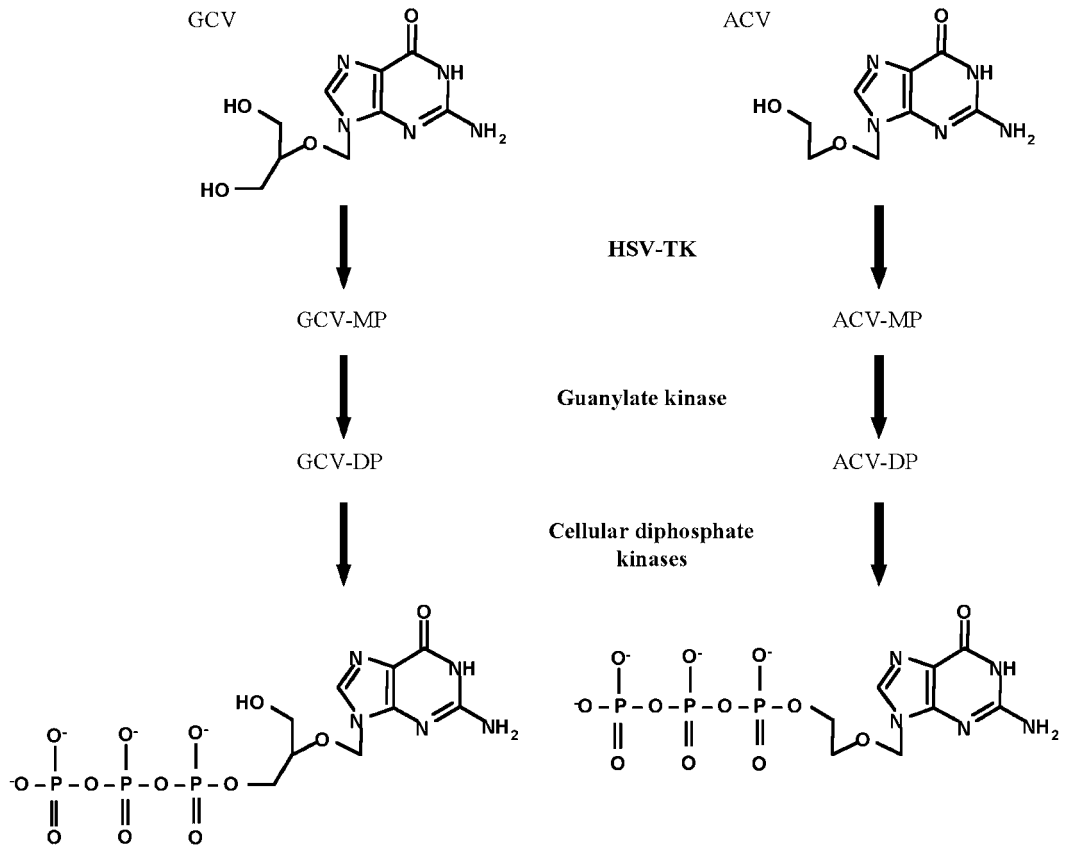
2.2.5. Viral TKs

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) and varicella zoster virus (VZV) encode TKs similar to the mammalian TKs but with a markedly different substrate specificity. HSV and VZV TK phosphorylate a wide range of antiherpetic nucleoside analogs in addition to the natural substrates Thd, dUrd, and dCyd. After the initial monophosphorylaton by herpes TK, the further phosphorylation to the active triphosphate form is carried out by cellular enzymes. GCV and ACV are purine nucleoside analogs that are specific substrates for herpesvirus TK (Fig. 6) with no toxicity in cells not expressing these enzymes.

Although both compounds are widely used as antiviral agents, GCV is the common prodrug used in HSV-TK suicide gene systems. The triphosphate of GCV has higher affinity for herpesvirus DNA polymerase than for human DNA polymerases, but several studies of HSV-1 TK/GCV gene therapy indicated that the tumor cells expressing HSV-1 TK generate enough levels of phosphorylated GCV to inhibit mammalian DNA polymerase *(99)*. The effect of GCV treatment on HSV-1 TK gene-transduced cells in terms of cell cycle progression and the mechanism of GCV-induced cell death have been investigated in several studies. GCV has been demonstrated to induce cell cycle arrest and to induce cell death by apoptosis *(100,101)*.

The first clinical trial using HSV-1 TK/GCV gene therapy for cancer was carried out in 1992 and involved patients with malignant brain tumors who had failed standard therapy *(102)*. The advantage of this kind of therapy for brain tumors is its high selectivity for tumor cells over healthy brain cells. Normal brain cells are postmitotic and are no longer dividing, so interfering with DNA synthesis is not nearly as harmful as it is for the rapidly dividing cancer cells. Although the tumor volume slightly

Fig. 6. *(Opposite page)* The activation of ganciclovir (GCV) and acyclovir (ACV) by herpes simplex thymidine kinase (HSV-TK). GCV and ACV are converted into their monophosphate forms by HSV-TK, and thereafter cellular mono- and diphosphate kinases add additional phosphate groups to form active GCV and ACV triphosphates.


GCV
ACV
HSV-TK
GCV-MP
ACV-MP
Guanylate kinase
GCV-DP
ACV-DP
Cellular diphosphate kinases

decreased in 50% of the patients, the efficiency of HSV-1 TK gene transfer was very low. Since then, numerous clinical trials have been carried out using adenovirus or retrovirus vectors to deliver HSV-TK to different tumors. Even though some patients show tumor regression with HSV-TK/GCV therapy, further improvements are necessary to obtain better therapeutic efficiency.

The major hurdle in these trials, as in most gene therapy protocols, is the low in vivo efficiency of gene delivery to a sufficient number of tumor cells *(103)*. The transduction difficulty is more pronounced for larger tumors than for small tumors, and subsequently the best tumor-killing effect has been for the small tumors. Another drawback is the pronounced hematopoietic toxicity exerted by GCV, which limits the dose that can be administered to patients. Possible improvements of the HSV-1 TK/GCV system include the development of novel routes of GCV administration, superior HSV-1 TK mutants, pharmacological enhancers of the bystander effects, more efficient and less-toxic prodrugs, and combinations with other anticancer approaches.

The VZV-TK has different substrate specificity compared to HSV-TK and has been investigated in cell cultures as a potential suicide gene. Several very potent pyrimidine nucleoside analogs have been demonstrated as inhibitors of the proliferation of VZV-TK gene-transduced cells *(104)*. Although they have high potency, in cell culture assays the specific VZV-TK pyrimidine substrates exhibit very poor bystander effect, which may limit the in vivo use of this system for cancer therapy.

The use of suicide gene therapy has been extended to allogeneic stem cell transplantation *(105)*. Donor T-cells are genetically modified by insertion of HSV-TK, which can activate GCV to eliminate the transplanted T-cells in the event of graft-vs-host disease. Because GCV is widely used to treat cytomegalovirus infection in immunosuppressed patients, another suicide gene/prodrug combination that does not include an antiviral compound should be developed for this clinical application. Furthermore, a bystander effect is not desired in circulating T-cells, compared to the importance of bystander effect in solid tumors, giving a broader range of prodrugs for the selection of the optimal compound.

2.2.6. Thymidine Phosphorylase

TP (also known as platelet-derived endothelial cell growth factor or gliostatin; EC 2.4.2.4) catalyzes the phospholytic cleavage of Thd or dUrd to deoxyribose-1-phosphate. TP also converts 5′-DFUR to the toxic 5-FU (Fig. 7). Because of its reversible phosphorolytic activity, this enzyme also converts 5-FU to its anabolite 5-fluoro-2′-deoxyuridine (FdURD) if sufficient cofactor is available *(106)*; however, usually the equilibrium of this reaction is at the side of the base.

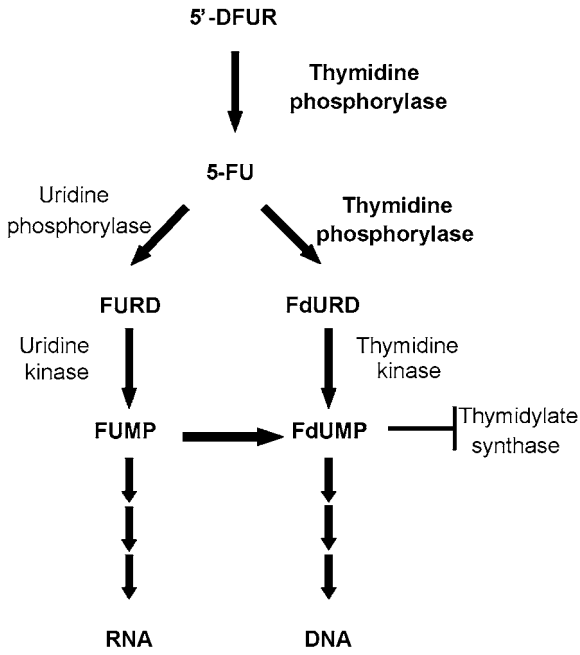


Fig. 7. Metabolic activation pathway for fluoropyrimidines. 5′-Deoxy-5-fluorouridine (5′-DFUR) is converted to toxic 5-fluorouracil (5-FU), which can be integrated into both RNA and DNA.

These pyrimidine analogs were developed for selective inhibition of tumor growth because the levels of TP are much higher in tumors than in normal tissues. However, tumors are not necessarily sensitive to these drugs as the levels of TP vary considerably from cell to cell. Therefore, raising the TP levels within the tumor is expected to enhance the sensitivity of the tumor to the prodrug significantly.

Indeed, this suicide system has been shown to sensitize the human MCF-7 breast cancer cell line 1000-fold to 5′-DFUR *(24)*. Similarly, when the TP gene was transfected into human colon cancer cells, the sensitivity to 5′-DFUR was increased by 100-fold *(107)*, although this effect might be cell line specific *(108)*. The TP/5′-DFUR system can also be modulated with 2′-deoxyinosine, which provides TP cofactor deoxyribose 1-phosphate. An almost 4000 fold increase of cell sensitivity in vitro was detected after combined TP/5′-DFUR/2′-deoxyinosine treatment *(109)*. A substantial bystander killing effect was observed when small portions of TP-expressing cells were mixed with parental cells.

The bystander effect of the TP system seems to be comparable to that of the CD system as the active product 5-FU is freely diffusible. However,

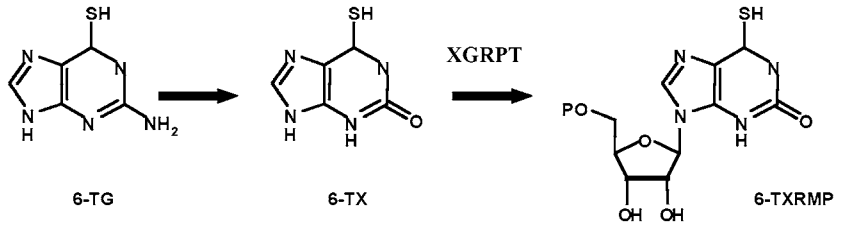


Fig. 8. Activation of 6-thioguanine (6-TG) and 6-thioxanthine (6-TX) by XGPRT.

5-FU is not always effective for every type of tumor as the levels of the enzymes responsible for further drug metabolism vary significantly in each tissue. Combining the TP and CD systems in murine gliosarcoma cells showed an increased sensitivity to 5-FC and prolonged the survival of mice bearing intracerebral tumors after in vivo CD gene therapy *(110)*. The combination of different suicide gene systems, such as CD/5-FC and HSV-TK/GCV, seems to be more effective than individual treatments *(111,112)*, although a single report contradicted these findings *(113)*.

The TP strategy has much in common with the CD suicide system, and it is questionable whether the TP system offers any advantages over the *E. coli* CD suicide system. For example, the activation rates and bystander effect are very similar. The crystal structures of both the TP *(114)* and the CD *(37)* enzymes are available, which makes enzyme optimization possible. The human TP gene (identified also as the endothelial cell growth factor–ECGF1) is quite large and includes 10 exons spanning more than 4.3 kb. The complementary DNA is 1449 bp long and codes for a protein of 482 aa with predicted molecular weight of approx 50 kDa. The bacterial CD gene is slightly smaller (427 aa), which might be advantageous when using delivery vectors for which size of the transgene is the limiting factor (e.g., replicative adenoviruses). On the other hand, the human TP gene is less likely to provoke an immunoresponse, although the enzyme might be more susceptible to metabolic inhibition in human cells.

2.2.7. Xanthine-Guanine Phosphorybosyl Transferase

XGPRT (EC 2.4.2.22) catalyzes the transfer of the phosphoribosyl group from 5-phospho-α-D-ribosyl 1-pyrophosphate to the 6-oxopurine bases. In contrast to its human homolog, the hypoxanthine phosphoribosyltransferase, the *E. coli* enzyme can convert xanthine to xanthine monophosphate *(115)*. Cells that produce *E. coli* XGPRT are also able to transfer the deoxyribose moiety to 6-mercaptopurine, which is further activated to its highly cytotoxic triphosphate analog *(22)*. 6-Thioguanine (6-TG), a potent agent for therapy of acute nonlymphocytic leukemias, can also be used as

an analog because it is deaminated to 6-thioxanthine (6-TX) by endogenous enzymes. 6-TX, an inactive compound having negligible antitumor activity, is thereafter converted into 6-TX monophosphate (Fig. 8).

The *E. coli* enzyme is a tetramer with 152 residues per subunit. The small gene size and available structure *(116)* may facilitate optimization of XGPRT and improve its substrate/inhibitor properties. A 10- to 20-fold degree of activation was obtained in rat C6 glioma cells for both 6-TG and 6-TX *(117)*. The effects of 6-TX on cell proliferation required at least 10 d of exposure. Bystander effect was also observed; cells expressing the XGPRT gene efficiently transferred 6-TX sensitivity to XGPRT-negative cells at ratios as low as 1:9. Oddly, the bystander effect was not present the first 4 d of prodrug exposure *(118)*. Trypsinization and replating of the cocultures were required for the bystander effect to take place in this case. In vivo, both 6-TX and 6-TG significantly inhibited the growth of subcutaneously and intracerebrally transplanted tumors.

The XGPRT gene provides a unique system because it not only sensitizes cells to the prodrugs but also provides resistance to a different course of therapy, such as mycophenolic acid. This provides a means to select for XGPRT-positive cells, which is advantageous for ex vivo gene therapy applications, such as bone marrow transplantation or the gene therapy of gliomas. Therefore, the XGPRT/6-TX system seems to be a promising alternative to the HSV-TK/GCV combination for gene therapy of gliomas. The XGPRT system still has not been tested in any clinical trials.

2.3. Bystander Effect

A major advantage of many suicide gene therapy strategies is that not only the tumor cells that contain the suicide gene are destroyed, but also the surrounding "nontransduced" cells are killed *(119)*. The mechanism by which nontransduced neighboring cells are killed is known as the *bystander effect* (Fig. 9). The bystander effect is dependent on cell contact and on intercellular communication. Cells close to those expressing the suicide gene can acquire activated prodrug by several ways. Small molecules, such as 5-FU, can freely cross cell membranes. In this case, the bystander effect is determined by the drug half-life and diffusion range. On the other hand, the phosphorylated nucleoside analogs must pass through gap junctions into noninfected cells. Gap junctions are protein channels connecting cells and allowing passage of small molecules and ions up to 1000 Da. Formation of gap junctions requires cell-to-cell contact. Although many tumor cells readily form gap junctions, some tumor tissues actually downregulate gap junction formation *(120)*. Transfer of phosphorylated prodrugs could be inhibited by the addition of α-glycyrrhetinic acid *(121)*, which is a gap junction inhibitor, suggesting that gap junction

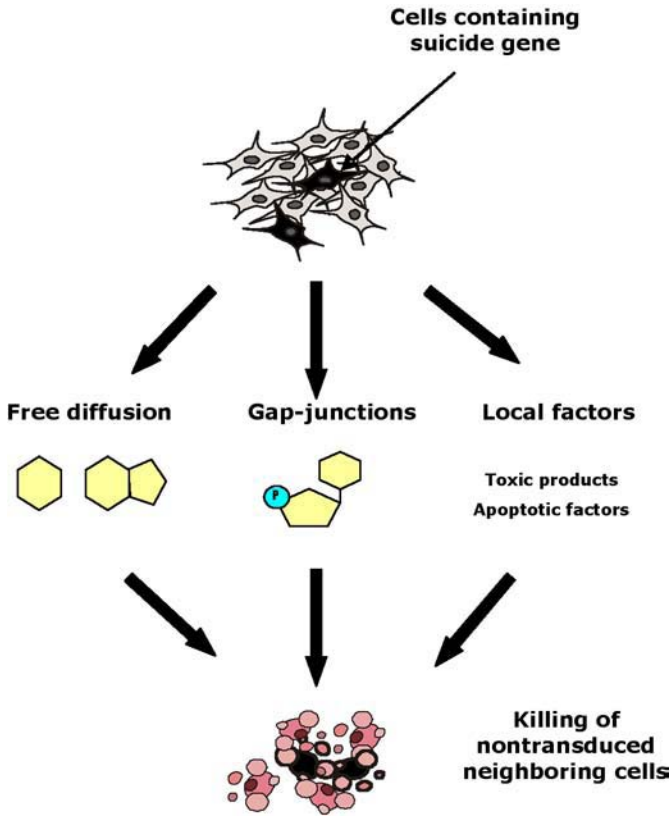


Fig. 9. Bystander effect allows the eradication of neighboring cells that do not contain suicide gene.

formation between neighboring cells is an important contributing factor in bystander effect. The local bystander effect can also be induced by the transfer of toxic products via apoptotic vesicles *(122)* or by contact with dead cells caused by the transfer of apoptotic factors *(123)*.

The bystander effect is extremely helpful as it addresses one of the main obstacles for efficient gene therapy, the transduction of the tumor cells. Not all tumor cells need to be transduced to reach a sufficient proportion of cells with the suicide gene for the therapy to be effective. Numerous studies have shown near-complete regression in small tumors, when as few as 10% of the cells contained the HSV-TK gene *(124–126)*.

Several strategies to improve the bystander effect have been considered. One way to enhance the bystander effect is the expression of the connexin (Cx) gene *(127)*. Cxs are membrane proteins considered to be building blocks of gap junctions. The recombinant expression of Cx proteins, or

chemically induced Cx overexpression, enhance the bystander effect in suicide gene therapy, both in vitro and in vivo settings *(128,129)*. However, it seems unlikely this approach will provide a feasible way of mediating the bystander effect in clinical settings. Studies of histone deacetylase inhibitors in combination with suicide gene therapy might provide an alternative strategy *(130)*. Histone acetylase and deacetylases regulate the level of transcription by modifying chromatin structure *(131)*. Numerous compounds that inhibit histone deacetylation have been identified *(132)*. Although the real synergistic mechanism between these agents and suicide genes remains elusive, results suggest that either increased expression, bystander effect, and tumor immunity all play important roles *(130)*.

Apart from local bystander effect, immune-mediated bystander effect also exists. In vivo models suggested that systemic immune response may play a role in inducing the bystander effect *(133)*. The HSV-TK/GCV system can induce an immune response, which could contribute to the bystander effect *(134)*. Tumor-specific killer T-cells were present in relatively high concentration in the tumors, and major histocompatibility complex class I expression increased on the tumor cells containing HSV-TK system. The immune stimulation not only enhances local tumor killing but also has almost a vaccination effect, inducing the regression of distant tumor deposits and preventing the recurrence of tumors *(135)*.

3. GENE DELIVERY AND TARGETING

The selection of a specific suicide system and prodrug combination is important to the success of the therapy. An equally important factor is the vector or delivery vehicle by which the suicide gene is delivered to the tumor cells. The effect of suicide gene therapy is linearly related to the efficiency of gene transfer. The ideal gene therapy vector should be administrated systemically and should specifically deliver the gene to the target cells, minimizing exposure to normal, nonmalignant cells. In addition, the vector should be biodegradable, nontoxic, nonimmunogenic, and stable during storage and administration. Not surprisingly, not a single gene delivery vector has all these properties at once.

Replication-deficient vectors derived from viruses are considered the most efficient for gene delivery. They are made by removing all or part of the viral genes and replacing these with therapeutic genes. The problems associated with replication-deficient vectors are low virus titers (limited quantities), toxicity, and potentially regaining replication ability.

The most-used virus vectors for suicide gene therapy are retroviruses and adenoviruses. A retrovirus is a eukaryotic RNA virus that uses viral enzymes to copy its genome into DNA and integrate it into the host

genome. Only dividing cells can be transduced with these vectors *(136)*, but the development of lentivirus vectors has also enabled the transduction of nondividing cells *(137)*. Some of the major disadvantages associated with retroviruses are the difficulties in obtaining high virus titers and insertional mutagenesis.

Compared to the retrovirus vectors, adenoviruses possess a number of properties that make them an ideal means of cell transduction. An adenovirus is an icosahedral virus that contains a large (35–36 kb) DNA genome. They can infect both dividing and nondividing cells, and high viral titers can be obtained. However, gene expression from adenoviruses is short as the adenoviral genome is not integrated into the host DNA, and adenoviruses induce inflammatory responses, which limits their repeated administration *(138)*.

The capacity of an adenovirus to infect a given cell is determined by the attachment receptor CAR (Coxsackie and adenovirus receptor) and the integrin expression levels of the host cell. A number of cell types, such as fibroblasts, lymphocytes, and endothelial cells, are partially or completely resistant to adenovirus infection *(139)*. In addition, many types of tumor cells, such as prostate and breast cancers, express CAR at undetectable levels *(140)*. To overcome these limitations, it is necessary to develop adenovirus vectors capable of infecting tumors in a CAR-independent fashion. A number of successful studies have shown the utility of receptor-modified adenovirus vectors. Simultaneously targeting two or more cell surface receptors could increase gene transfer in a heterogeneous population of tumor cells *(141)*. Current work is aimed at the development of truly targeted adenovirus vectors capable of highly efficient cell-specific delivery of suicide genes in vivo.

Replication-selective oncolytic adenoviruses represent a novel cancer treatment platform. These viruses are engineered to replicate selectively only in tumor cells, induce cell death, release tumor particles, and finally spread through tumor tissues. The problems of selectivity and intratumoral spreading are reduced using this delivery system. Several clinical studies have demonstrated the safety and feasibility of this approach, including systemic delivery *(142,143)*. It is an inherent capacity of replication-competent adenoviruses to sensitize tumor cells to chemotherapy. This opens the possibility of constructing novel adenoviruses expressing suicide genes to enhance even further the antitumor activity.

However, certain limitations do apply. Oncolytic adenoviruses can carry up to 7.5% extra DNA, which may limit the packaging size of the transgenes and promoters to be used. Active replication is needed for the virus to exert its therapeutic effect; therefore, the use of suicide systems based on antiviral prodrugs, such as GCV, will inhibit virus replication and eliminate their effect. To overcome this problem, the use of anticancer

drugs is recommended. Other virus species, including herpesvirus, vaccinia, reovirus, and measles virus are under development for oncolytic therapy *(144,145)*. So far, no virus species other than adenovirus has been combined with any of the suicide systems presented here.

Nonviral vectors represent an attractive alternative to virus vectors. These classes of vectors include naked DNA transfer, liposomes (lipoplex), polymers (polyplex), and the combination of the last two (liopopolyplex). They are all characterized by low toxicity and immunogenicity and can relatively easily be produced on a large scale. Disadvantages include low efficiency of gene transfer compared to the viral vectors. Nevertheless, many nonviral vectors are extensively used in combination with suicide genes and nucleoprodrugs *(146–148)*.

Specific targeting of the suicide genes to the tumor cells provides selectivity, which maximizes the therapeutic effect in cancer cells with a minimal and acceptable level of damage to normal, nonmalignant cells. Although the current vectors are still some distance from reaching this goal, different approaches exist by which this might be accomplished. Direct intratumoral injection is a simple way to introduce genes into a single tumor. Limited applications exist for this approach. Nevertheless, the method is widely employed for delivery into gliomas, although the insertion requires stereotactic injections to pinpoint the tumor *(149,150)*.

Tumor-specific vector binding, the so-called transductional targeting, is widely used for adenovirus vectors. An alternative way of obtaining selectivity is the use of tumor- or tissue-specific promoters. Many tumors overexpress specific genes, enabling the expression of the suicide genes to be linked to the transcriptional control of these genes. Tumor-specific expression of CD under control of the carcinoembryonic antigen promoter, for example, led to partial regression of a colorectal carcinoma after 5-FC treatment *(151)*.

Further developments and fine-tuning of existing systems and technologies will definitely make suicide gene therapy a clinically effective treatment for cancers.

4. CHALLENGES AND FUTURE ASPECTS

Several problems must be overcome before suicide gene therapy becomes an efficient cancer treatment. A number of other novel suicide gene/prodrug systems are currently under investigation. Unfortunately, most of these systems are not completely characterized or are only in early preclinical development. Another limiting factor is that the majority of suicide systems are based on nonapproved prodrugs, slowing their introduction into clinical trials. Fortunately, multiple options are available and are being tested. For example, double-suicide therapy, in which a combination of different suicide

systems is coexpressed, shows promising results. The activated prodrugs should preferably act by different mechanisms (i.e., termination of DNA synthesis and induction of apoptosis) or should have different bystander effects (free diffusion vs gap junction-mediated transfer). In this way, a synergistic effect can be achieved in the tumor cells, and the occurrence of resistant cell populations might be limited. Such a "combination" suicide gene therapy with HSV-TK and CD completely eliminated tumors, while expression of the individual genes did not provide the same result *(112)*.

Nevertheless, there is room for further improvement. The use of in vitro protein evolution provides a way to obtain both more efficient activation of prodrugs and better specificity for certain prodrugs. Numerous examples are available that demonstrate the power of this approach *(27,152,153)*. The prodrugs also can be redesigned to achieve better solubility or stronger bystander effect or simply to create better substrates for suicide enzymes. Finally, it will be useful to clarify ways in which different suicide systems synergize with each other or with other treatments, such as radiotherapy.

Cancer gene therapy based on suicide genes and nucleoanalogs will probably never produce a "magic bullet" for all types of malignant diseases. It is likely that single or combined gene therapy strategies in conjunction with existing treatments will be needed to accomplish a complete therapeutic effect. Nevertheless, the extraordinary progress witnessed in all areas of gene therapy will contribute to the development of specific suicide gene therapy applications and fully exploit the potential of this exciting technology.

REFERENCES

1. Conte, P., Gennari, A., Landucci, E., et al. New combinations with epirubicin in advanced breast cancer. Oncology (Huntingt.), 15, 24–27, 2001.
2. Oettle, H. and Riess, H. Gemcitabine in combination with 5-fluorouracil with or without folinic acid in the treatment of pancreatic cancer. Cancer, 95, 912–922, 2002.
3. Meropol, N. J., Niedzwiecki, D., Hollis, D., Schilsky, R. L., and Mayer, R. J. Phase II study of oral eniluracil, 5-fluorouracil, and leucovorin in patients with advanced colorectal carcinoma. Cancer, 91, 1256–1263, 2001.
4. Takechi, T., Fujioka, A., Matsushima, E., and Fukushima, M. Enhancement of the antitumour activity of 5-fluorouracil (5-FU) by inhibiting dihydropyrimidine dehydrogenase activity (DPD) using 5-chloro-2,4-dihydroxypyridine (CDHP) in human tumour cells. Eur. J. Cancer, 38, 1271–1277, 2002.
5. Henn, T. F., Garnett, M. C., Chhabra, S. R., Bycroft, B. W., and Baldwin, R. W. Synthesis of 2′-deoxyuridine and 5-fluoro-2′-deoxyuridine derivatives and evaluation in antibody targeting studies. J. Med. Chem., 36, 1570–1579, 1993.
6. Crosasso, P., Brusa, P., Dosio, F., et al. Antitumoral activity of liposomes and immunoliposomes containing 5-fluorouridine prodrugs. J. Pharm. Sci., 86, 832–839, 1997.

7. Mantripragada, S. A lipid based depot (DepoFoam technology) for sustained release drug delivery. Prog. Lipid Res., 41, 392–406, 2002.
8. Murry, D. J. and Blaney, S. M. Clinical pharmacology of encapsulated sustained-release cytarabine. Ann. Pharmacother., 34, 1173–1178, 2000.
9. Maxwell, I. H., Glode, L. M., and Maxwell, F. Expression of the diphtheria toxin A-chain coding sequence under the control of promoters and enhancers from immunoglobulin genes as a means of directing toxicity to B-lymphoid cells. Cancer Res., 51, 4299–4304, 1991.
10. Maxwell, I. H., Glode, L. M., and Maxwell, F. Expression of diphtheria toxin A-chain in mature B-cells: a potential approach to therapy of B-lymphoid malignancy. Leuk. Lymphoma, 7, 457–462, 1992.
11. Bundgaard H. Design of Prodrugs. Amsterdam: Elsevier; 1985.
12. Domin, B. A., Mahony, W. B., and Zimmerman, T. P. Transport of 5-fluorouracil and uracil into human erythrocytes. Biochem. Pharmacol., 46, 503–510, 1993.
13. Baldwin, S. A., Mackey, J. R., Cass, C. E., and Young, J. D. Nucleoside transporters: molecular biology and implications for therapeutic development. Mol. Med. Today, 5, 216–224, 1999.
14. Cass, C. E., Young, J. D., and Baldwin, S. A. Recent advances in the molecular biology of nucleoside transporters of mammalian cells. Biochem. Cell Biol., 76, 761–770, 1998.
15. Arner, E. S. and Eriksson, S. Mammalian deoxyribonucleoside kinases. Pharmacol. Ther., 67, 155–186, 1995.
16. Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H., and Blaese, R. M. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science, 256, 1550–1552, 1992.
17. Huber, B. E., Richards, C. A., and Krenitsky, T. A. Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. Proc. Natl. Acad. Sci. U. S. A, 88, 8039–8043, 1991.
18. Moolten, F. L., Wells, J. M., Heyman, R. A., and Evans, R. M. Lymphoma regression induced by ganciclovir in mice bearing a herpes thymidine kinase transgene. Hum. Gene Ther., 1, 125–134, 1990.
19. Mullen, C. A., Kilstrup, M., and Blaese, R. M. Transfer of the bacterial gene for cytosine deaminase to mammalian cells confers lethal sensitivity to 5-fluorocytosine: a negative selection system. Proc. Natl. Acad. Sci. U. S. A., 89, 33–37, 1992.
20. Parker, W. B., King, S. A., Allan, P. W., et al. In vivo gene therapy of cancer with *E. coli* purine nucleoside phosphorylase. Hum. Gene Ther., 8, 1637–1644, 1997.
21. Zheng, X., Johansson, M., and Karlsson, A. Retroviral transduction of cancer cell lines with the gene encoding *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase. J. Biol. Chem., 275, 39,125–39,129, 2000.
22. Mulligan, R. C. and Berg, P. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. U. S. A., 78, 2072–2076, 1981.
23. Manome, Y., Wen, P. Y., Dong, Y., et al. Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effects of cytosine arabinoside in vitro and in vivo. Nat. Med., 2, 567–573, 1996.
24. Patterson, A. V., Zhang, H., Moghaddam, A., et al. Increased sensitivity to the prodrug 5′-deoxy-5-fluorouridine and modulation of 5-fluoro-2′-deoxyuridine sensitivity in MCF-7 cells transfected with thymidine phosphorylase. Br. J. Cancer, 72, 669–675, 1995.

25. Rainov, N. G., Kramm, C. M., Banning, U., et al. Immune response induced by retrovirus-mediated HSV-tk/GCV pharmacogene therapy in patients with glioblastoma multiforme. Gene Ther., 7, 1853–1858, 2000.
26. Connors, T. A. The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. Gene Ther., 2, 702–709, 1995.
27. Christians, F. C., Scapozza, L., Crameri, A., Folkers, G., and Stemmer, W. P. Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling. Nat. Biotechnol., 17, 259–264, 1999.
28. Springer, C. J. and Niculescu-Duvaz, I. Prodrug-activating systems in suicide gene therapy. J. Clin. Invest., 105, 1161–1167, 2000.
29. Ishii-Morita, H., Agbaria, R., Mullen, C. A., et al. Mechanism of "bystander effect" killing in the herpes simplex thymidine kinase gene therapy model of cancer treatment. Gene Ther., 4, 244–251, 1997.
30. Rubsam, L. Z., Boucher, P. D., Murphy, P. J., KuKuruga, M., and Shewach, D. S. Cytotoxicity and accumulation of ganciclovir triphosphate in bystander cells cocultured with herpes simplex virus type 1 thymidine kinase-expressing human glioblastoma cells. Cancer Res., 59, 669–675, 1999.
31. Kilstrup, M., Meng, L. M., Neuhard, J., and Nygaard, P. Genetic evidence for a repressor of synthesis of cytosine deaminase and purine biosynthesis enzymes in *Escherichia coli*. J. Bacteriol., 171, 2124–2127, 1989.
32. Andersen, L., Kilstrup, M., and Neuhard, J. Pyrimidine, purine and nitrogen control of cytosine deaminase synthesis in *Escherichia coli* K 12. Involvement of the glnLG and purR genes in the regulation of codA expression. Arch. Microbiol., 152, 115–118, 1989.
33. Diasio, R. B. and Harris, B. E. Clinical pharmacology of 5-fluorouracil. Clin. Pharmacokinet., 16, 215–237, 1989.
34. Diasio, R. B., Lakings, D. E., and Bennett, J. E. Evidence for conversion of 5-fluorocytosine to 5-fluorouracil in humans: possible factor in 5-fluorocytosine clinical toxicity. Antimicrob. Agents Chemother., 14, 903–908, 1978.
35. Hayden, M. S., Linsley, P. S., Wallace, A. R., Marquardt, H., and Kerr, D. E. Cloning, overexpression, and purification of cytosine deaminase from *Saccharomyces cerevisiae*. Protein Expr. Purif., 12, 173–184, 1998.
36. Porter, D. J. *Escherichia coli* cytosine deaminase: the kinetics and thermodynamics for binding of cytosine to the apoenzyme and the Zn(2+) holoenzyme are similar. Biochim. Biophys. Acta, 1476, 239–252, 2000.
37. Ireton, G. C., McDermott, G., Black, M. E., and Stoddard, B. L. The structure of *Escherichia coli* cytosine deaminase. J. Mol. Biol., 315, 687–697, 2002.
38. Ireton, G. C., Black, M. E., and Stoddard, B. L. The 1.14 A crystal structure of yeast cytosine deaminase: evolution of nucleotide salvage enzymes and implications for genetic chemotherapy. Structure. (Camb.), 11, 961–972, 2003.
39. Kievit, E., Bershad, E., Ng, E., et al. Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts. Cancer Res., 59, 1417–1421, 1999.
40. Huber, B. E., Austin, E. A., Good, S. S., Knick, V. C., Tibbels, S., and Richards, C. A. In vivo antitumor activity of 5-fluorocytosine on human colorectal carcinoma cells genetically modified to express cytosine deaminase. Cancer Res., 53, 4619–4626, 1993.
41. Peng, X. Y., Won, J. H., Rutherford, T., et al. The use of the L-plastin promoter for adenoviral-mediated, tumor-specific gene expression in ovarian and bladder cancer cell lines. Cancer Res., 61, 4405–4413, 2001.

42. Miller, C. R., Williams, C. R., Buchsbaum, D. J., and Gillespie, G. Y. Intratumoral 5-fluorouracil produced by cytosine deaminase/5-fluorocytosine gene therapy is effective for experimental human glioblastomas. Cancer Res., 62, 773–780, 2002.
43. Mullen, C. A., Coale, M. M., Lowe, R., and Blaese, R. M. Tumors expressing the cytosine deaminase suicide gene can be eliminated in vivo with 5-fluorocytosine and induce protective immunity to wild type tumor. Cancer Res., 54, 1503–1506, 1994.
44. Huber, B. E., Austin, E. A., Richards, C. A., Davis, S. T., and Good, S. S. Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. Proc. Natl. Acad. Sci. U. S. A., 91, 8302–8306, 1994.
45. Trinh, Q. T., Austin, E. A., Murray, D. M., Knick, V. C., and Huber, B. E. Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine vs thymidine kinase/ganciclovir enzyme/prodrug systems in a human colorectal carcinoma cell line. Cancer Res., 55, 4808–4812, 1995.
46. Hoganson, D. K., Batra, R. K., Olsen, J. C., and Boucher, R. C. Comparison of the effects of three different toxin genes and their levels of expression on cell growth and bystander effect in lung adenocarcinoma. Cancer Res., 56, 1315–1323, 1996.
47. Hirschowitz, E. A., Ohwada, A., Pascal, W. R., Russi, T. J., and Crystal, R. G. In vivo adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. Hum. Gene Ther., 6, 1055–1063, 1995.
48. Greco, O. and Dachs, G. U. Gene directed enzyme/prodrug therapy of cancer: historical appraisal and future prospectives. J. Cell Physiol., 187, 22–36, 2001.
49. Cunningham, C., and Nemunaitis, J. A phase I trial of genetically modified *Salmonella typhimurium* expressing cytosine deaminase (TAPET-CD, VNP20029) administered by intratumoral injection in combination with 5-fluorocytosine for patients with advanced or metastatic cancer. Protocol no: CL-017. Version: April 9, 2001. Hum. Gene Ther., 12, 1594–1596, 2001.
50. Freytag, S. O., Khil, M., Stricker, H., et al. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. Cancer Res., 62, 4968–4976, 2002.
51. Pandha, H. S., Martin, L. A., Rigg, A., et al. Genetic prodrug activation therapy for breast cancer: a phase I clinical trial of erbB-2-directed suicide gene expression. J. Clin. Oncol., 17, 2180–2189, 1999.
52. Freytag, S. O., Stricker, H., Pegg, J., et al. Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. Cancer Res., 63, 7497–7506, 2003.
53. Adachi, Y., Tamiya, T., Ichikawa, T., et al. Experimental gene therapy for brain tumors using adenovirus-mediated transfer of cytosine deaminase gene and uracil phosphoribosyltransferase gene with 5-fluorocytosine. Hum. Gene Ther., 11, 77–89, 2000.
54. Milano, G. and Etienne, M. C. Potential importance of dihydropyrimidine dehydrogenase (DPD) in cancer chemotherapy. Pharmacogenetics, 4, 301–306, 1994.
55. Eriksson, S., Munch-Petersen, B., Johansson, K., and Eklund, H. Structure and function of cellular deoxyribonucleoside kinases. Cell Mol. Life Sci., 59, 1327–1346, 2002.

56. Van Rompay, A. R., Johansson, M., and Karlsson, A. Substrate specificity and phosphorylation of antiviral and anticancer nucleoside analogues by human deoxyribonucleoside kinases and ribonucleoside kinases. Pharmacol. Ther., 100, 119–139, 2003.
57. Brockman, R. W., Cheng, Y. C., Schabel, F. M., Jr., and Montgomery, J. A. Metabolism and chemotherapeutic activity of 9-β-D-arabinofuranosyl-2-fluoroadenine against murine leukemia L1210 and evidence for its phosphorylation by deoxycytidine kinase. Cancer Res., 40, 3610–3615, 1980.
58. Carson, D. A., Wasson, D. B., Taetle, R., and Yu, A. Specific toxicity of 2-chlorodeoxyadenosine toward resting and proliferating human lymphocytes. Blood, 62, 737–743, 1983.
59. Bouffard, D. Y., Laliberte, J., and Momparler, R. L. Kinetic studies on 2′,2′-difluorodeoxycytidine (gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem. Pharmacol., 45, 1857–1861, 1993.
60. Ullman, B., Coons, T., Rockwell, S., and McCartan, K. Genetic analysis of 2′,3′-dideoxycytidine incorporation into cultured human T lymphoblasts. J. Biol. Chem., 263, 12,391–12,396, 1988.
61. Bergman, A. M., Giaccone, G., van Moorsel, C. J., et al. Cross-resistance in the 2′,2′-difluorodeoxycytidine (gemcitabine)-resistant human ovarian cancer cell line AG6000 to standard and investigational drugs. Eur. J. Cancer, 36, 1974–1983, 2000.
62. Dumontet, C., Fabianowska-Majewska, K., Mantincic, D., et al. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br. J. Haematol., 106, 78–85, 1999.
63. Blackstock, A. W., Lightfoot, H., Case, L. D., et al. Tumor uptake and elimination of 2′,2′-difluoro-2′-deoxycytidine (gemcitabine) after deoxycytidine kinase gene transfer: correlation with in vivo tumor response. Clin. Cancer Res., 7, 3263–3268, 2001.
64. Hapke, D. M., Stegmann, A. P., and Mitchell, B. S. Retroviral transfer of deoxycytidine kinase into tumor cell lines enhances nucleoside toxicity. Cancer Res., 56, 2343–2347, 1996.
65. Kojima, H., Iida, M., Miyazaki, H., Koga, T., Moriyama, H., and Manome, Y. Enhancement of cytarabine sensitivity in squamous cell carcinoma cell line transfected with deoxycytidine kinase. Arch. Otolaryngol. Head Neck Surg., 128, 708–713, 2002.
66. Sabini, E., Ort, S., Monnerjahn, C., Konrad, M., and Lavie, A. Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. Nat. Struct. Biol., 10, 513–519, 2003.
67. Wang, L., Munch-Petersen, B., Herrstrom, S. A., et al. Human thymidine kinase 2: molecular cloning and characterisation of the enzyme activity with antiviral and cytostatic nucleoside substrates. FEBS Lett., 443, 170–174, 1999.
68. Sjoberg, A. H., Wang, L., and Eriksson, S. Substrate specificity of human recombinant mitochondrial deoxyguanosine kinase with cytostatic and antiviral purine and pyrimidine analogs. Mol. Pharmacol., 53, 270–273, 1998.
69. Zhu, C., Johansson, M., Permert, J., and Karlsson, A. Enhanced cytotoxicity of nucleoside analogs by overexpression of mitochondrial deoxyguanosine kinase in cancer cell lines. J. Biol. Chem., 273, 14,707–14,711, 1998.
70. Malspeis, L., Grever, M. R., Staubus, A. E., and Young, D. Pharmacokinetics of 2-F-ara-A (9-β-D-arabinofuranosyl-2-fluoroadenine) in cancer patients during the phase I clinical investigation of fludarabine phosphate. Semin. Oncol., 17, 18–32, 1990.

71. Xu, Y. Z. and Plunkett, W. Modulation of deoxycytidylate deaminase in intact human leukemia cells. Action of 2′,2′-difluorodeoxycytidine. Biochem. Pharmacol., 44, 1819–1827, 1992.
72. Frewin, R. J., and Johnson, S. A. The role of purine analogue combinations in the management of acute leukemias. Hematol. Oncol., 19, 151–157, 2001.
73. Robak, T. Purine nucleoside analogues in the treatment of myleoid leukemias. Leuk. Lymphoma, 44, 391–409, 2003.
74. Larsson, R., Fridborg, H., Liliemark, J., et al. In vitro activity of 2-chlorodeoxyadenosine (CdA) in primary cultures of human haematological and solid tumours. Eur. J. Cancer, 30A: 1022–1026, 1994.
75. Nabhan, C., Krett, N., Gandhi, V., and Rosen, S. Gemcitabine in hematologic malignancies. Curr. Opin. Oncol., 13, 514–521, 2001.
76. Csoka, K., Liliemark, J., Larsson, R., and Nygren, P. Evaluation of the cytotoxic activity of gemcitabine in primary cultures of tumor cells from patients with hematologic or solid tumors. Semin. Oncol., 22, 47–53, 1995.
77. Huang, P. and Plunkett, W. Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. Cancer Chemother. Pharmacol., 36, 181–188, 1995.
78. Huang, P. and Plunkett, W. Induction of apoptosis by gemcitabine. Semin. Oncol., 22, 19–25, 1995.
79. Robertson, L. E., Chubb, S., Meyn, R. E., et al. Induction of apoptotic cell death in chronic lymphocytic leukemia by 2-chloro-2′-deoxyadenosine and 9-β-D-arabinosyl-2-fluoroadenine. Blood, 81, 143–150, 1993.
80. Dionne, C. A., Camoratto, A. M., Jani, J. P., et al. Cell cycle-independent death of prostate adenocarcinoma is induced by the trk tyrosine kinase inhibitor CEP-751 (KT6587). Clin. Cancer Res., 4, 1887–1898, 1998.
81. Sadi, M. V. and Barrack, E. R. Determination of growth fraction in advanced prostate cancer by Ki-67 immunostaining and its relationship to the time to tumor progression after hormonal therapy. Cancer, 67, 3065–3071, 1991.
82. Munch-Petersen, B., Piskur, J., and Sondergaard, L. Four deoxynucleoside kinase activities from *Drosophila melanogaster* are contained within a single monomeric enzyme, a new multifunctional deoxynucleoside kinase. J. Biol. Chem., 273, 3926–3931, 1998.
83. Johansson, M., Van Rompay, A. R., Degreve, B., Balzarini, J., and Karlsson, A. Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster*. J. Biol. Chem., 274, 23,814–23,819, 1999.
84. Degreve, B., De Clercq, E., and Balzarini, J. Bystander effect of purine nucleoside analogues in HSV-1 tk suicide gene therapy is superior to that of pyrimidine nucleoside analogues. Gene Ther., 6, 162–170, 1999.
85. Zheng, X., Johansson, M., and Karlsson, A. Bystander effects of cancer cell lines transduced with the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster* and synergistic enhancement by hydroxyurea. Mol. Pharmacol., 60, 262–266, 2001.
86. Piskur, J., Sandrini, M. P., Knecht, W., and Munch-Petersen, B. Animal deoxyribonucleoside kinases: “forward” and “retrograde” evolution of their substrate specificity. FEBS Lett., 560, 3–6, 2004.
87. Johansson, K., Ramaswamy, S., Ljungcrantz, C., et al. Structural basis for substrate specificities of cellular deoxyribonucleoside kinases. Nat. Struct. Biol., 8, 616–620, 2001.

88. Knecht, W., Sandrini, M. P., Johansson, K., Eklund, H., Munch-Petersen, B., and Piskur, J. A few amino acid substitutions can convert deoxyribonucleoside kinase specificity from pyrimidines to purines. EMBO J., 21, 1873–1880, 2002.
89. Solaroli, N., Bjerke, M., Amiri, M. H., Johansson, M., and Karlsson, A. Active site mutants of *Drosophila melanogaster* multisubstrate deoxyribonucleoside kinase. Eur. J. Biochem., 270, 2879–2884, 2003.
90. Knecht, W., Petersen, G. E., Munch-Petersen, B., and Piskur, J. Deoxyribonucleoside kinases belonging to the thymidine kinase 2 (TK2)-like group vary significantly in substrate specificity, kinetics and feed-back regulation. J. Mol. Biol., 315, 529–540, 2002.
91. Knecht, W., Petersen, G. E., Sandrini, M. P., Sondergaard, L., Munch-Petersen, B., and Piskur, J. Mosquito has a single multisubstrate deoxyribonucleoside kinase characterized by unique substrate specificity. Nucleic Acids Res., 31, 1665–1672, 2003.
92. Jensen, K. F. Two purine nucleoside phosphorylases in *Bacillus subtilis*. Purification and some properties of the adenosine-specific phosphorylase. Biochim. Biophys. Acta, 525, 346–356, 1978.
93. Stoeckler, J. D., Cambor, C., and Parks, R. E., Jr. Human erythrocytic purine nucleoside phosphorylase: reaction with sugar-modified nucleoside substrates. Biochemistry, 19, 102–107, 1980.
94. Curlee, K. V., Parker, W. B., and Sorscher, E. J. Tumor sensitization to purine analogs by *E. coli* PNP. Methods Mol. Med., 90, 223–245, 2004.
95. Gadi, V. K., Alexander, S. D., Waud, W. R., Allan, P. W., Parker, W. B., and Sorscher, E. J. A long-acting suicide gene toxin, 6-methylpurine, inhibits slow growing tumors after a single administration. J. Pharmacol. Exp. Ther., 304, 1280–1284, 2003.
96. Martiniello-Wilks, R., Garcia-Aragon, J., Daja, M. M., et al. In vivo gene therapy for prostate cancer: preclinical evaluation of two different enzyme-directed prodrug therapy systems delivered by identical adenovirus vectors. Hum. Gene Ther., 9, 1617–1626, 1998.
97. Krohne, T. U., Shankara, S., Geissler, M., et al. Mechanisms of cell death induced by suicide genes encoding purine nucleoside phosphorylase and thymidine kinase in human hepatocellular carcinoma cells in vitro. Hepatology, 34, 511–518, 2001.
98. Mohr, L., Shankara, S., Yoon, S. K., et al. Gene therapy of hepatocellular carcinoma in vitro and in vivo in nude mice by adenoviral transfer of the *Escherichia coli* purine nucleoside phosphorylase gene. Hepatology, 31, 606–614, 2000.
99. Moolten, F. L. and Wells, J. M. Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. J. Natl. Cancer Inst., 82, 297–300, 1990.
100. Beltinger, C., Fulda, S., Kammertoens, T., Meyer, E., Uckert, W., and Debatin, K. M. Herpes simplex virus thymidine kinase/ganciclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases. Proc. Natl. Acad. Sci. U. S. A., 96, 8699–8704, 1999.
101. Wei, S. J., Chao, Y., Hung, Y. M., et al. S- and G2-phase cell cycle arrests and apoptosis induced by ganciclovir in murine melanoma cells transduced with herpes simplex virus thymidine kinase. Exp. Cell Res., 241, 66–75, 1998.
102. Oldfield, E. H., Ram, Z., Culver, K. W., Blaese, R. M., DeVroom, H. L., and Anderson, W. F. Gene therapy for the treatment of brain tumors using intratumoral transduction with the thymidine kinase gene and intravenous ganciclovir. Hum. Gene Ther., 4, 39–69, 1993.

103. Smythe, W. R. Prodrug/drug sensitivity gene therapy: current status. Curr. Oncol. Rep., 2, 17–22, 2000.
104. Degreve, B., Andrei, G., Izquierdo, M., et al. *Varicella-zoster* virus thymidine kinase gene and antiherpetic pyrimidine nucleoside analogues in a combined gene/chemotherapy treatment for cancer. Gene Ther., 4, 1107–1114, 1997.
105. Bonini, C., Ferrari, G., Verzeletti, S., et al. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-vs-leukemia. Science, 276, 1719–1724, 1997.
106. Ackland, S. P. and Peters, G. J. Thymidine phosphorylase: its role in sensitivity and resistance to anticancer drugs. Drug Resist. Updat., 2, 205–214, 1999.
107. Kanyama, H., Tomita, N., Yamano, T., et al. Enhancement of the anti-tumor effect of 5′-deoxy-5-fluorouridine by transfection of thymidine phosphorylase gene into human colon cancer cells. Jpn. J. Cancer Res., 90, 454–459, 1999.
108. Evrard, A., Cuq, P., Ciccolini, J., Vian, L., and Cano, J. P. Increased cytotoxicity and bystander effect of 5-fluorouracil and 5-deoxy-5-fluorouridine in human colorectal cancer cells transfected with thymidine phosphorylase. Br. J. Cancer, 80, 1726–1733, 1999.
109. Ciccolini, J., Cuq, P., Evrard, A., et al. Combination of thymidine phosphorylase gene transfer and deoxyinosine treatment greatly enhances 5-fluorouracil antitumor activity in vitro and in vivo. Mol. Cancer Ther., 1, 133–139, 2001.
110. Manome, Y., Watanabe, M., Abe, T., et al. Transduction of thymidine phosphorylase cDNA facilitates efficacy of cytosine deaminase/5-FC gene therapy for malignant brain tumor. Anticancer Res., 21, 2265–2272, 2001.
111. Rogulski, K. R., Wing, M. S., Paielli, D. L., Gilbert, J. D., Kim, J. H., and Freytag, S. O. Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization. Hum. Gene Ther., 11, 67–76, 2000.
112. Uckert, W., Kammertons, T., Haack, K., et al. Double suicide gene (cytosine deaminase and herpes simplex virus thymidine kinase) but not single gene transfer allows reliable elimination of tumor cells in vivo. Hum. Gene Ther., 9, 855–865, 1998.
113. Moriuchi, S., Wolfe, D., Tamura, M., et al. Double suicide gene therapy using a replication defective herpes simplex virus vector reveals reciprocal interference in a malignant glioma model. Gene Ther., 9, 584–591, 2002.
114. Norman, R. A., Barry, S. T., Bate, M., et al. Crystal structure of human thymidine phosphorylase in complex with a small molecule inhibitor. Structure. (Camb.), 12, 75–84, 2004.
115. Krenitsky, T. A., Neil, S. M., and Miller, R. L. Guanine and xanthine phosphoribosyltransfer activities of *Lactobacillus casei* and *Escherichia coli*. Their relationship to hypoxanthine and adenine phosphoribosyltransfer activities. J. Biol. Chem., 245, 2605–2611, 1970.
116. Vos, S., de Jersey, J., and Martin, J. L. Crystal structure of *Escherichia coli* xanthine phosphoribosyltransferase. Biochemistry, 36, 4125–4134, 1997.
117. Tamiya, T., Ono, Y., Wei, M. X., Mroz, P. J., Moolten, F. L., and Chiocca, E. A. *Escherichia coli* gpt gene sensitizes rat glioma cells to killing by 6-thioxanthine or 6-thioguanine. Cancer Gene Ther., 3, 155–162, 1996.
118. Ono, Y., Ikeda, K., Wei, M. X., Harsh, G. R., Tamiya, T., and Chiocca, E. A. Regression of experimental brain tumors with 6-thioxanthine and *Escherichia coli* gpt gene therapy. Hum. Gene Ther., 8, 2043–2055, 1997.

119. Freeman, S. M., Abboud, C. N., Whartenby, K. A., et al. The "bystander effect": tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res., 53, 5274–5283, 1993.
120. Holder, J. W., Elmore, E., and Barrett, J. C. Gap junction function and cancer. Cancer Res., 53, 3475–3485, 1993.
121. Asklund, T., Appelskog, I. B., Ammerpohl, O., et al. Gap junction-mediated bystander effect in primary cultures of human malignant gliomas with recombinant expression of the HSVtk gene. Exp. Cell Res., 284, 185–195, 2003.
122. Colombo, B. M., Benedetti, S., Ottolenghi, S., et al. The "bystander effect": association of U-87 cell death with ganciclovir-mediated apoptosis of nearby cells and lack of effect in athymic mice. Hum. Gene Ther., 6, 763–772, 1995.
123. Frank, D. K., Frederick, M. J., Liu, T. J., and Clayman, G. L. Bystander effect in the adenovirus-mediated wild-type p53 gene therapy model of human squamous cell carcinoma of the head and neck. Clin. Cancer Res., 4, 2521–2528, 1998.
124. Burrows, F. J., Gore, M., Smiley, W. R., et al. Purified herpes simplex virus thymidine kinase retroviral particles: III. Characterization of bystander killing mechanisms in transfected tumor cells. Cancer Gene Ther., 9, 87–95, 2002.
125. Kwong, Y. L., Chen, S. H., Kosai, K., Finegold, M. J., and Woo, S. L. Adenoviral-mediated suicide gene therapy for hepatic metastases of breast cancer. Cancer Gene Ther., 3, 339–344, 1996.
126. Rosenfeld, M. E., Feng, M., Michael, S. I., Siegal, G. P., Alvarez, R. D., and Curiel, D. T. Adenoviral-mediated delivery of the herpes simplex virus thymidine kinase gene selectively sensitizes human ovarian carcinoma cells to ganciclovir. Clin. Cancer Res., 1, 1571–1580, 1995.
127. Estin, D., Li, M., Spray, D., and Wu, J. K. Connexins are expressed in primary brain tumors and enhance the bystander effect in gene therapy. Neurosurgery, 44, 361–368, 1999.
128. Dilber, M. S., Abedi, M. R., Christensson, B., et al. Gap junctions promote the bystander effect of herpes simplex virus thymidine kinase in vivo. Cancer Res., 57, 1523–1528, 1997.
129. Kunishige, I., Samejima, Y., Moriyama, A., Saji, F., and Murata, Y. cAMP stimulates the bystander effect in suicide gene therapy of human choriocarcinoma. Anticancer Res., 18, 3411–3419, 1998.
130. Yamamoto, S., Yamano, T., Tanaka, M., et al. A novel combination of suicide gene therapy and histone deacetylase inhibitor for treatment of malignant melanoma. Cancer Gene Ther., 10, 179–186, 2003.
131. Grunstein, M. Histone acetylation in chromatin structure and transcription. Nature, 389, 349–352, 1997.
132. Yoshida, M., Horinouchi, S., and Beppu, T. Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. Bioessays, 17, 423–430, 1995.
133. Freeman, S. M., Ramesh, R., and Marrogi, A. J. Immune system in suicide-gene therapy. Lancet, 349, 2, 3, 1997.
134. Yamamoto, S., Suzuki, S., Hoshino, A., Akimoto, M., and Shimada, T. Herpes simplex virus thymidine kinase/ganciclovir-mediated killing of tumor cell induces tumor-specific cytotoxic T cells in mice. Cancer Gene Ther., 4, 91–96, 1997.
135. Dilber, M. S. and Smith, C. I. Suicide genes and bystander killing: local and distant effects. Gene Ther., 4, 273, 274, 1997.
136. Anderson, W. F. Human gene therapy. Science, 256, 808–813, 1992.

137. Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc. Natl. Acad. Sci. U. S. A., 93, 11,382–11,388, 1996.
138. Schneider, M. D. and French, B. A. The advent of adenovirus. Gene therapy for cardiovascular disease. Circulation, 88, 1937–1942, 1993.
139. Leon, R. P., Hedlund, T., Meech, S. J., et al. Adenoviral-mediated gene transfer in lymphocytes. Proc. Natl. Acad. Sci. U. S. A., 95, 13,159–13,164, 1998.
140. Okegawa, T., Li, Y., Pong, R. C., Bergelson, J. M., Zhou, J., and Hsieh, J. T. The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. Cancer Res., 60, 5031–5036, 2000.
141. Grill, J., Van Beusechem, V. W., Van, D. V., et al. Combined targeting of adenoviruses to integrins and epidermal growth factor receptors increases gene transfer into primary glioma cells and spheroids. Clin. Cancer Res., 7, 641–650, 2001.
142. Heise, C. C., Williams, A. M., Xue, S., Propst, M., and Kirn, D. H. Intravenous administration of ONYX-015, a selectively replicating adenovirus, induces antitumoral efficacy. Cancer Res., 59, 2623–2628, 1999.
143. Nemunaitis, J., Cunningham, C., Buchanan, A., et al. Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. Gene Ther., 8, 746–759, 2001.
144. Lou, E. Oncolytic herpes viruses as a potential mechanism for cancer therapy. Acta Oncol., 42, 660–671, 2003.
145. Wildner, O. Comparison of replication-selective, oncolytic viruses for the treatment of human cancers. Curr. Opin. Mol. Ther., 5, 351–361, 2003.
146. Karara, A. L., Bumaschny, V. F., Fiszman, G. L., Casais, C. C., Glikin, G. C., and Finocchiaro, L. M. Lipofection of early passages of cell cultures derived from murine adenocarcinomas: in vitro and ex vivo testing of the thymidine kinase/ganciclovir system. Cancer Gene Ther., 9, 96–99, 2002.
147. Zheng, X., Lundberg, M., Karlsson, A., and Johansson, M. Lipid-mediated protein delivery of suicide nucleoside kinases. Cancer Res., 63, 6909–6913, 2003.
148. Hasegawa, H., Shimada, M., Yonemitsu, Y., et al. Preclinical and therapeutic utility of HVJ liposomes as a gene transfer vector for hepatocellular carcinoma using herpes simplex virus thymidine kinase. Cancer Gene Ther., 8, 252–258, 2001.
149. Harsh, G. R., Deisboeck, T. S., Louis, D. N., et al. Thymidine kinase activation of ganciclovir in recurrent malignant gliomas: a gene-marking and neuropathological study. J. Neurosurg., 92, 804–811, 2000.
150. Hassenbusch, S. J., Nardone, E. M., Levin, V. A., Leeds, N., and Pietronigro, D. Stereotactic injection of DTI-015 into recurrent malignant gliomas: phase I/II trial. Neoplasia, 5, 9–16, 2003.
151. Richards, C. A., Austin, E. A., and Huber, B. E. Transcriptional regulatory sequences of carcinoembryonic antigen: identification and use with cytosine deaminase for tumor-specific gene therapy. Hum. Gene Ther., 6, 881–893, 1995.
152. Black, M. E., Kokoris, M. S., and Sabo, P. Herpes simplex virus-1 thymidine kinase mutants created by semi-random sequence mutagenesis improve prodrug-mediated tumor cell killing. Cancer Res., 61, 3022–3026, 2001.
153. Knecht, W., Munch-Petersen, B., and Piskur, J. Identification of residues involved in the specificity and regulation of the highly efficient multisubstrate deoxyribonucleoside kinase from *Drosophila melanogaster*. J. Mol. Biol., 301, 827–837, 2000.

17 3′-Deoxy-3′-Fluorothymidine as a Tracer of Proliferation in Positron Emission Tomography

Wieteke G. E. Direcks, MSc, Adriaan A. Lammertsma, PhD, and Carla F. M. Molthoff, PhD

CONTENTS

SUMMARY

3′-Deoxy-3′-fluorothymidine (FLT), a thymidine analog, is a relative new PET tracer for imaging proliferation. Thymidine is transported into the cell, and after several phosphorylation steps, it will be incorporated into DNA. FLT is transported into the cell by the same mechanism but is then trapped in the cell after the first phosphorylation step. The cell cycle-dependent enzyme TK1 is responsible for the first phosphorylation step of thymidine and FLT. Consequently, FLT labeled with ^{18}F is a PET tracer of TK1 activity, and as such an indirect marker of DNA synthesizing activity and cell proliferation. Potential applications of FLT are in detecting and staging tumors.

Key Words: 3′-Deoxy-3′-fluorothymidine; FLT; PET; positron emission tomography; proliferation marker; thymidine kinase.

From: *Cancer Drug Discovery and Development: Deoxynucleoside Analogs in Cancer Therapy*
Edited by: G. J. Peters © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

3′-Deoxy-3′-fluorothymidine (FLT; alovudine) belongs to the group of 3′-modified deoxynucleoside analogs (Fig. 1). The best-known compound in this group is 3′-azido-3′-deoxythymidine (AZT), which is one of the most widely used agents against human immunodeficiency virus (HIV) *(1–3)*. After phosphorylation by a thymidine kinase (TK) to a monophosphate, these compounds are further phosphorylated to a triphosphate, and they may either be incorporated into DNA *(3)* or inhibit viral reverse transcriptase. Incorporation into DNA leads to blockage of chain elongation because of the lack of the hydroxyl group on the 3′ position, thereby inhibiting viral replication.

Viral replication of HIV-1 is not completely suppressed by the deoxynucleoside analogs. One of the problems is to maintain a sufficiently high level at the site of action to inhibit viral replication. AZT is among the most potent agents against HIV-1 replication. Long-term treatment with AZT, however, is limited by its toxicity and development of drug resistance *(2–4)*. In the search for other active agents against HIV-1, 3′-fluoro-pyrimidine nucleoside analogs such as FLT have been developed. FLT proved to be at least as active as AZT in vitro, and in clinical studies efficacy was demonstrated *(5)*. Clinical results for FLT treatment provided an estimate of the therapeutic range in HIV-1-infected subjects. A narrow therapeutic window between 100 and 200 ng·h/mL showed acceptable hematological toxicity and antiretroviral activity.

Initially, FLT was labeled with ^{18}F with the objective to be able to monitor HIV infection. However, it was never used for this purpose *(6)*. Many years later, Shields et al. developed a method for [^{18}F]FLT synthesis for imaging tumor proliferation *(7,8)*.

Positron emission tomography (PET) is an imaging technique based on annihilation coincidence detection, which requires administration of a positron emitter. In contrast to imaging techniques such as computed tomography, magnetic resonance imaging, and ultrasound, which are primarily used for anatomical imaging, PET is able to visualize and quantify metabolic processes.

A positron emitter is a radionuclide that emits a positively charged β-particle (called a *positron*) and a neutrino on conversion of a proton into a neutron ($p^+ \rightarrow n + \beta^+ + \nu$). The emitted positron will travel a distance of a few millimeters, depending on its initial energy and the density of the surrounding tissue. After losing its kinetic energy, the positron combines with an electron. During this event, the masses of the two particles convert into their energy equivalent according to $E = mc^2$, resulting in the formation of two 511-KeV photons that are emitted simultaneously in opposite directions. This process is called *annihilation* (Fig. 2). After administration

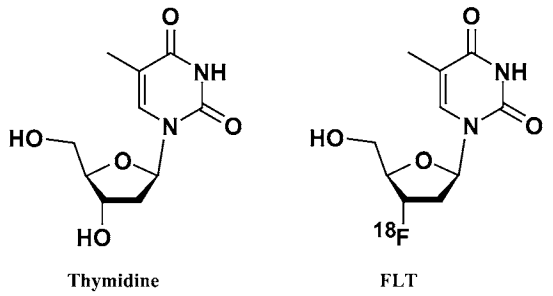


Fig. 1. Structural formulas of thymidine and FLT.

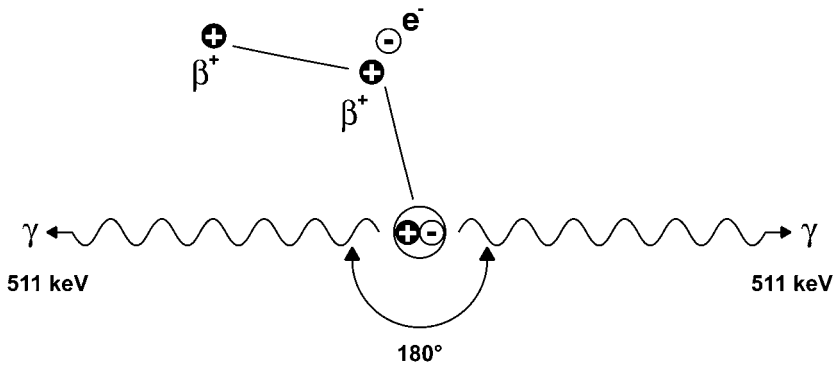


Fig. 2. Annihilation process.

of a positron-labeled molecule to a patient, its distribution can be monitored by detection of the annihilation photon pairs. For this purpose, a PET camera is used that consists of a ring of detectors placed around the body of the patient. If, within a very short time, two photons are recorded by opposing detectors, it is assumed that somewhere along the line between the two detectors an annihilation event has taken place. This line is referred to as the line of response and the simultaneous detection as a coincidence event. From the information of all lines of response, the distribution of radioactivity in the body can be reconstructed *(9)*.

Important positron-emitting radionuclides are ^{18}F, ^{11}C, and ^{15}O with half-lives of 110, 20, and 2 min, respectively. These radionuclides can be incorporated into virtually all biological elements, such as water and the glucose analog fluorodeoxyglucose (FDG).

^{18}FDG is the most commonly used PET tracer in oncology, with diagnostic, staging, and response monitoring applications *(10,11)*. ^{18}FDG is an analog of glucose in which the hydroxyl on the C2-position has been replaced by ^{18}F *(11)*. The pathway for FDG uptake is similar to that for

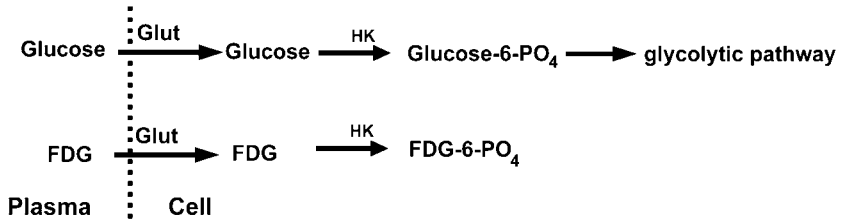


Fig. 3. FDG trapping.

glucose uptake. Both glucose and FDG are transported into the cell by glucose transporters. In the cells, the enzyme hexokinase phosphorylates glucose and FDG to glucose-6-phosphate and FDG-6-phosphate, respectively. Glucose-6-phosphate is a substrate for the next enzyme in the glycolytic pathway. In contrast, FDG-6-phosphate is not a substrate and consequently will be trapped in the cell (Fig. 3).

Tissues with high glucose consumption also have high FDG uptake. In 1930, Warburg et al. *(12)* discovered that tumors are characterized by the production of lactate (caused by glycolysis) despite the presence of sufficient oxygen. Tumors demonstrate higher glucose consumption than normal tissues, and FDG PET has been used for tumor detection and staging to obtain long-term prognostic information and to identify tumor response to chemotherapy at an early phase of treatment *(13)*. A disadvantage of FDG PET is the uptake in other tissues with physiologically high glucose consumption, such as brain, muscle, inflammatory tissue, macrophages, and lymphocytes *(14)*.

Therefore, other tracers have been developed. FLT is one of these tracers as it is considered to be more specific for proliferation, another characteristic for tumor tissue. Besides ^{18}FLT, other tracers for imaging proliferation, such as ^{11}C-thymidine and ^{11}C-choline, were developed. ^{11}C-choline is incorporated in lipids of the cell membrane, and choline kinase, involved in cellular choline uptake, is upregulated in tumor cells. However, conflicting results have been reported concerning correlations between ^{11}C-choline uptake and proliferation *(15,16)*.

^{11}C-thymidine is incorporated in DNA and therefore is a direct marker for DNA synthesis. Disadvantages of ^{11}C-thymidine have been its rapid catabolism and short half-life (20 min) *(17)*.

2. 3′-DEOXY-3′-FLUOROTHYMIDINE

2.1. Synthesis

Currently, several methods have been described for the synthesis of FLT starting from thymidine and bringing about [^{18}F]FLT with a fluor atom at

the 3′ position of the ribose (Fig. 1) *(18,19)*. The most frequently used method is that of Grierson and Shields *(18)*, a two-step process starting with thymidine and resulting in approx 500 MBq FLT within 2 h (Fig. 4). A more simplified method is that of Machulla et al. *(19)*. The precursor for the synthesis is easily (commercially) available, and the average yield is 1.5 GBq.

2.2. Pathway

Thymidine can be transported into cells by two different pathways: the salvage and the *de novo* pathways (Fig. 5). Pyrimidine precursors are predominantly supplied via the salvage pathway *(20,21)*. Exogenous thymidine is transported from the blood into cells by facilitated diffusion. Two different transporters for nucleosides, the *ei* (equilibrative protein transporter insensitive to nitrobenzylmercaptopurine) and *es* (equilibrative protein transporter sensitive to nitrobenzylmercaptopurine), control cellular influx and efflux *[22,23]*). Besides this facilitated transport, sodium-dependent concentrative carriers also mediate influx of nucleosides. In normal tissues, the latter type of transport is dominant, but in tumor cells, the equilibrium transporters prevail, especially the *es* transporter *(23)*.

Intracellular thymidine is phosphorylated by the cytosolic enzyme thymidine kinase 1 (TK1). Other enzymes are responsible for the phosphorylation of deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP) and deoxythymidine triphosphate (dTTP). Thymidine triphosphate is incorporated into DNA.

In the *de novo* pathway, small molecules are the basis for dTMP synthesis. From glutamine and adenosine triphosphate (ATP), deoxyuridine monophosphate is formed in several steps, and thymidylate synthase (TS) methylates deoxyuridine monophosphate to dTMP. Further phosphorylation steps take place in the same pathway as in the salvage pathway. In rapidly growing cells, high TS levels are found *(24)*. In resting cells, there is a lack of *de novo* synthesis *(20)*.

After transport into the cell, FLT is phosphorylated by salvage pathway enzymes. TK1 phosphorylates FLT to FLT monophosphate (Fig. 6), which is negatively charged. Controversy exists about the further phosphorylation steps of FLT monophosphate. In vitro research focused on HIV mainly reported phosphorylation into FLT triphosphate *(3,25)*, but [^{18}F]FLT studies could not confirm this and instead described trapping of FLT monophosphate *(26)*. FLT monophosphate contributes most to the radioactivity signal after FLT incubation, but a significant increase of phosphorylation into FLT triphosphate after 2 h of FLT incubation has been reported *(27)*.

Studies with ^{3}H-FLT hardly showed incorporation into DNA (0.2% in various cell lines). This would imply that FLT is not a direct marker for

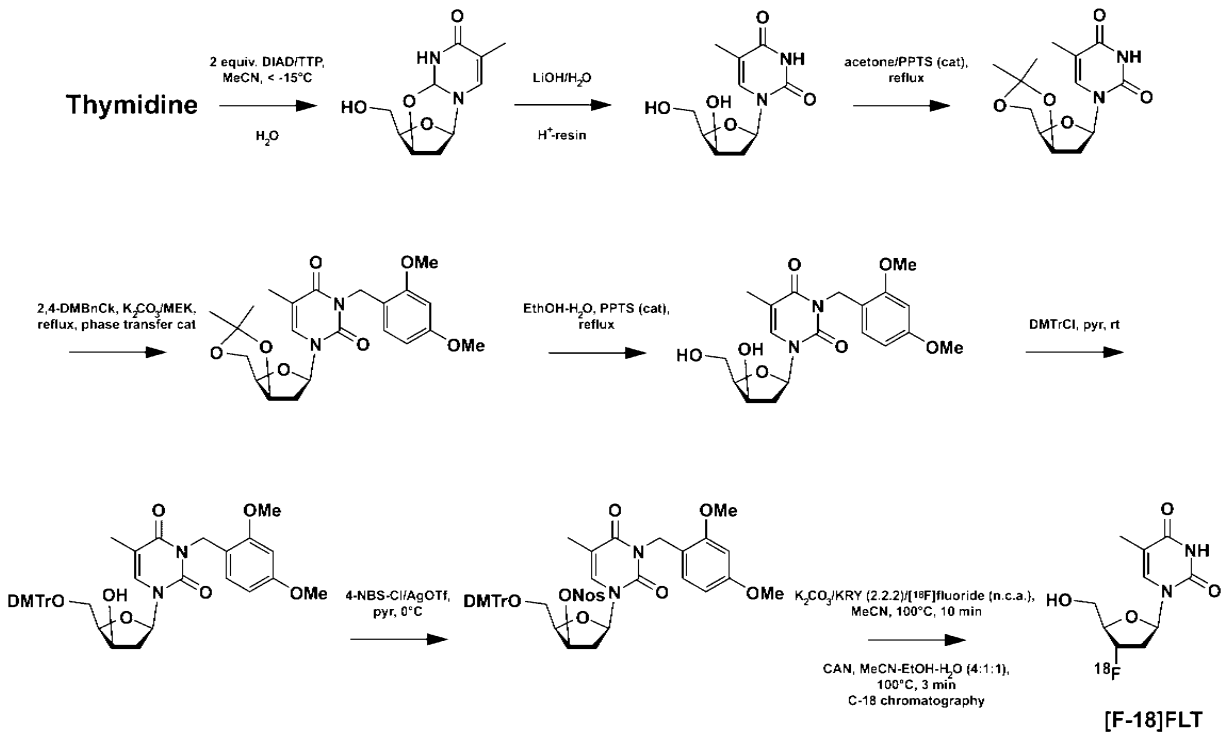


Fig. 4. Reaction steps in FLT synthesis. (Modified from ref. *18*.)

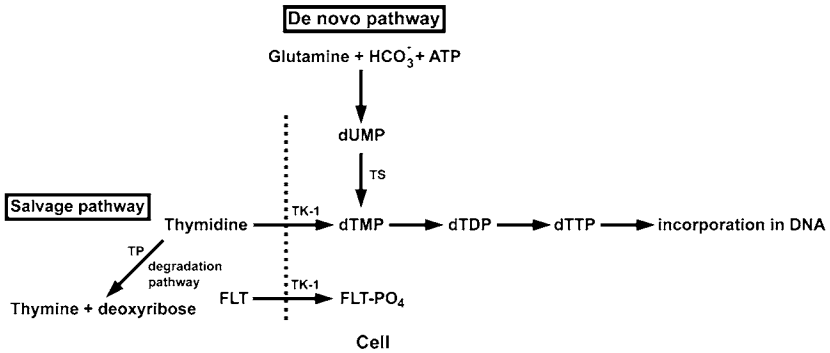


Fig. 5. De novo and salvage pathway.

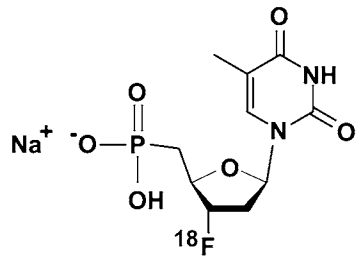


Fig. 6. Structure of FLT monophosphate *(8)*.

DNA synthesis *(28)*. This was confirmed in vivo by uptake experiments of ^{18}F-FLT in rats, with <2% DNA-incorporated activity (spleen and intestine) *(29)*. In bone marrow of dogs, low FLT incorporation also was demonstrated *(30)*.

2.3. Metabolism

Degradation of thymidine into thymine and deoxyribose-1-phosphate occurs under the influence of the enzyme thymidine phosphorylase (TP). However, FLT is resistant to catabolism by TP *(28,31)*. In most species, no metabolites of FLT, besides the nucleotide, can be demonstrated, with the exception of glucuronidation of FLT in monkeys and in humans *(30)*.

Several studies have been performed with respect to biodistribution and pharmacokinetics of FLT in vitro and in animal models. As demonstrated in a human B-cell line by high-performance liquid chromatographic (HPLC) analysis of cell lysates, most of the radioactivity appeared as FLT monophosphate, and very little was parent FLT *(32)*. In a subcutaneous xenograft tumor model of this cell line, tumor tissue displayed a time-dependent accumulation of FLT, reaching a maximum of 7.4% of the injected dose/gram at

60 min postinjection (pi). Highest FLT uptake in normal mouse tissues measured at most 5% 15 min pi with fast washout thereafter, with the exception of high uptake in the spleen (~threefold of tumor uptake with comparable kinetics) *(32)*.

In mice with fibrosarcoma tumors, the biodistribution of FDG and FLT was compared. In blood, plasma, liver, kidneys, and small intestine, FLT accumulation was higher compared with that of FDG, but was lower in brain, spinal cord, heart, and muscle *(33)*.

In another mouse tumor model for prostate cancer, fast clearance of FLT from the circulation was shown; 5 min pi, 3.8% of the injected dose/gram was present, but no comparison with FDG was performed *(34)*. Highest FLT uptake was shown 2 h pi as compared with normal tissues in this model. Ex vivo determination of plasma and liver extracts showed that 96% and 90% of total activity in plasma and liver, respectively, was caused by parent compound. A minor peak was seen in urine and in tumor tissue. Further investigations of these products showed that, in tumor tissue, phosphorylated FLT could be demonstrated *(33)*.

In non-tumor-bearing rats, at 80 min pi the highest FLT uptake was observed in kidney and intestine; spleen uptake increased up to 2 h pi *(29)*. In the same study, the effect of cimetidine was investigated, leading to increased uptake of FLT in these tissues. Radioactivity was excreted via urine, and analysis of urine 80 min pi with and without cimetidine showed that 62% and 40%, respectively, was eliminated. From the HIV literature, it was known that most unlabeled FLT administered in rats was excreted via urine, and no glucuronidated metabolites were ever detected in the urine *(35)*.

Analysis of dogs' urine and blood after FLT administration showed that more than 95% of the total radioactivity in the urine was caused by intact FLT *(8)*. The major tissue sites for FLT accumulation were bone marrow, kidneys, and bladder. In dogs, no glucuronidation of FLT has been observed *(30)*. Hardly any uptake was seen in the brain. A dog with soft tissue carcinoma showed increased uptake in the rim of proliferative tumor but no uptake in the central area of necrosis *(30)*. HPLC analysis of the supernatant of femoral marrow contained 22% unmetabolized FLT and 78% phosphorylated FLT. The HPLC conditions used could not separate mono-, di-, and triphosphate.

Pharmacokinetic studies with unlabeled FLT in rhesus monkeys did reveal glucuronidated metabolites in urine samples. A ratio of 1.45 of glucuronidated FLT/parent FLT was found 2 h pi *(36)*. In another study in monkeys with ^{3}H-FLT, most FLT found in urine was parent compound (40–60%), with 3–7% glucuronidated FLT and 3% unidentified activity (8 and 48 h pi, respectively) *(3)*. It is assumed that ^{18}F-FLT and ^{3}H-FLT behave similarly in vivo, but this has never been examined.

In human studies, glucuronidation could be expected, and in serum of patients with lymphoma, urine HPLC analysis demonstrated an additional peak with a shorter retention time than for FLT monophosphate, probably caused by 5′-glucuronide of FLT *(32)*. The parent compound made up 35% of the total urinary activity. In the blood of these patients, no other radioactive metabolites could be found up to 30 min pi. In contrast, HPLC chromatograms of arterial plasma of patients with colorectal cancer demonstrated two polar metabolites eluted before the parent compound (parent FLT measured 55–80% of the total radioactivity 60 min pi). The concentration of these metabolites increased from approx 4% at 5 min pi to 30% at 60 min pi *(37)*. At 60 min pi, 75% of radioactivity was parent FLT, and although the rate of glucuronidation is relatively slow, FLT glucuronide should be measured and taken into account for kinetic modeling *(38)*.

In most human studies, increased FLT accumulation can be observed in bone marrow, a tissue with a physiological high level of proliferation. Higher uptake in liver, kidneys, and bladder, however, is a consequence of metabolism and excretion *(39,40)*.

Probenecid has been used to decrease metabolism of AZT and to prolong its retention to improve imaging of the liver. Unfortunately, administration of probenecid prior to FLT injection did not decrease FLT glucuronidation, and no improvement of liver imaging was demonstrated *(38)*.

2.4. Thymidine Kinases and the Cell Cycle

TK1 is one of the key cytosolic enzymes in the synthesis of DNA. This enzyme catalyzes the transfer of a phosphate group from a nucleoside triphosphate to the 5′-hydroxyl group of thymidine. Only thymidine and deoxyuridine are substrates for TK1 *(41)*. The favorable phosphate donor is ATP. In the presence of ATP, TK1 is more stable and has a greater affinity for the substrate *(20)*. TK1 activity is high in proliferating cells. TK1 activity is low in the G0/G1 phase and increases during the S phase (Fig. 7.). Some cells show high TK1 levels in the M phase. After cell division, the level of TK1 drops again to the low levels in the G1/G0 phase *(31)*. In malignant tumor cells, no relation has been found between TK1 expression and cell cycle phase *(42)*. Hence, the activity of TK1 seems to be uncoupled with DNA synthesis *(28)*. Similarly, in a study of human and murine cell lines, it was demonstrated that the uptake of ^{3}H-thymidine was higher than that of ^{3}H-FLT. Again, a strong correlation ($0.41 < r^2 < 0.96$ for six different cell lines) was found between cell proliferation and TK1 *(13)*. In pancreatic cancer cell lines with overexpression of TK1 messenger RNA, TS expression also was increased *(24)*. The expression of TS showed a pattern comparable with that of TK1, suggesting an increased capacity for utilization of thymidine metabolites from both the salvage and the *de novo* pathways *(24)*.

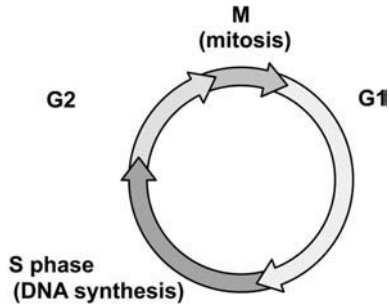


Fig. 7. Cell cycle.

Thymidine kinase 2 (TK2) is a mitochondrial enzyme. TK2 is hardly able to phosphorylate FLT. The relative affinity of TK2 for FLT was less than 0.01 compared with thymidine; for comparison, the affinity of purified human TK1 for FLT measured 0.3 compared with thymidine *(20)*. TK2 is the only TK present in nondividing cells, and the activity of TK2 is not cell cycle dependent. In dividing cells, only 1–5% of the TK activity is TK2 activity *(43)*.

Considering species differences with respect to FLT uptake, it was shown that rats had a 10-fold higher thymidine blood level than humans. Because thymidine is a competing substrate for TK1, less accumulation of FLT will be found in rats. Therefore, an FLT study in rats has to be designed with care. One way to deal with the high levels of thymidine in rat blood is the injection of TP prior to experimentation. Thymidine will be degraded to deoxyribose-1-phosphate and thymine, leading to increased uptake of FLT in tissue *(44)*.

In 22 different tumor cell lines, uptake of ^{3}H-FLT was analyzed with respect to the number of individual cells in the S phase (Fig. 8). These cell lines demonstrated predefined differences in cell proliferation genes *(28)*. Results showed a direct correlation between the percentage of cells in S phase and FLT accumulation ($r = 0.76$, $p < 0.0001$), implying that FLT reflected proliferation despite genetic differences between various cell lines *(45)*. The investigators also examined the uptake of arabinothymidine, a substrate for TK2 (not correlated with S phase, $r = 0.19$, $p = 0.39$).

3. APPLICATIONS

3.1. Oncology

At present, FDG is the most widely used tracer in oncology for tumor detection, staging, and response monitoring *(10)*. A disadvantage of FDG is its uptake in macrophages, lymphocytes, and inflammatory tissues.

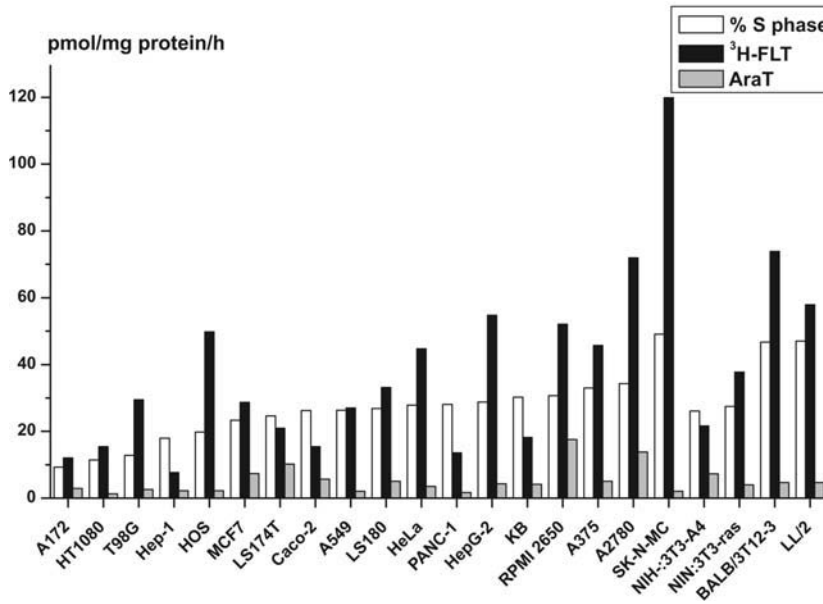


Fig. 8. Uptake of ^{3}H-FLT and arabinothymidine in various cell lines. (Modified from ref. *45*.)

Tumor growth and DNA synthesis are attractive alternative targets for imaging tumors *(46)*. FLT is an indirect marker of DNA synthesis and proliferation. Tumors usually are characterized by a high degree of proliferation, and therefore high FLT uptake can be expected.

Primary colorectal cancer and malignant soft tissue carcinomas were detected with a sensitivity of 100% *(26,47)*, but sensitivity was lower in patients with bronchial carcinoma *(48)*. For detecting laryngeal cancer, equal sensitivities of 88% for FDG and FLT were obtained *(49)*. Hardly any uptake of FLT was seen in recurrent tumor *(49)*.

In both lung and esophageal tumors, FLT uptake was higher in the squamous cell type than in adenocarcinomas *(50)*. Squamous cell carcinoma of non-small cell lung cancer (NSCLC) is also more sensitive to chemotherapy, indicating that FLT could be used as a prognostic marker in untreated NSCLC *(50)*. However, low sensitivity could result in false-negative scans and understaging of patients *(42)*.

Detection of tumors with FDG can result in false positive-findings caused by FDG uptake in inflammatory tissue. FLT uptake in inflammatory tissues was investigated by van Waarde et al. in an experimental rat model and showed significant differences between tumor and inflammatory tissue *(44)*. Because of its lower sensitivity, FLT is not a substitute for

FDG in detecting well-differentiated pulmonary or slowly proliferating tumors *(46,48)*. Although FLT uptake in liver and bone metastases is slightly higher than in surrounding tissues, detection of these metastases is difficult because of the high uptake of FLT in these organs *(50)*.

FLT has high potential for the detection of brain tumors based on low uptake of FLT in normal brain caused by both low extraction and a low proliferation index of normal brain cells *(50,51)*. FLT appeared more sensitive (100%) in identifying recurrent high-grade glioma than FDG (72%), and FLT also better predicted tumor progression. Furthermore, significant differences in FLT uptake were found between high-grade and low-grade glioma. FDG showed the same results, but the tumor/nontumor ratio was higher for FLT (3.54 for FLT vs 1.28 for FDG) *(51,52)*.

Staging with FLT is possible in pulmonary nodules because FLT uptake is observed only in malignant and not in benign tumors. It should be noted, however, that FDG is more sensitive *(53,54)*, and FLT is considered not suitable for staging in NSCLC *(55)*. In soft tissue carcinomas, FLT has the potential to distinguish between low- (grade 1) or high- (grade 2 or higher) grade soft tissue carcinomas, whereas FDG is not able to differentiate between these grades *(47)*.

3.2. Response Monitoring

FLT is an indirect marker of DNA synthesis activity, which can be a target for chemotherapy *(28)*. Chemotherapeutic agents may affect DNA synthesis in various ways. The chemotherapeutic drug 5-fluorouracil (5-FU) is a *de novo* pathway inhibitor by inhibiting the enzyme TS *(56)*. The *de novo* pathway is also inhibited by the drug methotrexate (MTX) by depleting nucleotide pools. Cisplatin (CCDP) does not interfere with this pathway but binds to DNA and makes internal crosslinks or links with other DNA strands, causing local denatured DNA. Within the cell, gemcitabine (GEM, 2′-2′-difluorodeoxycytidine) is converted into the 5′-triphosphate form before it is incorporated into DNA, terminating strand elongation *(56)*.

The effects of these four different chemotherapeutic agents on FLT uptake were tested in vitro. Esophageal carcinoma cells were incubated with different concentrations of the drugs. Cells treated with CCDP showed S-phase arrest, and FLT uptake was decreased; after treatment with 5-FU and MTX, the amount of FLT increased in the cells. Cells treated with 5-FU and MTX showed cell cycle arrest at the beginning of the S phase. Both 5-FU and MTX are inhibiting the *de novo* pathway, causing cell cycle arrest in the early S phase. The salvage pathway will compensate for inhibition of the *de novo* pathway, and increased TK1 activity may be responsible for increased accumulation of FLT *(56)*. FLT uptake hardly changed after gemcitabine treatment, probably because of the salvage pathway activation *(56)*.

In mice, uptake of FLT in fibrosarcoma was lower after treatment with 5-FU *(33)* than in nontreated mice, in contrast to the in vitro results with esophageal carcinoma cells *(56)*. This decrease in FLT uptake was correlated with decreases in tumor volume of 25% and 33%, at 24 and 48 h after treatment, respectively *(33)*. The 5-FU-induced reduction in tumor FLT uptake was significantly more pronounced than that of FDG *(33)*. The levels of TK1 decreased 24 h after treatment with 5-FU, but at 48 h an increase was observed, which is in contrast to the lower uptake of FLT observed at 48 h. The levels of the cofactor ATP were lower in 5-FU-treated tumors than in control mice. The decrease in FLT uptake, combined with the increased TK1 levels, at 48 h after treatment could be caused by a decreased level of ATP *(33)*. In mice bearing TK1 +/– cells in one flank and TK1 –/– cells in the other flank, higher FLT uptake was shown in the TK1 +/– tumor. This could be explained by the higher TK1 concentration of 48% in the TK1 +/– tumor. ATP levels were the same in both types of tumor. Not only TK1 expression is important in the uptake of FLT, but also the levels of TK1 and the cofactor ATP are important *(57)*.

An in vivo fibrosarcoma mouse model was also used to determine the FLT uptake after CCDP treatment. In concordance with the 5-FU treatment, FLT uptake decreased after CCDP treatment. Also, TK1 levels showed a similar pattern as for 5-FU: a decrease after 24 h followed by an increase at 48 h and ATP levels decreased in time. In this model, FLT was superior to FDG for imaging changes in proliferation associated with early response after chemotherapy *(58)*. For other anticancer drugs involved in cell cycle control, a decrease in FLT uptake might actually be caused by the corresponding decrease in TK1 levels *(33)*.

Apart from chemotherapy, it may also be possible to use FLT for response monitoring of other types of therapy. Mice with androgen-dependent prostate tumor were treated with surgical castration or diethylstilbestrol, and FLT uptake was determined. A decrease in FLT uptake was observed 2 and 3 wk after treatment. Diethylstilbestrol and castration ensured a decrease in serum testosterone levels, and this will affect TK1 activity and hence FLT uptake, making it suitable for response monitoring *(34)*.

The use of protein kinase inhibitors in the treatment of solid tumors is a promising form of targeted therapy. Mice with ErbB-overexpressing A431 xenografts treated with an ErbB inhibitor showed a 79% decrease in FLT tumor uptake during the first week of treatment. Histological evaluation of cellular proliferation by staining for proliferating cell nuclear antigen (PCNA) correlated with FLT uptake. FLT PET may become a valuable test to determine clinically the effectiveness of ErbB kinase inhibitors *(59)*.

In mice bearing a SCCVII tumor (squamous cell carcinoma), ^{18}FLT and ^{3}H-thymidine uptake (both markers for proliferation) decreased rapidly after radiotherapy. In HeLa-bearing mice, the same result was observed

after photodynamic therapy. A tendency for ^{18}FLT uptake to increase 7 d after radiotherapy indicated that the role of FLT as an indicator of tumor regrowth is not clear *(60)*.

Thus far, no patient studies have been published on response monitoring after chemotherapy using FLT PET. At present, in our department a response monitoring study is under way in patients with (locally) advanced breast cancer to compare FDG and FLT before and after one course of high-dose chemotherapy.

4. PET IMAGING

4.1. Imaging and Kinetic Model

Most studies used the standardized uptake value (SUV) to compare uptake of FDG and FLT. SUV is a semiquantitative method and does not take into account tracer kinetics *(61)*. It is defined as the tissue concentration of tracer divided by the injected dose *(40,62)*, normalized for patient weight:

$$SUV = C/(ID/W)$$

where C is the tissue concentration (Bq/mL), ID is the injected dose (Bq), and W is the patient weight (kg).

In theory, tracer uptake should reach a constant value to obtain correct SUV measurements. For an irreversible tracer, this usually is not the case, and therefore SUV will depend on the time between tracer injection and PET scanning *(62)*. This time difference may vary between studies; consequently, comparing SUV values from different studies should be performed with great care. More important, the error because no equilibrium will be reached might be different before and after chemotherapy, thereby complicating the interpretation of changes in SUV.

The calculation of SUV does take into account differences in patient weight. If, however, uptake in fat is different from that in nonfatty tissues, a simple correction for weight might not be optimal. For FDG, this has led to alternative normalizations (i.e., for body surface area or lean body mass) *(62)*, although it is still not clear which normalization is best. This is certainly the case for FLT as data are lacking.

The SUVs for FLT and FDG were compared in colorectal cancer, soft tissue carcinoma, pulmonary nodules, esophageal cancer, laryngeal cancer, thoracic tumors, lung cancer, gliomas, and breast cancer. SUV values for FLT were always lower than for FDG *(26,47–51,53,63,64)*. In breast cancer, it was found that, because of the very low FLT uptake in surrounding normal breast tissue, tumor contrast was comparable to that with FDG *(63)*. Because mediastinum demonstrated low FLT uptake, the tumor-to-mediastinum ratio was even higher than that of FDG, facilitating detection of small tumor foci in this area *(63)*.

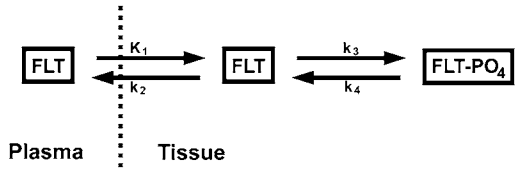


Fig. 9. Tracer kinetic model for FLT in dogs. (Modified from ref. *30*)

Because of low FLT background signal in normal tissue (i.e., low rate of proliferation), tumors can be imaged with high contrast *(50)*. Lower contrast because of higher background levels has only been in liver (glucuronidation) and bone marrow (proliferating tissue).

FLT is a relatively new tracer, and a three-compartment model has been proposed for its kinetics, analogous to the FDG model (Fig. 9). The rate constant of the transport of FLT into tissue is described by K_1; k_2 represents outflow, and k_3 describes the rate constant of phosphorylation of FLT into FLT monophosphate. Dephosphorylation of FLT, represented by k_4, is assumed to be negligible during the time of a PET study (60 min in general). For interpreting FLT metabolism, estimation of k_4 may be important, especially in tumors, where therapy may have an effect on reutilization of nucleotides. Elimination of k_4 in lung tumor simulation leads to a bias of 30% in the K_{FLT} (flux constant for FLT from plasma into phosphorylated state in tissue), which could mask a biological response to therapy. Simulations suggest that longer imaging times (>60 min) reduce the bias and associated error in the estimation of K_{FLT} *(65,66)*. In dogs, application of this three-compartment model predicted that at 60 min pi 63% of total activity would be present as phosphorylated FLT (because of chromatographic conditions, no differences could be made between the various phosphorylated forms of FLT) and 37% as free FLT. The measured values were 78% and 22%, respectively *(30)* and consistent with the predicted values. In colorectal cancer patients, the same kinetic model was applied *(37)*. The quantitative behavior of FLT could be characterized up to 60 min pi using a 3k model or Patlak analysis in combination with image-derived input functions *(37)*.

4.2. Safety and Toxicity

FLT is a relatively new PET tracer and information about radiation-related risks is useful. Information about toxicity is readily available from acquired immunodeficiency syndrome research. For PET studies, FLT is administered in tracer quantities. As these doses are orders of magnitude lower than those given (as antiviral drug) in the treatment of HIV-positive patients, there are no toxicity problems in PET *(67)*. The biodistribution of ^{18}F-FLT in patients with (suspected) lung cancer was used for estimating the doses for different organs. The mean dose was the highest for the bladder.

Voiding 2 h after injection, however, will decrease this dose ($7.91*e^{-2}$ mGy/MBq) more than 50% instead of voiding only 6 h pi ($1.79*e^{-1}$ mGy/MBq) *(68)*. The mean organ doses for ^{18}F-FLT are comparable with those for ^{18}F-FDG *(68,69)*.

4.3. Biomarkers

In pathology, proliferation is often studied by the amount of Ki-67 staining, a nuclear antigen only expressed in the G1, G2, and S phases of the cell cycle *(70,71)*. It has also been indicated as a prognostic marker *(71–74)*.

Ki-67 staining in solitary pulmonary nodules *(75)*, glioma, and lung cancer *(40,50,51,53)* was positive in all malignant tumor lesions, positively correlating with FLT uptake. In benign tumors, proliferative activity as indicated by Ki-67 staining was less than 5%, with no FLT uptake *(75)*. In contrast, in primary breast and esophageal cancer and thoracic tumors, no correlation between FLT uptake and the amount of Ki-67 could be demonstrated *(50,63,64)*. This lack of correlation might be explained by the known differences in tumor growth pattern of breast tumors compared with lung cancer *(63)*. Ki-67 levels were assessed in the proliferating part of the esophageal cancer and compared with tumor SUV values. This comparison will influence outcome *(64)*. For thoracic tumors, it might be caused by the diversity of tumor types included in the study *(50)*.

P53, a tumor suppressor protein, is involved at the checkpoint between the G1 and S phases of the cell cycle *(76)*. Cells with DNA damage, caused by hypoxia, radiation, or chemotherapy *(77)*, show a rapid increase of p53 levels *(76)*. This results in G1 arrest or apoptosis *(76,77)*. In cell lines, activation of p53 is involved in maintaining the common relationship between TK1 activity and S phase by regulating TK1 activity. In human cancers, often mutations of p53 are seen with subsequent deregulations of the cell cycle. It also has been reported that, in the absence of p53, TK1 activity and FLT uptake can remain high even when the fraction of cells in the S phase is low *(77)*. Another characteristic of tumors is the presence of regions with low oxygen concentration because of hypoxia. FLT uptake in these regions may be p53 dependent, resulting in lower tracer uptake *(77)*.

5. SUMMARY AND CONCLUSIONS

FLT, a thymidine analog, is a relative new PET tracer for imaging proliferation. Thymidine is transported into the cell, and after several phosphorylation steps, it will be incorporated into DNA. FLT is transported into the cell by the same mechanism but is then trapped in the cell after the first phosphorylation step. The cell cycle-dependent enzyme TK1 is responsible for the first phosphorylation step of thymidine and FLT. Consequently, FLT labeled with ^{18}F is a PET tracer of TK1 activity, and as such an indirect marker of DNA synthesizing activity and cell proliferation.

Potential applications of FLT are in detecting and staging tumors. At present, in the reported studies the sensitivity of detecting tumors is comparable with FDG, although in general the uptake of FLT is lower than that of FDG. A disadvantage of FLT is the difficulty of detecting liver and bone metastases because of high physiological uptake in liver and bone marrow. FLT has great potential in detecting brain metastases because there is low physiological uptake in normal brain. An important advantage of FLT over FDG is that it can discriminate between tumor and inflammatory tissue.

Treatment with chemotherapeutic drugs such as 5-FU and CCDP resulted in a change of FLT uptake both in vitro and in vivo. Experiments in which physiological processes in animals are studied should be designed with care. Knowledge of the specific animal used is of great importance to interpret the results and extrapolate them to humans *(78)*. At present, no credible patient studies have been published on response monitoring after chemotherapy. As a tracer of proliferation, however, FLT could become an important tool for early response monitoring. At present, in our department such a study is operational in breast cancer patients comparing FDG and FLT before and after one course of high-dose chemotherapy. Furthermore, a simultaneous dynamic FLT PET study in head-and-neck cancer patients will give insight into the best kinetic model for analyzing quantitative behavior of FLT.

Glucuronidated FLT is the only labeled metabolite found in monkeys and humans. FLT is primarily excreted via the urine. Therefore, high physiological uptake of FLT (or its metabolite) is seen in liver (glucuronidation), bladder, and kidneys. In addition, high uptake is seen in bone marrow as a result of the physiological high level of proliferation. The radiation dose for different organs is comparable with that of ^{18}F-FDG.

As a tracer of proliferation, FLT could be a more specific tumor marker than FDG. Its potential for early response monitoring warrants further studies.

REFERENCES

1. Isel C, Ehresmann C, Walter P, Ehresmann B, Marquet R. The emergence of different resistance mechanisms toward nucleoside inhibitors is explained by the properties of the wild type HIV-1 reverse transcriptase. J Biol Chem 2001;276: 48,725–48,732.
2. Johnston MI, Hoth DF. Present status and future prospects for HIV therapies. Science 1993;260:1286–1293.
3. Kong XB, Zhu QY, Vidal PM, et al. Comparisons of anti-human immunodeficiency virus activities, cellular transport, and plasma and intracellular pharmacokinetics of 3′-fluoro-3′-deoxythymidine and 3′-azido-3′-deoxythymidine. Antimicrob Agents Chemother 1992;36:808–818.
4. Kumar R, Wang L, Wiebe LI, Knaus EE. Synthesis and antiviral (HIV-1, HBV) activities of 5-halo-6-methoxy(or azido)-5,6-dihydro-3′-fluoro-3′-deoxythymidine

diastereomers. Potential prodrugs to 3′-fluoro-3′-deoxythymidine. J Med Chem 1994;37:3554–3560.
5. Flexner C, van der Horst C, Jacobson MA, et al. Relationship between plasma concentrations of 3′-deoxy-3′-fluorothymidine (alovudine) and antiretroviral activity in two concentration-controlled trials. J Infect Dis 1994;170:1394–1403.
6. Wilson IK, Chatterjee S, Wolf W. Synthesis of 3′-fluoro-3′-deoxythymidine and studies of its ^{18}F-radiolabeling, as a tracer for the noninvasive monitoring of the biodistribution of drugs against AIDS. J Fluorine Chem 1991;55:283–289.
7. Shields AF, Grierson JR, Kozawa SM, Zheng M. Development of labeled thymidine analogs for imaging tumor proliferation. Nucl Med Biol 1996;23:17–22.
8. Shields AF, Grierson JR, Dohmen BM, et al. Imaging proliferation in vivo with [F-18]FLT and positron emission tomography. Nat Med 1998;4:1334–1336.
9. Townsend DW. Physical principles and technology of clinical PET imaging. Ann Acad Med Singapore 2004;33:133–145.
10. Klabbers BM, Lammertsma AA, Slotman BJ. The value of positron emission tomography for monitoring response to radiotherapy in head and neck cancer. Mol Imaging Biol 2003;5:257–270.
11. Pauwels EK, Sturm EJ, Bombardieri E, Cleton FJ, Stokkel MP. Positron-emission tomography with [^{18}F]fluorodeoxyglucose. Part I. Biochemical uptake mechanism and its implication for clinical studies. J Cancer Res Clin Oncol 2000;126: 549–559.
12. Warburg O, Wind F, Negalein E. The metabolism of tumours in the body. J Physiol 1927;8:519–530.
13. Schwartz JL, Tamura Y, Jordan R, Grierson JR, Krohn KA. Monitoring tumor cell proliferation by targeting DNA synthetic processes with thymidine and thymidine analogs. J Nucl Med 2003;44:2027–2032.
14. Maschauer S, Prante O, Hoffmann M, Deichen JT, Kuwert T. Characterization of 18F-FDG uptake in human endothelial cells in vitro. J Nucl Med 2004;45:455–460.
15. Breeuwsma AJ, Pruim J, Jongen MM, et al. In vivo uptake of [^{11}C]choline does not correlate with cell proliferation in human prostate cancer. Eur J Nucl Med Mol Imaging 2005;32:668–673.
16. Al Saeedi F, Welch AE, Smith TA. [Methyl-3H]choline incorporation into MCF7 tumour cells: correlation with proliferation. Eur J Nucl Med Mol Imaging 2005; 32:660–667.
17. Wells P, West C, Jones T, Harris A, Price P. Measuring tumor pharmacodynamic response using PET proliferation probes: the case for 2-[(11)C]-thymidine. Biochim Biophys Acta 2004;1705:91–102.
18. Grierson JR, Shields AF. Radiosynthesis of 3′-deoxy-3′-[(18)F]fluorothymidine: [(18)F]FLT for imaging of cellular proliferation in vivo. Nucl Med Biol 2000;27:143–156.
19. Machulla H-J, Blocher A, Kuntzsch M, Piert M, Wie R, Grierson JR. Simplified labeling approach for synthesizing 3′-deoxy-3′-[^{18}F]fluorothymidine ([18F]FLT). J Radioanalyt Nucl Chem 2000;243:843–846.
20. Arner ES, Eriksson S. Mammalian deoxyribonucleoside kinases. Pharmacol Ther 1995;67:155–186.
21. Cohen A, Barankiewicz J, Lederman HM, Gelfand EW. Purine and pyrimidine metabolism in human T lymphocytes. Regulation of deoxyribonucleotide metabolism. J Biol Chem 1983;258:12,334–12,340.
22. Belt JA, Marina NM, Phelps DA, Crawford CR. Nucleoside transport in normal and neoplastic cells. Adv Enzyme Regul 1993;33:235–252.

23. Mier W, Haberkorn U, Eisenhut M. [^{18}F]FLT; portrait of a proliferation marker. Eur J Nucl Med Mol Imaging 2002;29:165–169.
24. Seitz U, Wagner M, Neumaier B, et al. Evaluation of pyrimidine metabolising enzymes and in vitro uptake of 3′-[(18)F]fluoro-3′-deoxythymidine ([(18)F]FLT) in pancreatic cancer cell lines. Eur J Nucl Med Mol Imaging 2002;29:1174–1181.
25. Sundseth R, Joyner SS, Moore JT, Dornsife RE, Dev IK. The anti-human immunodeficiency virus agent 3′-fluorothymidine induces DNA damage and apoptosis in human lymphoblastoid cells. Antimicrob Agents Chemother 1996;40:331–335.
26. Francis DL, Visvikis D, Costa DC, et al. Potential impact of [^{18}F]3′-deoxy-3′-fluorothymidine vs [^{18}F]fluoro-2-deoxy-D-glucose in positron emission tomography for colorectal cancer. Eur J Nucl Med Mol Imaging 2003;30:988–994.
27. Grierson JR, Schwartz JL, Muzi M, Jordan R, Krohn KA. Metabolism of 3′-deoxy-3′-[F-18]fluorothymidine in proliferating A549 cells: Validations for positron emission tomography. Nucl Med Biol 2004;31:829–837.
28. Toyohara J, Fujibayashi Y. Trends in nucleoside tracers for PET imaging of cell proliferation. Nucl Med Biol 2003;30:681–685.
29. Lu L, Samuelsson L, Bergstrom M, Sato K, Fasth KJ, Langstrom B. Rat studies comparing ^{11}C-FMAU, ^{18}F-FLT, and ^{76}Br-BFU as proliferation markers. J Nucl Med 2002;43:1688–1698.
30. Shields AF, Grierson JR, Muzik O, et al. Kinetics of 3′-deoxy-3′-[F-18]fluorothymidine uptake and retention in dogs. Mol Imaging Biol 2002;4:83–89.
31. Rasey JS, Grierson JR, Wiens LW, Kolb PD, Schwartz JL. Validation of FLT uptake as a measure of thymidine kinase-1 activity in A549 carcinoma cells. J Nucl Med 2002;43:1210–1217.
32. Wagner M, Seitz U, Buck A, et al. 3′-[^{18}F]fluoro-3′-deoxythymidine ([^{18}F]-FLT) as positron emission tomography tracer for imaging proliferation in a murine B-cell lymphoma model and in the human disease. Cancer Res 2003;63:2681–2687.
33. Barthel H, Cleij MC, Collingridge DR, et al. 3′-Deoxy-3′-[^{18}F]fluorothymidine as a new marker for monitoring tumor response to antiproliferative therapy in vivo with positron emission tomography. Cancer Res 2003;63:3791–3798.
34. Oyama N, Ponde DE, Dence C, Kim J, Tai YC, Welch MJ. Monitoring of therapy in androgen-dependent prostate tumor model by measuring tumor proliferation. J Nucl Med 2004;45:519–525.
35. Boudinot FD, Smith SG, Funderburg ED, Schinazi RF. Pharmacokinetics of 3′-fluoro-3′-deoxythymidine and 3′-deoxy-2′,3′-didehydrothymidine in rats. Antimicrob Agents Chemother 1991;35:747–749.
36. Schinazi RF, Boudinot FD, Ibrahim SS, Manning C, McClure HM, Liotta DC. Pharmacokinetics and metabolism of racemic 2′,3′-dideoxy-5-fluoro-3′-thiacytidine in rhesus monkeys. Antimicrob Agents Chemother 1992;36:2432–2438.
37. Visvikis D, Francis D, Mulligan R, et al. Comparison of methodologies for the in vivo assessment of (18)FLT utilisation in colorectal cancer. Eur J Nucl Med Mol Imaging 2003.
38. Shields AF, Briston DA, Chandupatla S, et al. A simplified analysis of [(18)F] 3′-deoxy-3′-fluorothymidine metabolism and retention. Eur J Nucl Med Mol Imaging 2005.
39. Cobben DC, Jager PL, Elsinga PH, Maas B, Suurmeijer AJ, Hoekstra HJ. 3′-^{18}F-Fluoro-3′-deoxy-L-thymidine: a new tracer for staging metastatic melanoma? J Nucl Med 2003;44:1927–1932.
40. Vesselle H, Grierson J, Muzi M, et al. In vivo validation of 3′deoxy-3′-[(18)F]fluorothymidine ([(18)F]FLT) as a proliferation imaging tracer in

humans: correlation of [(18)F]FLT uptake by positron emission tomography with Ki-67 immunohistochemistry and flow cytometry in human lung tumors. Clin Cancer Res 2002;8:3315–3323.
41. Eriksson S, Munch-Petersen B, Johansson K, Eklund H. Structure and function of cellular deoxyribonucleoside kinases. Cell Mol Life Sci 2002;59:1327–1346.
42. Been LB, Suurmeijer AJ, Cobben DC, Jager PL, Hoekstra HJ, Elsinga PH. [(18)F]FLT-PET in oncology: current status and opportunities. Eur J Nucl Med Mol Imaging 2004;31:1659–1672.
43. Munch-Petersen B, Cloos L, Jensen HK, Tyrsted G. Human thymidine kinase 1. Regulation in normal and malignant cells. Adv Enzyme Regul 1995;35:69–89.
44. van Waarde A, Cobben DC, Suurmeijer AJ, et al. Selectivity of ^{18}F-FLT and ^{18}F-FDG for differentiating tumor from inflammation in a rodent model. J Nucl Med 2004;45:695–700.
45. Toyohara J, Waki A, Takamatsu S, Yonekura Y, Magata Y, Fujibayashi Y. Basis of FLT as a cell proliferation marker: comparative uptake studies with [^{3}H]thymidine and [^{3}H]arabinothymidine, and cell-analysis in 22 asynchronously growing tumor cell lines. Nucl Med Biol 2002;29:281–287.
46. Shields AF. PET imaging with 18F-FLT and thymidine analogs: promise and pitfalls. J Nucl Med 2003;44:1432–1434.
47. Cobben DC, Elsinga PH, Suurmeijer AJ, et al. Detection and grading of soft tissue sarcomas of the extremities with (18)F-3′-fluoro-3′-deoxy-L-thymidine. Clin Cancer Res 2004;10:1685–1690.
48. Halter G, Buck AK, Schirrmeister H, et al. [^{18}F]3-Deoxy-3′-fluorothymidine positron emission tomography: alternative or diagnostic adjunct to 2-[^{18}F]-fluoro-2-deoxy-D-glucose positron emission tomography in the workup of suspicious central focal lesions? J Thorac Cardiovasc Surg 2004;127:1093–1099.
49. Cobben DC, van der Laan BF, Maas B, et al. ^{18}F-FLT PET for visualization of laryngeal cancer: comparison with ^{18}F-FDG PET. J Nucl Med 2004;45:226–231.
50. Dittmann H, Dohmen BM, Paulsen F, et al. [^{18}F]FLT PET for diagnosis and staging of thoracic tumours. Eur J Nucl Med Mol Imaging 2003;30:1407–1412.
51. Chen W, Cloughesy T, Kamdar N, et al. Imaging proliferation in brain tumors with ^{18}F-FLT PET: comparison with ^{18}F-FDG. J Nucl Med 2005;46:945–952.
52. Choi SJ, Kim JS, Kim JH, et al. [^{18}F]3′-Deoxy-3′-fluorothymidine PET for the diagnosis and grading of brain tumors. Eur J Nucl Med Mol Imaging 2005;32:653–659.
53. Buck AK, Halter G, Schirrmeister H, et al. Imaging proliferation in lung tumors with PET: ^{18}F-FLT vs ^{18}F-FDG. J Nucl Med 2003;44:1426–1431.
54. Buck AK, Hetzel M, Schirrmeister H, et al. Clinical relevance of imaging proliferative activity in lung nodules. Eur J Nucl Med Mol Imaging 2005;32:525–533.
55. Cobben DC, Elsinga PH, Hoekstra HJ, et al. Is ^{18}F-3′-fluoro-3′-deoxy-L-thymidine useful for the staging and restaging of non-small cell lung cancer? J Nucl Med 2004;45:1677–1682.
56. Dittmann H, Dohmen BM, Kehlbach R, et al. Early changes in [^{18}F]FLT uptake after chemotherapy: an experimental study. Eur J Nucl Med Mol Imaging 2002;29:1462–1469.
57. Barthel H, Perumal M, Latigo J, et al. The uptake of 3′-deoxy-3′-[(18)F]fluorothymidine into L5178Y tumours in vivo is dependent on thymidine kinase 1 protein levels. J Nucl Med Mol Imaging 2005;32:257–263.
58. Leyton J, Latigo JR, Perumal M, Dhaliwal H, He Q, Aboagye EO. Early detection of tumor response to chemotherapy by 3′-deoxy-3′-[^{18}F]fluorothymidine

positron emission tomography: the effect of cisplatin on a fibrosarcoma tumor model in vivo. Cancer Res 2005;65:4202–4210.
59. Waldherr C, Mellinghoff IK, Tran C, et al. Monitoring antiproliferative responses to kinase inhibitor therapy in mice with 3′-deoxy-3′-^{18}F-fluorothymidine PET. J Nucl Med 2005;46:114–120.
60. Sugiyama M, Sakahara H, Sato K, et al. Evaluation of 3′-deoxy-3′-^{18}F-fluorothymidine for monitoring tumor response to radiotherapy and photodynamic therapy in mice. J Nucl Med 2004;45:1754–1758.
61. Krak NC, van der Hoeven JJ, Hoekstra OS, Twisk JW, van der Wall E, Lammertsma AA. Measuring [(18)F]FDG uptake in breast cancer during chemotherapy: comparison of analytical methods. Eur J Nucl Med Mol Imaging 2003;30:674–681.
62. Hoekstra CJ, Paglianiti I, Hoekstra OS, et al. Monitoring response to therapy in cancer using [^{18}F]-2-fluoro-2-deoxy-D-glucose and positron emission tomography: an overview of different analytical methods. Eur J Nucl Med 2000;27: 731–743.
63. Smyczek-Gargya B, Fersis N, Dittmann H, et al. PET with [^{18}F]fluorothymidine for imaging of primary breast cancer: a pilot study. Eur J Nucl Med Mol Imaging 2004;31:720–724.
64. van Westreenen HL, Cobben DC, Jager PL, et al. Comparison of ^{18}F-FLT PET and ^{18}F-FDG PET in esophageal cancer. J Nucl Med 2005;46:400–404.
65. Muzi M, Mankoff DA, Grierson JR, Wells JM, Vesselle H, Krohn KA. Kinetic modeling of 3′-deoxy-3′-fluorothymidine in somatic tumors: mathematical studies. J Nucl Med 2005;46:371–380.
66. Muzi M, Vesselle H, Grierson JR, et al. Kinetic analysis of 3′-deoxy-3′-fluorothymidine PET studies: validation studies in patients with lung cancer. J Nucl Med 2005;46:274–282.
67. Kenny LM, Aboagye EO, Price PM. Positron emission tomography imaging of cell proliferation in oncology. Clin Oncol (R Coll Radiol) 2004;16:176–185.
68. Vesselle H, Grierson J, Peterson LM, Muzi M, Mankoff DA, Krohn KA. ^{18}F-Fluorothymidine radiation dosimetry in human PET imaging studies. J Nucl Med 2003;44:1482–1488.
69. Hays MT, Watson EE, Thomas SR, Stabin M. MIRD dose estimate report no. 19: radiation absorbed dose estimates from (18)F-FDG. J Nucl Med 2002;43:210–214.
70. Buck AC, Schirrmeister HH, Guhlmann CA, et al. Ki-67 immunostaining in pancreatic cancer and chronic active pancreatitis: does in vivo FDG uptake correlate with proliferative activity? J Nucl Med 2001;42:721–725.
71. Francis DL, Freeman A, Visvikis D, et al. In vivo imaging of cellular proliferation in colorectal cancer using positron emission tomography. Gut 2003;52:1602–1606.
72. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311–322.
73. van Diest PJ, van der Wall E, Baak JP. Prognostic value of proliferation in invasive breast cancer: a review. J Clin Pathol 2004;57:675–681.
74. Bos R, van der Hoeven JJ, van der Wall E, et al. Biologic correlates of (18)fluorodeoxyglucose uptake in human breast cancer measured by positron emission tomography. J Clin Oncol 2002;20:379–387.
75. Buck AK, Schirrmeister H, Hetzel M, et al. 3-Deoxy-3-[(18)F]fluorothymidine-positron emission tomography for noninvasive assessment of proliferation in pulmonary nodules. Cancer Res 2002;62:3331–3334.

76. Hartwell LH, Kastan MB. Cell cycle control and cancer. Science 1994;266: 1821–1828.
77. Schwartz JL, Tamura Y, Jordan R, Grierson JR, Krohn KA. Effect of p53 activation on cell growth, thymidine kinase-1 activity, and 3′-deoxy-3′fluorothymidine uptake. Nucl Med Biol 2004;31:419–423.
78. Pakzad F, Ell PJ, Carrio I. Molecular imaging in animal models of disease—every little detail counts! Eur J Nucl Med Mol Imaging 2005;32:899, 900.

Index

A

D

E

H

N

T

CANCER DRUG DISCOVERY AND DEVELOPMENT

Series Editor: Beverly A. Teicher

Deoxynucleoside Analogs in Cancer Therapy

Edited by

Godefridus J. Peters, PhD

Professor of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands

Emerging as an important new volume in the renowned *Cancer Drug Discovery and Development*™ series, *Deoxynucleoside Analogs in Cancer Therapy* expertly summarizes the current status of development and application of deoxynucleoside analogs. Authoritative up-to-date reviews are presented by scientists well known in their specific areas and all contributions include valuable sound advice on structure and topics.

Organized into several sections, the book's first part covers general aspects of drug uptake and metabolism and explains how novel technology has enabled a rapid expansion of this field. The second part is concerned with a number of specific drugs, including cytarabine, gemcitabine, troxacitabine, clofarabine and ara-G. The final section covers pharmacokinetics, prodrugs, and specific applications such as radiosensitization, gene therapy, and the use of deoxynucleoside analogs as tracers.

Throughout *Deoxynucleoside Analogs in Cancer Therapy,* the focus is on novel aspects of deoxynucleoside analogs in the clinical context, as well as on unexpected targets of these compounds, such as their specific activity against cell cycle-dependent kinases or oncogenes. The wealth of information presented here can be used to design rational combinations aimed at inhibiting various cellular signaling pathways, or combining inhibition of various targets. *Deoxynucleoside Analogs in Cancer Therapy* has been designed specifically to facilitate such an interaction between various fields.

- **Identifies unexpected targets of deoxynucleoside analogs**
- **Explains the role of transporters and nucleotidases in resistance to nucleoside analogs**
- **Covers use of 3'-deoxy-3'-fluorothymidine as an active tracer in PET**
- **Authoritative up-to-date reviews**

Contents

ISBN 1-58829-327-0

90000

9 781588 293275

Cancer Drug Discovery and Development™
DEOXYNUCLEOSIDE ANALOGS IN CANCER THERAPY
ISBN: 1-58829-327-0
E-ISBN: 1-59745-148-7
humanapress.com