CANCER DRUG DISCOVERY AND DEVELOPMENT

Cancer Therapeutics

Experimental and Clinical Agents

Edited by

Beverly A. Teicher

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Cancer Therapeutics: Experimental and Clinical Agents, edited by Beverly A. Teicher, *1997*

Anticancer Drug Development Guide, edited by Beverly A. Teicher, *1997*

CANCER THERAPEUTICS

EXPERIMENTAL AND CLINICAL AGENTS

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For the beautiful ones
Joseph and Emily

Originally published by Humana Press Inc. in 1997
Softcover reprint of the hardcover 1st edition 1997

This publication is printed on acid-free paper. ♾
ANSI Z39.48-1984 (American Standards Institute) Permanence of Paper for Printed Library Materials.

Cover illustration: From Fig. 1 in Chapter 14, "Discovery of TNP-470 and Other Angiogenesis Inhibitors," by Donald E. Ingber.

Cover design by Patricia F. Cleary.

Library of Congress Cataloging-in-Publication Data

Cancer therapeutics: experimental and clinical agents / edited by
Beverly A. Teicher.
p. cm. -- (Cancer drug discovery and development)
Includes index.
ISBN 978-1-61737-046-5 ISBN 978-1-59259-717-8 (eBook)
DOI 10.1007/978-1-59259-717-8
1. Antineoplastic agents. I. Teicher, Beverly A., 1952–
II. Series.
[DNLM: 1. Antineoplastic Agents. QV 269 C2154 1997]
RC271.C5C3228 1997
616.99'4061--dc21
DNLM/DLC
for Library of Congress 96-48083
CIP

SERIES PREFACE

Cancer drug discovery has been and continues to be a process of ingenuity, serendipity, and dogged determination. In an effort to develop and discover better therapies against cancer, investigators all over the world have increased our knowledge of cell biology, biochemistry, and molecular biology. The goal has been to define therapeutically exploitable differences between normal and malignant cells. The result has been an increased understanding of cellular and whole-organism biology and an increased respect for the flexibility and resiliency of biologically systems. Thus, as some new therapeutic targets have been defined and new therapeutic strategies have been attempted, so have some new biological hurdles resulting from tumor evasion of the intended therapeutic attack been discovered.

Historically, anticancer drugs have originated from all available chemical sources. Synthetic molecules from the chemical industry, especially dyestuffs and warfare agents, and natural products from plants, microbes, and fungi have all been potential sources of pharmaceuticals, including anticancer agents. There is no shortage of molecules; the challenge has been and continues to be methods of identifying molecules that have the potential to be therapeutically important in human malignant disease. "Screening" remains the most important and most controversial method in cancer drug discovery. In vitro screens have generally focused on cytotoxicity and have identified several highly cytotoxic molecules. Other endpoints available in vitro are inhibition of proliferation, inhibition of [^{3}H]thymidine incorporation into DNA and various viability assays, based most frequently on dye exclusion or metabolism. The current National Cancer Institute in vitro screen attempts to take into account both cytotoxic potency and histological selectivity. In vitro screens may be viewed as limited to the discovery of only directly cytotoxic agents, thereby neglecting the fact that cancer is a disease occurring in a host organism.

The discovery of cancer drugs by in vivo screening has traditionally utilized syngeneic transplantable murine tumors. The earliest in vivo screens were the fast-growing murine leukemias (L1210 and/or P388) implanted intraperitoneally and growing as ascites. These tumors, with survival as an endpoint, provided a rapid, reproducible means for identifying potential anticancer drugs. It became evident more than 10 years ago that there were marked similarities in the drugs emerging from the murine leukemia screen. Panels of murine solid tumors and panels of human tumor xenografts were added to or have replaced the murine leukemias as anticancer drug screens. Each of these models has strengths and limitations. Most obviously, xenograft systems are not suitable for testing immunologically active agents or species-specific agents that involve host cell signaling cascades. The endpoints most frequently used with in vivo screens include tumor growth inhibition, tumor growth delay, increase in lifespan, and tumor cell survival.

In vivo systems also allow the opportunity to assess normal tissue damage by prospective agents. Murine dose-limiting toxicities are frequently useful, but studies in larger animals are often done to provide more definitive information on the potential clinically important toxicities of new anticancer agents. Spontaneous tumors in pets (dogs and cats) can provide useful populations in which to test new agents where efficacy and toxicity can be examined.

Having demonstrated activity and with a documented toxicity profile, a new agent enters clinical testing. Initial trials test the tolerance of patients to the drug and try to establish an appropriate dose for the drug in humans. The second phase of clinical testing seeks to demonstrate efficacy of the new drugs as a single agent. The third phase of clinical testing incorporates the new agent into current therapeutic regimens and seeks to demonstrate that the addition of the new agent to the combinations leads to better treatment outcomes than the conventional regimen. Final passage into medical use requires approval from the FDA in the United States and similar regulatory agencies in other countries.

The current volume traces the discovery, preclinical and clinical testing of anticancer agents currently available for routine use as well as the discovery, rationale for, and current status of potentially exciting new agents for cancer therapy. Current screening methods for cancer drug discovery and for determination of the activity of rationally designed agents are discussed with a focus on the strengths and limitations of the methods. The phases of clinical testing of new agents are discussed with a view toward presenting the strengths and limitations of that process. Finally, the requirements for approval of new anticancer drugs are presented.

The time from discovery to the time of approval for a new anticancer agent can be 10 years or more. The survival rate for compounds through this process is small.

We are entering a potentially very exciting period in anticancer agent discovery where the therapeutic focus may expand to include not only agents cytotoxic toward malignant cells, but agents that may be growth controlling, growth inhibitory, activating or deactivating toward stromal cells or malignant cells, or may alter signaling cascades from one cell type to another. At this important time in the development of cancer treatment, *Cancer Drug Discovery and Development* takes stock of what has been accomplished, where experimental therapeutics of cancer are going, and the continuing evolution of the means and methods of cancer drug discovery.

Beverly A. Teicher

PREFACE

The history of modern cancer therapy is contained within the 20th century beginning with the astute observations of physicians treating victims of mustard gas poisoning during World War I. It was the best rational medical and scientific thought that led to the application of nitrogen mustard to the treatment of lymphoma. It was also rational scientific thought toward means to improve on nitrogen mustard that motivated the chemical synthesis programs in Germany and the United States that ultimately produced cyclophosphamide and ifosfamide. The potential of the nitrosoureas first came to light from an early screening program in the 1960s searching for chemicals cytotoxic toward malignant cells and resulted in a large chemical synthesis program intended to improve the antitumor activity of that family of agents by reducing their toxicity. Again in the 1960s, actively searching for natural products with anticancer activity, the anthracyclines were discovered and developed in Italy and France. The antitumor alkylating agents and anthracyclines along with the antimetabolites such as 5-fluoracil formed the basis of medical oncology and are still some of the most widely used anticancer drugs.

The second generation of anticancer drugs included:

1. The fully inorganic molecule cis-diamminedichloroplatinum (II), a well-known inorganic complex championed for use as an anticancer agent by Dr. Barnet Rosenberg;
2. The camptothecins, discovered in the 1950s, whose clinical potential is just now being realized because of the initially unrecognized pH sensitivity of the parent molecule;
3. The epipodophyllotoxins that allowed elucidation of the topoisomerase enzymes; and
4. The taxanes, discovered in the 1960s, whose clinical development was delayed because of the difficulty in formulating these molecules for administration to patients.

Each of the second-generation lead molecules led to the exploration and development of congeners, the clinical potentials of which are still being explored.

In recent years, the scope of the search for anticancer drugs has markedly expanded so that the focus is not only on malignant cell proliferation. Though eradication of all malignant cells remains the goal of anticancer therapy, many new strategies seek to achieve growth control not only of malignant cells, but of endothelial cells and other stromal cells proliferating in response to chemical signals secreted by the malignant cells. Antiangiogenic agents and matrix metalloproteinase inhibitors focus on the inappropriate proliferation of endothelial cells and the inappropriate breakdown of the extracellular matrix that occurs in malignancy. Similarly, the premise for the use of many growth factor inhibitors and many oncogene-directed inhibitors is not only that proliferation of malignant cells will cease, but that the stimulatory signals from the malignant cells to the normal cells supporting growth of the tumor will cease. A greater understanding of the myriad components of the immune system is leading to much more effective use of interferons, cytokines, and immunoconjugates in cancer therapy and may enable the effective use of gene therapy and vaccines in cancer therapy.

This volume covers nearly 100 years of focused effort by scientists and physicians to develop pharmacologic means to cure the many diseases called cancer. This has been, and remains, a successful stepwise progression forward through which we have learned enormous amounts about the complex networks of cells from which we are composed and have steadily decreased the lethality of cancer. Our work is far from completed, but our commitment to the effort remains true to those who came before us.

I wish to thank each of the contributors to this volume for their scholarly efforts in the preparation of these chapters and for their continuing efforts in cancer therapeutics.

Beverly A. Teicher

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I CYTOTOXIC AGENTS: OLD AND NEW

1 Nitrogen Mustards

Gerald J. Goldenberg, MD, PhD
and Malcolm J. Moore, PhD

CONTENTS

1. HISTORY

The use of mustard gas in World War I dramatically demonstrated the biological potential of alkylating agents. In addition to the vesicant action of sulfur mustard to the skin, conjunctiva, and respiratory tract, the myelosuppressive and lymphocytolytic effects were first documented by Krumbhaar and Krumbhaar in 1919 (*1*).

The chemical and biological properties of nitrogen mustards, which are close structural analogs of sulfur mustard, were studied from that time until World War II, and the prototype of this group, nitrogen mustard, was known by the military code name HN2. The pronounced cytotoxic effect of these compounds on lymphoid tissue prompted Gilman, Goodman, Philips, and Dougherty to study the effect of nitrogen mustards against murine lymphosarcoma, and in 1942 patients with neoplastic disease were first treated (*1,2*). In 1946, studies on nitrogen mustards were declassified, and several reports appeared indicating clinical activity in Hodgkin's disease, lymphoma, and chronic lymphocytic and myelocytic leukemia, but not against a wide range of solid tumors (*3–9*). These studies are of historical interest in that they represented the first clear reports of chemical compounds with antitumor activity and ushered in the modern era of cancer chemotherapy.

An intensive search was begun for alkylating agents with more selective cytocidal activity, and these basic and clinical studies were spearheaded by Haddow, Bergel, Ross, Boyland, Timmis, and Galton at the Chester Beatty Research Institute in London, England. The contributions of this group included the synthesis of:

1. Chlorambucil (the phenylbutyric acid derivative of nitrogen mustard), which is relatively stable in aqueous solution, well absorbed from the gastrointestinal tract, and has the distinct advantage over HN2 of being active after oral administration;

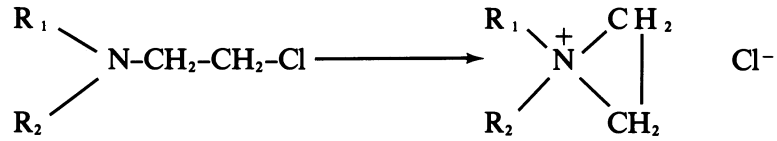
From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

2. Melphalan (the phenylalanine derivative of nitrogen mustard), which was synthesized with the objective of achieving selective concentration in tumors, such as multiple myeloma, that are actively engaged in protein synthesis; and
3. Myleran (Busulfan), which was particularly active in chronic myelocytic leukemia.

Although a large number of nitrogen mustard analogs and other alkylating agents have been synthesized with varying degrees of clinical effectiveness, the object of selective cytotoxicity for tumor cells has remained an elusive goal.

2. CHEMISTRY AND MECHANISM OF ACTION

Nitrogen mustards are alkylating agents, which are highly reactive chemically, that dissociate into positively charged carbonium ion intermediates. These electrophilic alkyl groups form covalent bonds with electron-rich nucleophiles, such as sulfhydryl, hydroxyl, carboxyl, acetyl, phosphoryl, amino, and imidazole groups present in normal and neoplastic cells. Thus, nitrogen mustards have the potential to enter into a large variety of chemical reactions with many important biological molecules, including DNA, RNA, and proteins. The mechanism for generation of the carbonium ion intermediate varies with the nature of the alkylating agent. Nitrogen mustards undergo an internal SN1 cyclization to form the highly reactive aziridinum ion (*10,11*):



There is considerable evidence linking alkylation of DNA to the cytotoxic, carcinogenic, and mutagenic effects of these agents (*12*). The superiority of bifunctional over monofunctional agents suggests that crosslinking of DNA is critical for cytotoxic activity (*10,13*). The N-7 position of guanine is the most common site of DNA alkylation, with fewer adducts forming at the N-1 and O-6 positions of guanine, the N-1, N-3, and N-7 positions of adenine, the N-3 position of cytosine, and the O-4 position of thymidine (*11*). DNA sequence selectivity for alkylation of the N-7 position of guanine by nitrogen mustard, uracil mustard, and quinacrine mustard has been reported (*14*). DNA extracted from drug-treated cells showed the same base selectivities observed following alkylation of DNA in vitro.

Formation of DNA-interstrand crosslinks has a direct inhibitory effect on DNA replication, repair, and transcription (*15*). Formation of DNA adducts may cause a variety of structural alterations, such as ring openings, base deletions, and strand breaks (*16,17*). Genomic and gene-specific DNA-interstrand crosslinks produced by HN2 were analyzed in the human tumor cell line Colo 320HSR (*18*). Crosslinks were detected in the amplified, overexpressed c-*myc* oncogene, but not in the weakly expressed N-*ras* gene. Furthermore, the crosslinks in the c-*myc* oncogene disappeared more rapidly than total genomic crosslinks. These findings suggested that HN2-induced DNA-interstrand crosslinks are produced and processed in the genome in a nonrandom fashion. Evidence has also been presented that alkylating agents may modulate expression of c-*myc* and c-*fos* with no apparent effect on the steady-state levels of N-*ras* or β-actin (*19*).

Removal of DNA-interstrand crosslinks was postulated as a prerequisite for the outflow of cells from G2 arrest after melphalan treatment (*20*). Treatment of the RPMI 8226 myeloma cell line with melphalan resulted in cell-cycle arrest, with preferential reduction in the outflow of cells from late S- and G2 phases. As cell arrest abated, all of the DNA-interstrand crosslinks, but only 50% of the DNA-protein crosslinks were removed. No DNA double-strand breaks as measured by neutral elution were formed during the observation period (*20*). In a study of Burkitt's lymphoma cell lines, no correlation was found between HN2 sensitivity and growth fraction, presence of Epstein-Barr virus, and location of (*8,14*) translocation breakpoints (*21*). No simple correlation was detected between HN2 sensitivity and the amount of DNA crosslinks. Evidence was also presented to suggest that HN2-sensitive Burkitt's lymphoma cells may be more susceptible to delay in S phase at a given level of DNA crosslinks and that prolongation of S phase appeared to correlate with apoptotic cell death (*21*). O'Connor et al. also studied the role of the cdc25C phosphatase in G2 arrest induced by HN2 (*22*). Evidence was presented that checkpoints guarding against mitotic entry in the presence of unreplicated or damaged DNA suppress formation of the cdc2-cdc25C autocatalytic feedback loop that normally brings about rapid activation of cdc2.

The cytotoxicity, DNA crosslinking ability, and DNA sequence selectivity of the aniline mustards melphalan, chlorambucil, and 4-(*bis*[2-chloroethyl] amino) benzoic acid were compared (*23*). The order of cytotoxicity against human colonic adenocarcinoma LS174T and leukemic K562 cell lines was chorambucil > melphalan > the benzoic acid derivative. Simple chemical reactivity or hydrolysis rate was not a good indicator of DNA reactivity or cytotoxicity, whereas DNA-interstrand crosslinking provided a good index of biological activity.

Since the alkylating agents are highly reactive chemically, it is not surprising that reports have appeared describing inhibition of protein synthesis. In a study of the synthesis of histone variants in the HEp-2 cancer cell line, chlorambucil was shown to inhibit histone synthesis with no significant decrease in total protein synthesis (*24*).

Estramustine chemically can be classified as a nitrogen mustard, since it is a conjugate of nor-nitrogen mustard and estradiol (*25*). However, the agent accumulates preferentially in cells containing estramustine binding protein and appears to exert its cytotoxic activity as an antimicrotubule agent, not as an alkylator. Accordingly, this compound will not be considered further in this chapter.

3. MECHANISM OF TRANSPORT

3.1. Mechanism of Transport of Nitrogen Mustard

A major complication of many of the early transport studies of alkylating agents was the failure to separate drug transport from alkylation reactions. The latter could result in drug binding to nucleophilic groups within the cell or on the cell membrane, with the formation of apparent cell/medium drug concentration gradients. Using the hydrolyzed derivative of nitrogen mustard HN2-OH, which is inactive as an alkylating agent, we showed that uptake was by an active, carrier-mediated mechanism (*26*). Furthermore, in studies of HN2 and HN2-OH transport, each acted as a mutual and reciprocal competitive inhibitor of the other providing strong evidence that in L5178Y lymphoblasts, both compounds shared the same single transport carrier system.

The chemical specificity of the transport carrier for HN2 was investigated. Strong evidence was obtained that transport of HN2 by L5178Y lymphoblasts was mediated by the transport carrier for choline (*27*). Choline influx in these cells was an active process proceeding "uphill" against a concentration gradient of at least 45-fold and was competitively inhibited by HN2, HN2-OH, ethanolamine, and hemicholinium-3, a specific inhibitor of choline transport. The similarity of K_m and K_i for each of choline, HN2, and HN2-OH suggested that each compound shared the same transport carrier. The relative affinity for the transport carrier was choline > HN2-OH > HN2 consistent with the notion that choline was the preferred transport substrate.

3.2. Mechanism of Transport of Melphalan

Our laboratory provided the first evidence that melphalan uptake was mediated by an active, carrier-mediated process, which was independent of that used by other alkylating agents (*28*). Melphalan transport by LPC-1 Plasmacytoma cells and L5178Y lymphoblasts was mediated by two distinct amino acid transport systems (*29,30*). At high melphalan concentrations, uptake was predominantly by the leucine-preferring System L, which was sodium-independent and inhibited by BCH (DL-β-2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid). At low drug concentrations, uptake was by a sodium-sensitive, BCH-independent ASC-like system that also transported the amino acids alanine, serine, and cystine. Vistica et al. (*31,32*) also reported that melphalan and the amino acid leucine share a common transport system in murine L1210 leukemia cells.

The effect of altering the ionic environment was investigated to determine the direct effects of hydrogen and calcium ions on these amino acid transport systems, as well as the indirect effects of modulators of intracellular calcium (*33*). Melphalan transport followed a bell-shaped distribution curve over a pH range from 3.0 to 9.0 with a pH optimum of 4.3 and 4.6 for transport by systems ASC and L, respectively. Agents that cause a decrease in cytosolic calcium, such as the calcium channel blockers verapamil, diltiazem, and nitrendipine, the calcium chelator EGTA and reduction of pH were found to augment melphalan uptake, whereas conditions that elevate intracellular calcium, such as the calcium ionophore A23187, the calcium channel agonist (−) Bay K 8644, the calcium pump inhibitor trifluoperazine, and elevation of extracellular calcium were all found to decrease melphalan uptake. These findings suggested that modification of ionic environment directly or indirectly by agents known to alter intracellular calcium can modulate melphalan uptake.

3.3. Mechanism of Transport of Chlorambucil

The uptake of [^{14}C]chlorambucil by L5178Y lymphoblasts was studied using thin-layer chromatography to identify the various radioactive components that enter or leave cells (*34*). Theoretical calculations predicted that entry of chlorambucil into cells by simple diffusion would be rapid and essentially complete in 45 s or less. Uptake of intact chlorambucil was rapid, reaching a cell/medium ratio of approx 1.5 in < 15 s at both 37 and 4 °C, consistent with a simple diffusion mechanism. In cells treated with [^{14}C]chlorambucil for 60 min, the intracellular level of intact drug decreased with time, and this decay was attributed to hydrolysis and alkylation. The level of intact drug in the medium decreased at a similar rate resulting in a nearly constant cell/medium distribution ratio. Intact chlorambucil in the cells was found to be entirely

ethanol- and trichloroacetic acid-soluble. Efflux of intact chlorambucil was very rapid and temperature-insensitive. These findings suggested that chlorambucil efflux, as well as influx, is by a simple diffusion mechanism.

A derivative of chlorambucil was found in ethanol solutions of the drug (*34*). This derivative, which may be the ethyl ester of chlorambucil, is highly concentrated in cells and may complicate pharmacological studies of the drug. Even minor contamination of chlorambucil solutions by this derivative may interfere with studies, since the derivative is rapidly concentrated by cells. This problem may be avoided by preparing drug solutions immediately before use or by using solvents other than ethanol.

4. MECHANISMS OF RESISTANCE

4.1. The Role of Transport in Resistance to Nitrogen Mustards

4.1.1. The Role of Transport in Resistance to Nitrogen Mustard

Uptake of ^{14}C-labeled HN2 was found to be reduced in drug-resistant L5178Y lymphoblasts (*26*). In HN2-resistant cells, drug influx was characterized by an increase in K_m and a decrease in V_{max}, suggesting that reduced influx was owing to a decrease in binding affinity, and a reduction in the number of transport sites and/or slower carrier mobility. HN2-resistant cells that were 18.5-fold resistant to HN2 were only two- to threefold resistant to other alkylating agents, including chlorambucil, melphalan, BCNU, mitomycin C, and trenimon (*35*). However, a major portion of resistance to HN2 was not shared by these alkylating agents. Furthermore, transport of HN2, HN2-OH, and choline was not competitively inhibited by any of these compounds, suggesting that transport of these other alkylating agents was by independent mechanisms.

The sensitivity of L5178Y lymphoblasts to the cytocidal action of HN2 was found to be a function of the proliferative state of the cells (*36*). Exponentially, dividing cells were approx 2.6-fold more sensitive to HN2 than resting cells. Drug transport was also more efficient in log-phase cells than in resting cells. The more efficient transport was owing to a higher binding affinity between carrier and drug and to a greater number of transport carriers and/or more rapid carrier mobility. The greater sensitivity of log-phase cells to HN2 to a large extent could be accounted for by these differences in drug transport.

4.1.2. The Role of Transport in Resistance to Melphalan

Redwood and Colvin reported decreased melphalan uptake in drug-resistant L1210 cells, which was attributed primarily to a decrease in binding affinity between drug and transport carrier (*37*). Dantzig et al. (*38*) isolated a leucine transport mutant of CHO cells that was resistant to melphalan.

Other investigators have not found differences in drug uptake in resistant cells. No difference in melphalan transport was found in human melanoma cell lines that were sensitive (MM253) or resistant (MM253-12M) to melphalan (*39*). In two clones of melphalan-resistant CHO cells, uptake of [^{14}C]melphalan was not significantly different from that observed in the sensitive parental line, suggesting that the clones were not membrane-altered mutants (*40*). No difference in drug uptake was observed in a melphalan-resistant human myeloma cell line that was partially cross-resistant to other alkylators and X-irradiation (*41*). Reduced DNA interstrand crosslinking was

noted in the resistant cells, but it was not determined if this was owing to decreased formation or increased rate of removal of the lesion. Conversely, reduced transport of melphalan by system L was reported in melphalan-resistant human breast cancer cells, in which there was no difference in GSH content, GST activity, or expression of pi class GST mRNA (*42*).

4.1.3. The Role of Transport in Resistance to Chlorambucil

Since chlorambucil uptake is by a passive diffusion process, one would not expect differences in drug transport between drug-sensitive and drug-resistant cells. In a study comparing untreated and treated resistant patients with chronic lymphocytic leukemia (CLL), no differences in chlorambucil transport or metabolism were detected (*43*).

4.2. The Role of Glutathione and Glutathione-S-Transferases in Resistance to Nitrogen Mustards

4.2.1. The Role of Glutathione-S-Transferases in Drug Resistance

Glutathione-*S*-transferases (GSTs) represent a multigene family of enzymes that catalyze the conjugation of glutathione to a broad range of electrophilic xenobiotics and carcinogens (*44–48*). By conjugating glutathione (GSH) to various xenobiotics, GSTs appear to play a role whereby cells develop resistance to antineoplastic agents (*47*). The existence of multiple GST isozymes with overlapping structural and functional features has made it difficult to distinguish among closely related forms and has made the design of a systematic nomenclature a challenge (*49*). Five distinct GST classes or gene families have been identified (*47*). The cytosolic GSTs are abundant and are classified on the basis of isoelectric point as basic (α class), neutral (μ class), and acidic (π class); the other two GST classes are the microsomal isoform and the more recently described θ class (*50*). The π, θ, and microsomal classes each consist of a single gene product, whereas the α and μ classes each contain more than one gene product. Each functional GST enzyme is a homodimer or heterodimer made up of subunits encoded by gene loci from within a given class.

Two major experimental approaches have been followed to establish the role of GSTs in resistance to antineoplastic agents. The first has involved correlative studies in which elevated levels of expression and activity of GSTs have been associated with increased levels of drug resistance (*45,47*). This approach has also included modulation of enzyme activity by inhibitors of GST, such as ethacrynic acid in order to circumvent or reverse drug resistance (*51*). The second approach has used in transfection studies to provide direct functional evidence that GSTs cause drug resistance.

Both approaches, including the more definitive transfection studies, have provided conflicting evidence. Transfection of human GSTπ was reported to increase resistance of Chinese hamster ovary cells (*52*), but not that of NIH-3T3 transfectants (*53*) to the alkylator cisplatinum. More consistently, GSTπ has been associated with resistance to Adriamycin (*53–55*) and GSTα with resistance to alkylating agents (*46*) not only in correlative, but also in transfection studies. Puchalski and Fahl (*56*) reported that GSTα conferred resistance to chlorambucil and melphalan, whereas GSTμ and π conferred resistance to cisplatinum and Adriamycin, respectively, in stably transfected mouse C3H cells and transiently transfected COS cells. Conversely, several attempts to transfect MCF-7 human breast cancer cells with either class π, α, or μ

GST have resulted in overexpression of the gene product and, occasionally, resistance to ethacrynic acid, benzpyrene, or benzpyrene epoxide, but not to antineoplastic agents (*57–60*).

We undertook a study of the chemical specificity of induction of GST isoforms using two related compounds—the aromatic alkylating agent aniline mustard (AM) and the close structural analog hydrolyzed benzoquinone mustard (HBM) in drug-sensitive and drug-resistant L5178Y murine lymphoblasts (*61*). The chloroethyl groups in AM make it an active alkylating agent, whereas in HBM, these groups are hydrolyzed and cytotoxicity is owing to the chemically reactive quinone moiety rather than to alkylation. In AM-resistant cells, there was evidence on Northern and Western blot of overexpression of all three cytosolic GST classes π, α, and μ, but with marked predominance of the α class. In HBM-resistant cells, once again there was increased expression of all three classes, but unlike AM-resistant cells, the π isoform was most dominant. These studies provided evidence that induction of GST isoforms in drug-resistant cells may have both a nonspecific as well as a selective component. The difference in isozyme profile between AM- and HBM-resistant cell lines emphasizes how structural differences, in particular, the nature of the electrophilic signal, may influence the pattern of induction of GST isozymes.

Several lines of evidence support a role for GST in resistance to nitrogen mustards:

1. Nitrogen mustards can form GSH conjugates in reactions catalyzed by GSTs (Hall, 1994);
2. Human tumor specimens and cell lines often overexpress GST isozymes;
3. Transient expression of GST genes in cultured cells has resulted in increased resistance to alkylating agents;
4. Transfection of some yeast and mammalian cells with GST isozymes will increase resistance to alkylating agents;
5. GST inhibitors cause sensitization of cultured cells to alkylating agents;
6. Cell-cycle-dependent sensitivity to melphalan correlates with the cell-cycle-dependent expression of GSTs; and
7. Elevation of GST-α occurs within several days of exposure to chlorambucil as part of the normal cellular response (*46,48,62–65*).

The α, μ, and π classes appear to be the major GST isozymes involved in anticancer drug resistance.

Evidence also exists suggesting that GSTs may not play a major role in drug resistance to nitrogen mustards. As mentioned above, transfection of cells with GST isozymes has not consistently increased the level of resistance to alkylating agents. In some cell lines selected for resistance to alkylating agents, no increase in GST protein or function has been noted (*66*). In studies of melphalan–glutathione conjugate formation in tumor cells, GST-catalyzed conjugation made only a minimal contribution to the overall rate of conjugation (*67*). One explanation for the contradictory results of the transfection experiments is that appropriate concentrations of cofactors, such as GSH, might be required to enable GST isozymes to confer resistance (*68*). Nevertheless, despite these contrary findings, there is reasonable evidence in the literature that high levels of GST isozymes are associated with resistance to the nitrogen mustards. Whether improvements in therapeutic index can be realized by inhibition of GST isozymes has not been clearly established.

4.2.2. The Role of GSH in Drug Resistance

GSH-mediated detoxification pathways play a central role in the inactivation and elimination of xenobiotics, including the nitrogen mustards. γ-Glutamylcysteine synthetase (GCS) catalyzes the rate-limiting step in *de novo* synthesis of GSH. The importance of the GSH pathway in the protection of normal tissue is reflected in its widespread distribution. Increased GSH levels can be related to increased activity of GCS. Studies of the relationship of drug sensitivity to GSH conjugation have examined intracellular GSH, the activity of GCS, and the GSTs. Modulation of GSH conjugation has been accomplished by depletion of cellular GSH using either an inhibitor of GSH synthesis, such as buthionine sulfoximine (BSO), or agents that inhibit the function of one or more GST isoforms. Thiols appeared to play a significant role in resistance, since nonprotein thiols were elevated in resistant cells and resistance dropped on reduction of the thiol content by treatment with BSO (*41*).

In three human prostate carcinoma cell sublines selected for resistance to melphalan, GSH levels were increased 1.7- to 2.8-fold, whereas resistance was increased 2- to 27-fold over the parental lines. However, depletion of GSH to baseline levels in the resistant lines using BSO restored melphalan responsiveness to that of control levels (*66*). A twofold increase in intracellular GSH concentration and a fivefold increase in GST activity were detected in a rat mamary carcinoma cell line selected in vitro for melphalan resistance (*69*). Melphalan responsiveness of these cells, which were crossresistant to chlorambucil and HN2, was also restored by intracellular depletion of GSH and inhibition of GST. Conversely, resistance was not circumvented in a melphalan-resistant human rhabdomyosarcoma cell line using BSO, despite the presence of elevated GSH levels (*70*). This cell line was crossresistant to other alkylating agents and to topoisomerase I active agents (*70*). Elevated levels of DNA repair enzymes and alterations of topoisomerase I and II were also seen and probably contributed to the resistance phenotype.

Elevated levels of GSH, GCS activity, and of GCS mRNA were observed in melphalan-resistant DU 145 human prostatic carcinoma cells (*71,72*). Nuclear run-on studies established that increased transcription of the GCS mRNA was responsible for the elevation of the steady-state level of the message.

4.2.3. Modulation of Glutathione Conjugation

Depletion of cellular GSH can be achieved using BSO. Several in vitro and in vivo studies have demonstrated that BSO treatment potentiates cytotoxicity induced by alkylating agents in cell lines with acquired drug resistance, as well as in previously untreated cells (*73,74*). In mice treated with BSO and melphalan, toxicity of normal tissue was also increased, but to a lesser extent than that of tumor cells, so that therapeutic index was enhanced (*75*). Although BSO potentiated the effects of melphalan, it did not influence the efficacy of other alkylating agents, such as cyclophosphamide or mitomycin C (*76*). Phase I clinical studies of BSO and melphalan are under way (*77,78*). These have demonstrated that BSO can deplete cellular GSH in mononuclear cells to a variable degree and that increased myelotoxicity is associated with GSH depletion.

A variety of GST inhibitors exist, and some have been tested for their ability to alter the cytotoxicity of alkylating agents. In a chlorambucil-resistant cell line with elevated GSH and GST, cytotoxicity was increased with the use of BSO, and/or the

GST inhibitors ethacrynic acid (EA) or indomethacin (*79*). Sensitivity could be restored to that of the parental line by a combination of both inhibitors. A chlorambucil-resistant cell line was established in which resistance was solely attributed to a 40-fold increase in GST α (*80*). Inhibition of GST α by indomethacin, but not by other cyclooxygenase inhibitors restored sensitivity to chlorambucil. EA potentiates the cytotoxicity of melphalan against MCF-7 breast cells when given in combination with BSO (*81*). In a melanoma cell line, a twofold increase in chemosensitivity to melphalan was observed when EA was given at concentrations sufficient to inhibit GST π, μ, and α by at least 50% (*82*). This was accompanied by a corresponding increase in DNA crosslinking. Chlorambucil-resistant Yoshida rat ascites hepatoma AH44 and AH66 cells contained higher levels of GSH and greater GST activity than the parental line. Both EA and BSO decreased chlorambucil resistance of AH44 and AH66 cells without influencing the sensitivity of the parental line; sensitivity was not influenced by inhibitors of P-glycoprotein (*83*).

A patient with chlorambucil-resistant CLL and high GST activity has been reported in whom EA partially circumvented chlorambucil resistance (*84*). Phase I clinical testing of alkylating agents with the GST inhibitor EA is under way (*85,86*). O'Dwyer et al. (*85*) used a schedule of EA orally every 6 h for three doses followed by thiotepa iv 1 h after the second dose of EA. Metabolic abnormalities secondary to the diuretic effects of EA were seen at doses of 75 mg/m^2, and the recommended dose for further studies was 50 mg/m^2. This dose reduced GST activity in mononuclear cells to 37% of the baseline level with recovery by 6 h after EA. Plasma concentrations of EA were not measured, and inhibition of specific isozymes was not determined.

4.2.4. Detoxification by Nonglutathione Compounds

Other sulfhydryl-containing compounds, such as metallothionein, can also form covalent linkages with nitrogen mustards and may assist in intracellular inactivation (*87*). However, the degree of resistance to HN2 in Chinese hamster ovary cells that had no detectable metallothionein was greater than in a cadmium-resistant variant that overexpressed metallothionein (*88*).

4.3. The Role of DNA Repair Enzymes in Resistance to Nitrogen Mustards

Alteration of DNA may occur as a result of DNA repair enzymes attacking the DNA adducts, which may either restore DNA integrity or create apurinic sites or DNA strand breaks (*89*). Apurinic or apyrimidinic endonucleases recognize and excise the defective sites together with neighboring or flanking nucleotides (*16,17,89*). DNA polymerase fills in the missing gap using the opposite DNA strand as template, and DNA ligase completes the repair process (*90*). Apurinic sites may persist if the repair process is defective, and result in mutations or chromosomal breakage or rearrangement. Agents that inhibit DNA repair, such as aphidicolin and cytosine arabinoside, have been reported to enhance the cytotoxic activity of melphalan and other alkylating agents (*91*).

Increased expression of DNA repair enzymes has been reported following treatment with alkylating agents. Overexpression of the ribosomal phosphoprotein PO gene, a putative DNA repair gene, was observed following treatment with HN2, melphalan, and 4-hydroperoxycyclophosphamide (*92*). Furthermore, constitutive expres-

sion of the PO gene in Mer− tumor cell lines, which lack *o*6-methylguanine methyltransferase activity, was 30- to 50-fold greater than that in Mer+ cells. These findings suggested that increased expression of the PO gene is linked to DNA repair, and may also compensate for the decreased *o*6-methylguanine DNA methyltransferase activity in Mer− cells.

DNA repair has been identified as an important component of HN2 sensitivity in other cell lines (*93*). In cells treated with melphalan, DNA damage is repaired quite rapidly (*91,94*). Drugs that inhibited DNA repair enhanced cell killing two- to threefold, although some of the agents, such as cytosine arabinoside, are also cytotoxic. Treatment of human ovarian carcinoma cells A2780 with aphidicolin or hydroxyurea, compounds that inhibit DNA repair, and melphalan alone or in combination with cisplatin or thiotepa, enhanced the cytotoxic action of the alkylators (*94*).

In patients with CLL, increased activity of the DNA repair enzyme 3-methyladenine-DNA glycosylase and increased expression of the nucleotide excision repair gene ERCC-1 occur with the development of resistance to the nitrogen mustards, suggesting that DNA repair is an important factor in drug resistance (*95*). In a study of drug resistance in B-lymphocytes of chronic lymphocytic leukemia, the MTT assay was used to measure resistance in vitro to nitrogen mustards and other agents (*96*). The resistant population was 5.6- and 4.1-fold more resistant to chlorambucil and melphalan, respectively. Neither GSH nor GST levels correlated with resistance; however, resistance to nitrogen mustards was associated with enhanced DNA repair. Crossresistance was observed against other alkylating agents, such as mitomycin C and cisplatin drugs that act by forming DNA crosslinks. This group also studied lymphocytes from patients with CLL resistant to chlorambucil and in newly diagnosed untreated patients. The level of DNA interstrand crosslinks in lymphocytes from treated resistant CLL patients was lower than that of untreated patients (*97*), and a kinetic analysis showed that the rate of removal of the crosslinks was greater in treated resistant than in untreated patients (*98*). A followup study by the same group of 11 untreated and 12 treated resistant CLL patients showed no difference in the level of expression of the DNA repair enzymes ERCC-1 or ERCC-2 or in expression of DNA polymerase β, GST-α, or topoisomerase I (*99*). However, two of the resistant patients displayed increased expression of all the DNA repair enzymes examined.

Flow cytometric analysis of cells stained with the monoclonal anti-DNA antibody MoAB F7-26, which binds to single-stranded regions of alkylated DNA, has been used to study DNA damage and repair in drug-resistant cells (*100*). Development of melphalan resistance in A2780 cells was associated with decreased immunoreactivity of DNA with MoAB F7-26; fluorescence was significantly lower in resistant cells than in sensitive cells. The enhancement of melphalan cytotoxicity by BSO and hyperthermia in resistant cells was accompanied by a proportional increase of MoAB binding to DNA.

The role of the enzyme poly (adenosine diphosphate-ribose) polymerase (PADPRP) in DNA repair was studied in human HeLa cells treated with PADPRP antisense transcripts under control of a dexamethasone-inducible promoter (*101*). DNA damage and repair were studied in these cells after dexamethasone induction reduced the level of PADPRP by 90%. Clonal survival studies showed that PADPRP-depleted cells demonstrated increased sensitivity to HN2, but not UV irradiation, suggesting that antisense-induced cells were deficient in DNA repair of HN2-induced lesions.

4.4. Other Factors Contributing to Resistance

The cytotoxicity of melphalan against animal and human tumor cell lines in vitro can be potentiated by acidic and hypoxic conditions (*102*). Although both an acidic microenvironment and cellular hypoxia can increase melphalan uptake, changes in transport were not considered sufficient to account for the observed differences. The combination in vivo of melphalan with agents that reduce intracellular pH has been demonstrated to increase cell kill compared to that observed with melphalan alone (*103,104*). In human A549 adenocarcinoma and mouse KHT sarcoma cells, hypoxia and acidic pH independently increased melphalan cytotoxicity, and the effect was greatest when both conditions were present (*105*). In studies using chlorambucil in vitro at low pH (≤ 7.0), a large potentiation of cytotoxicity was observed, whereas hypoxia alone resulted in minimal change (*106*). A much higher ratio of intracellular/extracellular drug concentration was observed at low pH, which likely accounted for the increase in cytotoxicity.

Mutations of p53 have been associated with resistance not only to nitrogen mustards, but also to a wide range of cytotoxic agents and to radiation therapy (*107*). Mutations of p53 and apoptosis will be considered further below (*see* Section 6.).

4.5. Multifactorial Resistance

We suggested that resistance of L5178Y murine lymphoblasts to nitrogen mustard was multifactorial, since HN2-resistant cells showed elevated sulfhydryl levels, decreased uptake of [^{14}C]HN2, and decreased binding of [^{14}C]HN2 to DNA, RNA, and protein (*26,35,108*). Additional evidence was that cells selected for resistance to HN2 were only partially crossresistant to other alkylating agents, suggesting that some of the factors contributing to resistance were active against alkylating agents generally, whereas other traits, such as transport mechanisms, were agent-specific (*35*).

Our group also provided indirect evidence that chlorambucil resistance in patients with CLL was multifactorial (*109,110*). Although an inverse correlation was observed between chlorambucil-induced DNA crosslinks and GST activity and/or GSH levels, there was no correlation between prior clinical exposure to alkylating agents and DNA crosslink formation (*109*). No correlation was observed between total or protein-bound sulfhydryl (PSH), GSH levels or GST activity, and clinical response to chlorambucil, although a slight positive correlation was noted between the ratio of PSH/GSH and clinical response (*110*). These findings also suggested that resistance to chlorambucil in CLL may be multifactorial.

Inhibition of DNA repair enzymes can in some instances restore sensitivity to nitrogen mustards, but more effective results have been observed with alteration of other resistance factors, such as intracellular GSH (*111*). This finding also supports the notion that multiple factors influence sensitivity to nitrogen mustards (*111*).

5. CARCINOGENIC ACTIVITY OF THE NITROGEN MUSTARDS

The acute effects of the nitrogen mustards are relatively predictable and manageable with appropriate drug dosing and supportive measures. Over the longer term, these drugs can cause permanent gonadal dysfunction and have been demonstrated to

be carcinogenic. The nitrogen mustards are DNA-damaging agents that are both mutagenic and carcinogenic in a variety of testing systems. They lead to positive results in the McCann and Ames test, they induce sister chromatid exchanges and other chromosomal changes, they lead to transformation in cell-culture studies, and induce tumors in laboratory animals. Since cohorts of patients treated with nitrogen mustards have now lived for many years following treatment, evidence is accumulating on the risk of development of second tumors. The absolute risk is difficult to determine accurately when based on retrospective analyses where different drug dosages and schedules were used. Often the nitrogen mustards are just one component of a combination chemotherapy regimen that may include other carcinogenic agents. In addition, these drugs may have been used in diseases, such as Hodgkin's disease or myeloma, where an increased incidence of second malignancies is seen regardless of treatment. Nevertheless, there is now sufficient information available in the literature to support the fact that these drugs are associated with a higher risk for the development of second neoplasms.

In patients with ovarian cancer treaated with melphalan, there are a number of different series that have implicated this drug as a cause of secondary acute leukemia (*112,113*). In a review of over 5000 patients with ovarian cancer treated with alkylating agents, predominantly melphalan, chlorambucil, or cyclophosphamide, there was a 36-fold increase in the risk of acute leukemia overall and a 170-fold increase in risk for patients surviving 2 yr following therapy (*114*). Other reviews have confirmed this increased risk of leukemia in women with ovarian cancer, and have suggested that melphalan and chlorambucil are more likely to lead to secondary leukemia than cyclophosphamide (*115,116*). The latency period to develop leukemia after the nitrogen mustards is 4–5 yr, but the risk remains higher than normal for up to 10 yr. There also appears to be a relationship between the dose of melphalan or chlorambucil and the risk of secondary leukemia. In breast cancer where melphalan was used in the earlier adjuvant regimens, there is approximately a fivefold increase in risk of developing leukemia, whereas no such increase in risk has been reported for women receiving adjuvant treatment with cyclophosphamide (*117*). In myeloma, where melphalan is one of the primary drugs used, the risk of developing acute leukemia within 4 yr of therapy is approx 200 times that expected (*118*). An increased risk of secondary leukemia has also been noted in patients treated for Hodgkin's disease. In a large case-control study, the cumulative dose of HN2 appeared to be the most important leukemogenic risk factor (*119*).

The carcinogenic risk of the nitrogen mustards has not been studied as thoroughly as the leukemogenic risk. The latency period for the development of solid tumors may be longer than that for leukemia. In Hodgkin's disease where the largest cohort of patients has been followed for the longest time, there is a higher incidence of solid tumors than would be expected. This has included non-Hodgkin's lymphoma, colon, lung, breast, and thyroid cancer as well as soft tissue sarcomas (*120–122*). Some of this increased risk may be related to the use of radiation therapy, but chemotherapy, and specifically nitrogen mustards, appear to be the most significant factor. Although the increase in relative risk is two- to threefold, which is less than that for acute leukemia, increases in the more common solid tumors account for most of the absolute increase in cancer cases in these patients.

6. CLINICAL CONSIDERATIONS

Alkylating agents remain an integral component of cancer treatment, although the role of the nonoxazaphosphorine nitrogen mustards has diminished somewhat over time. HN2 has been largely replaced by other less reactive agents with the exception of its use in standard regimens to treat Hodgkin's disease. Melphalan is active against breast and ovarian cancer, but is no longer a first-line drug for either of those diseases. Melphalan continues to be an important drug for the treatment of multiple myeloma; whether any treatment has been demonstrated to be superior to melphalan and prednisone in this disease is still a controversial subject despite a large number of randomized trials. Melphalan is also given intra-arterially using limb perfusion for the treatment of melanoma of the extremities (*123*). Melphalan given intrathecally may be useful in the treatment of meningeal carcinomatosis (*124*). Chlorambucil is commonly used in the treatment of low-grade lymphomas and CLL, and has also been used in the treatment of some immunological diseases, such as glomerulonephritis and rheumatoid arthritis.

In CLL, p53 gene mutations are associated with aggressive disease and a high likelihood of resistance to chlorambucil (*125*). Others have found that mutant p53 is associated with resistance not only to nitrogen mustards, but also to a wide range of cytotoxic agents and to radiation therapy (*107*). Chlorambucil may produce its antitumor effect in CLL by inducing apoptosis-associated membrane changes that result in rapid clearance of the apoptotic cells by the immune system (*126*). In cells transfected with bcl-2, HN2 produced only 50% of the cell kill observed in control cells, whereas similar levels of interstrand crosslink formation, DNA repair, and cell-cycle progression were seen in both cell lines (*127*). Transfection with bcl-2 may represent a different mechanism of resistance to nitrogen mustards, which may be mediated by a decrease in drug-induced apoptosis.

Apoptosis has been observed in lymphocytes isolated from patients with CLL and incubated in vitro for 72 h, following a clinical course of chlorambucil (*126*). The level of apoptosis was greater in cells isolated from patients after than before chlorambucil therapy. Because a large increase in drug-induced apoptosis in vitro was followed by a significant decrease in the patient's lymphocyte count, it was suggested that chlorambucil may produce its antitumor activity in CLL by inducing apoptosis-associated membrane changes.

The chemosensitivity of lymphocytes from patients with B-cell CLL was evalutated in vitro using the MTT assay, and patients were arbitrarily designated as "sensitive" or "resistant" to chlorambucil if the IC_{50} was $<$ or $>$ 61 μmol/L, respectively (*125*). The response of "sensitive" patients to a clinical course of chlorambucil therapy was significantly better than that of "resistant" patients, and there was a significant degree of crossresistance between chlorambucil and fludarabine. Mutations of p53 were associated with aggressive disease, a poor prognosis, and resistance to the two drugs. Mutations of p53 have also been associated with resistance of Burkitt's lymphoma and lymphoblastoid cells to HN2, cisplatin, and other agents (*107*).

The effect of melphalan on terminal divisions and self-renewal capacity of acute myeloblastic (AML) progenitors was compared to that of a cyclophosphamide analog (*128*). Melphalan was equally effective in inhibiting terminal divisions and self-renewal

as assayed by primary (PE1) and secondary (PE2) colony formation, respectively, whereas the cyclophosphamide analog was less effective in the self-renewal assay. The authors suggested that melphalan might offer a greater potential than cyclophosphamide in the therapy of AML, since chemotherapy should be preferentially directed against the self-renewal of leukemic progenitors.

Owing to the steep dose-response curve of all the nitrogen mustard alkylating agents, they remain an important component of most high-dose autologous or allogeneic bone marrow transplantation regimens. The most common drugs used are melphalan, as well as the oxazaphosphorines cyclophosphamide and ifosfamide (*129,130*). Early myeloablative therapy of multiple myeloma supported by autologous bone marrow transplantation was evaluated in 72 patients with myeloma within 1 yr of their initial therapy (*131*). In patients responding to previous therapy, myeloablative therapy increased the rate of complete remission from 5 to 45%, but with no effect on survival. However, in patients with *de novo* drug resistance, ablative therapy induced remissions in 70% of individuals and prolonged the median survival from 37 to 83%.

REFERENCES

1. Calabresi P, Parks RE Jr. Alkylating agents, antimetabolites, hormones and other antiproliferative agents. In: Goodman LS, Gilman A, eds. *The Pharmacological Bases of Therapeutics.* New York: MacMillan. 1975:1254-1268.
2. Karnofsky DA. Summary of results obtained with nitrogen mustard in the treatment of neoplastic disease. *Ann NY Acad Sci* 1958; 68:899-914.
3. Rhoads CP. Nitrogen mustards in the treatment of neoplastic disease. *J Am Med Assoc* 1946; 131:656-658.
4. Goodman LS, Wintrobe MM, Dameshek W, Goodman MJ, Gilman A, McLennan MT. Nitrogen mustard therapy; use of methyl-bis(beta-chloroethyl)amine hydrochloride and tris (betachloroethyl) amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. *J Am Med Assoc* 1946; 132:126-132.
5. Goodman LS, Wintrobe MM, McLennan MR, Dameshek W, Goodman MJ, Gilman A. The use of methyl-bis(beta-chloroethyl)amine hydrochloride and tris(betachloroethyl)amine hydrochloride ("nitrogen-mustards") in the therapy of Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. In: *Approaches to Tumor Chemotherapy.* Washington, DC. 1947:338-346.
6. Jacobson LO Spurr CL, Guzman-Barron ES, Smith T, Lushbaugh C, Dick GF. Nitrogen mustard therapy: Studies on effect of methyl-bis(beta-chloroethyl)amine hydrochloride on neoplastic disease and allied disorders of hemopoietic system. *J Am Med Assoc* 1946; 132: 263-271.
7. Spurr CL, Jacobson LO, Smith TR, Guzman-Barron ES. The clinical application of methyl-bis(beta-chloroethyl) amine hydrochloride to the treatment of lymphomas and allied dyscrasias. In: *Approaches to Tumor Chemotherapy.* Washington, DC: Am Assoc Adv Sci. 1947: 306-318.
8. Karnofsky DA, Craver LF, Rhoads CP, Abels JC. Evaluation of methyl-bis(beta-chloroethyl)-amine hydrochloride and tris(beta-chloroethyl)amine hydrochloride (nitrogen mustards) in the treatment of lymphomas, leukemia and allied diseases. In: *Approaches to Tumor Chemotherapy.* Washington, DC: Am Assoc Adv Sci. 1947:319-337.
9. Wilkinson JF, Fletcher F. Effect of beta-chloroethylamine hydrochlorides in leukemia, Hodgkin's disease and polycythemia vera; report on 18 cases. *Lancet* 1947; 2:540-545.
10. Ross WCJ. *Biological Alkylating Agents. Fundamental Chemistry and the Design of Compounds for Selective Toxicity.* London: Butterworths. 1962.
11. Ludlum DB. Alkylating agents and the nitrosoureas. In: Becker F.F, ed. *Cancer, a Comprehensive Treatise,* vol 5. Plenum. 1977:285-387.

12. Ludlum DB. Molecular biology of alkylating agents: An overview, In: Sartorelli AC and Johns DG, eds. *Antineoplastic and Immunosuppressive Agents,* part II. New York: Springer. 1975: 6–17.
13. Goldenberg GJ, Alexander PA. The effects of nitrogen mustard and dimethyl myleran on murine leukemia cell lines of different radiosensitivity in vitro. *Cancer Res* 1965; 25:1401–1409.
14. Hartley JA, Bingham JP, Souhami RL. DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards is preserved in intact cells. *Nucleic Acids Res* 1992; 20:3175–3178.
15. Lawley PD, Brookes P. Molecular mechanism of the cytotoxic action of difunctional alkylating agents and of resistance to this action. *Nature* 1965; 206:480–483.
16. Hanawalt PC, Cooper PK, Ganesan AK, Smith CA. DNA repair in bacteria and mammalian cells. *Ann Rev Biochem* 1979; 48:783–836.
17. Bohr VA, Philips DH, Hanawalt PC. Heterogeneous DNA damage and repair in the mammaliangenome. *Cancer Res* 1987; 47:6426–6436.
18. Futscher BW, Pieper RO, Dalton WS, Erickson LC. Gene-specific DNA interstrand crosslinks produced by nitrogen mustard in the human tumor cell line Colo 32OHSR. *Cell Growth Differ* 1992; 3:217–223.
19. Futscher BW, Erickson LC. Changes in c-myc and c-fos expression in a human tumor cell line following exposure to bifunctional alkylating agents. *Cancer Res* 1990; 50:62–66.
20. Fernberg JO, Levensohn R, Skog S. Cell cycle arrest and DNA damage after melphalan treatment of the human myloma cell line RPMI 8226, *Eur J Haematol* 1991; 47:161–167.
21. O'Connor PM, Wassermann K, Sarang M, Magrath I, Bohr VA, Kohn KW. Relationship between DNA cross-links, cell cycle, and apoptosis in Burkitt's lymphoma cell lines differing in sensitivity to nitrogen mustard. *Cancer Res* 1991; 51:6550–6557.
22. O'Connor PM, Ferris DK, Hoffman I, Jackman J, Draetta G, Kohn KW. Role of the cdc25c phosphatase in G2 arrest induced by nitrogen mustard. *P.N.A.S.* 1994; 91:9480–9484.
23. Sunters A, Springer CJ, Bagshawe KD, Souhami RL, Hartley JA. The cytotoxicity, DNA crosslinking ability and DNA sequence selectivity of the aniline mustards melphalan, chlorambucil and 4-[bis(2-chloroethyl)amino] benzoic acid. *Biochem Pharmacol* 1992; 44:59–64.
24. Sourlingas TG, Aleporou-Marinou V, Pataryas TA, Sekeri-Pataryas KE. Influence of chlorambucil, a bifunctional alkylating agent, on the histone variant biosynthesis of HEp-2 cell, *Biochemica Biophys Acta* 1991; 1092:298–303.
25. Speicher LA, Barone LR, Chapman AE, Hudes GR, Laing N, Smith CD, Tew DK. P glycoprotein binding and modulation of the multidrug resistant phenotype of Estramustine. *JNCI* 1994; 86:688–694.
26. Goldenberg GJ, Vanstone CL, Israels LG, Ilse D, Bihler I. Evidence for a transport carrier of nitrogen mustard in nitrogen mustard-sensitive and -resistant L5178Y lymphoblasts. *Cancer Res* 1970; 30:2285–2291.
27. Goldenberg GJ, Vanstone CL, Bihler I. Transport of nitrogen mustard on the transport carrier for choline in L5178Y lymphoblasts. *Science* 1971; 172:1148–1149.
28. Goldenberg GJ, Lee M, Lam HYP, Begleiter A. Evidence for carrier-mediated transport of melphalan by L5178Y lymphoblasts in vitro. *Cancer Res* 1977; 37:755–760.
29. Goldenberg GJ, Lam HYP, Begleiter A. Active carrier-mediated transport of melphalan by two separate amino acid transport systems in LPC-1 plasmacytoma cells in vitro. *J Biol Chem* 1979; 25:1057–1064.
30. Begleiter A, Lam HYP, Grover J, Froese E, Goldenberg GJ. Evidence for active transport of melphalan by amino acid carriers in L5178Y lymphoblasts in vitro. *Cancer Res* 1979; 39: 353–359.
31. Vistica DT, Toal JN, Rabinowitz M. Amino acids affecting melphalan transport and cytotoxicity in cultured L1210 cells. *Proc Am Assoc Cancer Res* 1977; 18:26.
32. Vistica DT, Rabon A, Rabinowitz M. Interference with melphalan transport and therapy in the L1210 murine leukemia system, its significance and prevention. *Proc Am Assoc Cancer Res* 1978; 19:44.
33. Miller L, Deffie AM, Bose R, Goldenberg GJ. Modulation of melphalan uptake in murine L5178Y lymphoblasts in vitro by changes in ionic environment. *Biochem Pharm* 1992; 43: 1154–1158.
34. Begleiter A, Goldenberg GJ. Uptake and decomposition of chlorambucil by L5178Y lymphoblasts in vitro. *Biochem Pharm* 1983; 32:535–539.

35. Goldenberg GJ. The role of drug transport in resistance to nitrogen mustard and other alkylating agents in L5178Y lymphoblasts. *Cancer Res* 1975; 35:1687–1692.
36. Goldenberg GJ, Lyons RM, Lepp JA, Vanstone CL. Sensitivity to nitrogen mustard as a function of transport activity and proliferative rate in L5178Y lymphoblasts. *Cancer Res* 1971; 31:1618–1619.
37. Redwood WR, Colvin M. Trnasport of melphalan by sensitive and resistant L1210 cells. *Cancer Res* 1980; 40:1144–1149.
38. Dantzig AH, Slayman CW, Adelberg EA. An amino acid transport mutant of Chinese hamster ovary cells resistant to the chemotherapeutic agent melphalan. *Fed Proc* 1981; 40:1894.
39. Parsons PG, Carter FB, Morrison L, SR Regius Mary. Mechanism of melphalan resistance developed in vitro in human melanoma cells. *Cancer Res* 1981; 41:1525–1534.
40. Elliott EM, Ling V. Selection and characterization of Chinese hamster ovary cell mutants resistant to melphalan (L-phenylalanine mustard). *Cancer Res* 1981; 41:393–400.
41. Bellamy WT, Dalton WS, Gleason MC, Grogan TM, Trent JM. Development and characterization of a melphalan-resistant human multiple myeloma cell line. *Cancer Res* 1991; 51: 995–1002.
42. Moscow JA, Swanson CA, Cowan KH. Decreased melphalan accumulation in a human breast cancer cell line selected for resistance to melphalan. *BR J Cancer* 1993; 68:732–737.
43. Bank BB, Kanganis D, Liebes LF, Silber R. Chlorambucil pharmaco-kinetics and DNA binding in chronic lymphocytic leukemia. *Cancer Res* 1989; 49:554–559.
44. Mannervik B, Danielson UH. Glutathione S-transferases-structure and catalytic activity. *CRC Crit Rev Biochem* 1988; 23:283–337.
45. Pickett CB, Lu AY. Glutathione S-transferases:gene structure, regulation and biological function. *Ann Rev Biochem* 1989; 743–764.
46. Waxman DJ. Glutathione S-transferases: role in alkylating agent resistance and possible target for modulation chemotherapy—a review. *Cancer Res* 1990; 50:6449–6454.
47. Waxman DJ, Sundseth SS, Srivastava PK, Lapenson DP. Gene-specific oligonucleotide probes for α, μ, π and microsomal rat glutathione S-transferases: analysis of liver transferase expression and its modulation by hepatic enzyme inducers and platinum and anticancer drugs. *Cancer Res* 1992; 52:5797–5802.
48. Rushmore TH, Pickett CB. Glutathione S-transferases, structure, regulation, and therapeutic implications. *J Biol Chem* 1993; 268:11,475–11,478.
49. Mannervik B, Awasthi YC, Board PG, et al. Nomenclature for human glutathione transferases. *Biochem J* 1992; 282:305–306.
50. Meyer DJ, Coles B, Pemble SE, Gilmore KS, Fraser GM, Ketterer B. θ a new class of glutathione transferases purified from rat and man. *Biochem J* 1991; 274:409–414.
51. Tew KD, Bomber AM, Hoffman SJ. Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines. *Cancer Res* 1988; 48:3622–3625.
52. Miyazaki M, Kohno K, Saburi Y, Matsuo K, Ono M, Kuwano M, Tsuchida S, Sata K, Sakai M, Muramatsu M. Drug resistance to cis-diammine-dichloroplatinum (II) in Chinese hamster ovary cell lines transfected with glutathione S-transferase π gene. *Biochem Biophys Res Commun* 1990; 166:1358–1364.
53. Nakagawa K, Sajo N, Tsuchida S, Sakai M, Tsunokawa Y, Yokota J, Muramatsu M, Sato K, Terada M, Tew KD. Glutathione S-transferase π as a determinant of drug resistance in transfectant cell lines. *J Biol Chem* 1990; 265:4296–4301.
54. Batist G, Tulpule A, Sinha BK, Katki AG, Myers CE, Cowan KH. Over-expression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. *J Biol Chem* 1986; 261:15,544–15,549.
55. Deffie AM, Alam T, Seneviratne C, Beenken SW, Batra JK, Shea TC, Henner WD, Goldenberg GJ. Multifactorial resistance in Adriamycin: relationship of DNA repair, glutathione transferase activity, drug efflux and P-glycoprotein in cloned cell lines of Adriamycin-sensitive- and resistant P388 leukemia. *Cancer Res* 1988; 48:3595–3602.
56. Puchalski RB, Fahl WE. Expression of recombinant glutathion S-transferase π, Ya or Ybl confers resistance to alkylating ageints. *Proc Natl Acad Sci* 1990; 87:2443–2447.
57. Moscow JA, Townsend AJ, Cowan KH. Elevation of π class glutathione S-transferase activity in human breast cancer cells by transfection of the GST π gene and its effect on sensitivity to toxins. *Mol Pharmacol* 1989; 36:22–28.

58. Fairchild CR, Moscow JA, O'Brien EE, Cowan KH. Multidrug resistance in cells transfected with human genes encoding a variant P-glycoprotein and glutathione S-transferase-π. *Mol Pharmacol* 1990; 37:801–809.
59. Leyland-Jones BR, Townsend AJ, Tu CPD, Cowan KH, Goldsmith ME. Antineoplastic drug sensitivity to cytotoxic agents. *Cancer Res* 51; 1991:587–594.
60. Townsend AJ, Tu DP, Cowan KH. Expression of human μ or α class glutathione S-transferases in stably transfected human MCF-7 breast cancer cells: effect on cellular sensitivity to cytotoxic agents. *Mol Pharmacol* 1992; 41:230–236.
61. Stelmack GL, Goldenberg GJ. Increased expression of cytosolic glutathione S-transferases in drug-resistant L5178Y murine lymphoblasts: chemical selectivity and molecular mechanisms. *Cancer Res* 1993; 53:3530–3535.
62. Jungnelius U, Hao XY, Skog S, Castro VM, Mannervik B, Ringborg U. Cell cycle dependent sensitivity of human melanoma cells to melphalan is correlated with the activity and cellular concentration of glutathione transferases. *Carcinogenesis* 1994; 15(1):99–103.
63. Morrow CS, Cowan KH. Glutathione S-transferases and drug resistance. *Cancer Cells* 1990; 2:15–22.
64. Wolf CR, Wareing CJ, Black SM, et al. Glutathione S-transferases in resistance to chemotherapeutic drugs. In: Hayes JD, Pickett CB, Mantle TJ, eds. *Glutathione S-transferases and Drug Resistance.* London: Taylor & Francis. 1990:295–307.
65. Clapper ML, Kuzmich S, Seestaller LM, Tew KD. Time course of glutathione S-transferase elevation in Walker mammary carcinoma cells following chlorambucil exposure. *Biochem Pharmacol* 1993; 45(3):683–690.
66. Ripple M, Mulcahy RT, Wilding G. Characteristics of the glutathione/glutathione-S-transferase detoxification system in melphalan-resistant human prostate cancer cells. *J Urol* 1993; 150(1):209–214.
67. Guenthner TM, Whalen R, Jevtovic-Todorovic V. Direct measurement of melphalan conjugation with glutathione: studies with human melanoma cells and mammalian liver. *J Pharmacol Exp Ther* 1992; 260(3):1331–1336.
68. Tew KD. Glutathione-associated enzymes in anticancer drug resistance. *Cancer Res* 1994; 54:4313–4320.
69. Alaoui-Jamali MA, Panasci L, Centurioni GM, Schecter R, Lehnert S, Batist G. Nitrogen mustard-DNA interaction in melphalan-resistant mammary carcinoma cells with elevated intracellular glutathione and glutathione-S-transferase activity. *Cancer Chemother Pharmacol* 1992; 330(5):341–347.
70. Friedman HS, Dolan ME, Kaufmann SH, Colvin OM, Griffith OW, Moschel RC, Schold SC, Bigner DD, Ali-Osman F. Elevated DNA polymerase alpha, DNA polymerase beta, and DNA topoisomerase II in a melphalan-resistant rhabdomyosarcoma xenograft that is cross-resistant to nitrosoureas and topotecan. *Cancer Res* 1994; 54(13):3487–3493.
71. Bailey HH, Gipp JJ, Ripple M, Wilding G, Mulcahy RT. Increase in gamma-glutamylcysteine synthetase activity and steady-state messenger RNA levels in melphalan-resistant DU-145 human prostate carcinoma cells expressing elevated glutathione levels. *Cancer Res* 1992; 52: 5115–5118.
72. Mulcahy RT, Untawale S, Gipp JJ. Transcriptional up-regulation of gamma-cysteine synthetase gene expression in melphalan-resistant human prostate carcinoma cells, *Mol Pharmacol* 1994; 46:909–914.
73. Harris AL, Hochhauser D. Mechanisms of multidrug resistance in cancer treatment. *Acta Oncologica* 1992; 31:205–213.
74. Canada A, Herman L, Kidd K, Robertson C, Trump D. Glutathione depletion increases the cytotoxicity of melphalan to PC-3, an androgen-insensitive prostate cancer cell line. *Cancer Chemother Pharmacol* 1993; 32(1):73–77.
75. Kramer RA, Greene K, Ahmad S, et al. Chemosensitization of melphalan by the thiol modulating agent BSO. *Cancer Res* 1987; 47:1593–1597.
76. Siemann DW, Beyers KL. In vivo therapeutic potential of combination thiol depletion and alkylating chemotherapy. *Br J Cancer* 1993; 68(6):1071–1079.
77. O'Dwyer PJ, Hamilton TC, Young RC, et al. Depletion of GSH in normal and malignant human cells in vivo by BSO: clinical and biochemical results. *JNCI* 1992; 84:264–267.
78. Bailey HH, Mulcahy RT, Tutsch KD, et al. GSH levels, GCS activity and BSO pharmaco-

kinetics in patients undergoing phase I treatment with BSO and melphalan. *Proc Am Assoc Cancer Res* 1993; 34:1697.
79. Yang WZ, Begleiter A, Johnston JB, et al. Role of glutathione and glutathione S-transferase in chlorambucil resistance. *Mol Pharmacol* 1992; 41:625–630.
80. Hall A, Robson CN, Hickson ID, et al. Possible role of inhibition of glutathione S-transferase in the partial reversal of chlorambucil resistance by indomethacin in a Chinese hamster ovary cell line. *Cancer Res* 1989; 49:6265–6268.
81. Chen G, Waxman DJ. Role of cellular glutathione and glutathione S-transferase in the expression of alkylating agent cytotoxicity in human breast cancer cells. *Biochem Pharmacol* 1994; 47:1079–1087.
82. Hansson J, Berhane K, Castro VM, Jungnelius U, Mannervik B, Ringborg U. Sensitization of human melanoma cells to the cytotoxic effect of melphalan by the glutathione transferase inhibitor ethacrynic acid. *Cancer Res* 1991; 51(1):94–98.
83. Miyamoto K, Wakabayashi D, Minamino T, Nomura M. Glutathione-S-transferase P-form dependent chlorambucil resistance in Yoshida rat ascites hepatoma cell lines. *Cancer Lett* 1994; 78(1-3):77–83.
84. Petrini M, Conte A, Caracciolo F, Sabbatini A, Grassi B, Ronca G. Reversing of chlorambucil resistance by ethacrynic acid in a B-CLL patient. *Br J Hematol* 1993; 85(2):409–410.
85. O'Dwyer PJ, LaCreta F, Nash S, et al. Phase I study of thiotepa in combination with the GST inhibitor EA. *Cancer Res* 1991; 51:6059–6065.
86. Hantel A, Nelson J, Delknap S, et al. Phase I study of melphalan and the GST inhibitor EA. Minutes of the NCI Phase I working group. 1991:359–379.
87. Yu X, Wu Z, Fenselau C. Covalent sequestration of melphalan by metallothionein and selective alkylation of cysteines. *Biochemistry* 1995; 34(10):3377–3385.
88. Wassermann K, Pirsel M, Bohr VA. Overexpression of metallothionein in Chinese hamster ovary cells and its effect on nitrogen mustard-induced cytotoxicity: role of gene-specific damage and repair. *Cancer Res* 1992; 52(24):6853–6859.
89. Cathcart R, Goldthwait DA. Enzymatic excision of 3-methyladenine and 7-methylguanine by a rat liver nuclear fraction. *Biochemistry* 1981; 20:273–280.
90. Soderhall S, Lindahl T. DNA ligases of eukaryotes. *FEBS Lett* 1976; 67:1–8.
91. Frankfurt OS. Inhibition of DNA repair and the enhancement of cytotoxicity of alklylating agents. *Int J Cancer* 1991; 48:916–923.
92. Grabowski DI, Pieper RO, Futscher BW, Deutsch WA, Erickson LC, Kelley MR. Expression of ribosomal phosphoprotein PO is induced by antitumor agents and increased in Mer-human tumor cell lines. *Carcinogenesis* 1992; 13:259–263.
93. Li Z, Brendel M. Sensitivity to nitrogen mustard in Saccharomyces cerevisiae is independently determined by regulated choline permease and DNA repair. *Mutat Res* 1994; 315(2):139–145.
94. Frankfurt OS, Seckinger D, Sugarbaker EV. Inhibition of DNA repair in cells treated with a combination of alkylating agents. *Anticancer Res* 1993; 13:947–952.
95. Geleziunas R, McQuillan A, Malapetsa A, Hutchinson M, Kopriva D, Wainberg MA, Hiscott J, Bramson J, Panasci L. Increased DNA synthesis and repair-enzyme expression in lymphocytes from patients with chronic lymphocytic leukemia resistant to nitrogen mustards. *J Nat Cancer Inst* 1991; 83(8):557–564.
96. Bramson J, McQuillan A, Aubin R, Alaoui-Jamali M, Batist G, Christodoulopoulos G, Panasci LC. Nitrogen mustard drug resistant B-cell chronic lymphocytic leukemia as an in vivo model for cross-linking agent resistance. *Mutat Res* 1995; 336(3):269–278.
97. Panasci L, Henderson D, Torres-Garcia SJ, Skalski V, Caplan S, Hutchinson M. Transport metabolism and DNA interaction of melphalan in lymphocytes from patients with chronic lymphocytic leukemia. *Cancer Res* 1988; 48:1972–1976.
98. Torres-Garcia SJ, Cousineau L, Caplan S, Panasci L. Correlation of resistance to nitrogen mustards in chronic lymphocytic leukemia with enhanced removal of melphalan-induced DNA crosslinks. *Biochem Pharmacol* 1989; 38:3122–3123.
99. Bramson J, McQuillan A, Panasci LC. DNA repair enzyme expression in chronic lymphocytic leukemia vis-a-vis nitrogen mustard drug resistance. *Cancer Lett* 1995; 90(2):139–148.
100. Frankfurt OS, Seckinger D, Sugarbaker EV. Flow cytometric analysis of DNA damage and repair in the cells resistant to alkylating agents. *Cancer Res* 1990; 50:4453–4457.

101. Stevnsner T, Ding R, Smulson M, Bohr VA. Inhibition of gene-specific repair of alkylation damage in cells depleted of poly(ADP-ribose) polymerase. *Nucleic Acids Res* 1994; 22: 4620–4624.
102. Skarsgard LD, Skwarchuk MW, Vinczan A, Kristl J, Chaplin DJ. The cytotoxicity of melphalan and its relationship to pH, hypoxia and drug uptake. *Anticancer Res* 1995; 15(1): 219–223.
103. Wood PJ, Sansom JM, Newell K, Tannock IF, Stratford IJ. Reduction of tumour intracellular pH and enhancement of melphalan cytotoxicity by the ionophore Nigericin. *Int J Cancer* 1995; 60(2):264–268.
104. Bump EA, Coleman CN, Cerce BA, McGinnis DJ. Sensitization of Chinese hamster ovary cells to melphalan by etanidazole under intermittent hypoxia. *Int J Radiat Oncol Biol Phys* 1992: 22(4):731–735.
105. Siemann DW, Chapman M, Beikirch A. Effects of oxygenation and pH on tumor cell response to alkylating chemotherapy. *Int J Radiat Oncol,Biol, Phy* 1991; 20(2):287–289.
106. Skarsgard LD, Chaplin DJ, Wilson DJ, Skwarchuk MW, Vinczan A, Kristl J. The effect of hypoxia and low pH on the cytotoxicity of chlorambucil. *Int J Radiat Oncol Biol Phys* 1992; 22(4):737–741.
107. Fan S, el-Deiry WS, Bae I, Freeman J, Jondle D, Bhatia K, Fornace AJ Jr, Magrath I, Kohn KW, O'Connor PM. p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res* 1994; 54(22):5824–5830.
108. Goldenberg GJ. Properties of L5178Y lymphoblasts highly resistant to nitrogen mustard. *Ann NY Acad Sci* 1969; 163:936–953.
109. Johnston JB, Israels LG, Goldenberg GJ, Anhalt CD, Verburg L, Mowat MRA, Begleiter A. Glutathione S-transferase activity, sulfhydryl group and glutathione levels, and DNA cross-linking activity with chlorambucil in chronic lymphocytic leukemia. *JNCI* 1990; 82:776–779.
110. Begleiter A, Goldenberg GJ, Anhalt CD, Lee K, Mowat MRA, Israels LG, Johnston JB. Mechanisms of resistance to chlorambucil in chronic lymphocytic leukemia. *Leukemia Res* 1991; 15:1019–1027.
111. Alaoui-Jamali M, Loubaba BB, Robyn S, Tapiero H, Batist G. Effect of DNA-repair-enzyme modulators on cytotoxicity of L-phenylalanine mustard and cis-diamminedichloroplatinum (II) in mammary carcinoma cells resistant to alkylating drugs. *Cancer Chemother Pharmacol* 1994; 34(2):153–158.
112. Einhorn N. Acute leukemia after chemotherapy (melphalan). *Cancer* 1978; 41:444–447.
113. Sotrel G, Jafari K, Lash AF, et al. Acute leukemi in advanced ovarian carcinoma after treatment with alkylating agents. *Obstet Gynecol* 1976; 47:67S–71S.
114. Reimer PR, Hoover R, Fraumeni JF Jr, et al. Acute leukemia after alkylating-agent therapy of ovarian cancer. *New Engl J Med* 1977; 297:177–181.
115. Greene MH, Harris EL, Gershenson DM, et al. Melphalan may be a more potent leukemogen than cyclophosphamide. *Ann Intern Med* 1986; 105:360–367.
116. Kaldor JM, Day NE, Pettersson F, Clarke EA, Pedersen D, Mehnert W, Bell J, Host H, Prior P, Karjalainen S, et al. Leukemia following chemotherapy for ovarian cancer. *New Engl J Med* 1990; 322(1):1–6.
117. Valagussa P, Tancini G, Bonadonna G. Second malignancies after CMF for resectable breast cancer. *J. Clin Oncol* 1987; 5:1138–1142.
118. Sieber SM, Adamson RH. Toxicity of antineoplastic agents in man, chromosomal aberrations, antifertility effects, congenital malformations, and carcinogenic potential. *Adv Cancer Res* 1975; 22:57–155.
119. van Leeuwen FE, Chorus AM, van den Belt-Dusebout AW, Hagenbeek A, Noyon R, van Kerkhoff EH, Pinedo HM, Somers R. Leukemia risk following Hodgkin's disease: relation to cumulative dose of alkylating agents, treatment with teniposide combinations, number of episodes of chemotharapy, and bone marrow damage. *J Clin Oncol* 1994; 12(5):1063–1073.
120. Sont JK, van Stiphout WA, Noordijk EM, Molenaar J, Zwetsloot-Schonk JH, Willemze R, Vandenbroucke JP. Increased risk of second cancers in managing Hodgkin's disease: the 20-year Leiden experience. *Ann Hematol* 1992; 65(5):213–218.
121. Swerdlow AJ, Douglas AJ, Vaughan Hudson G, et al. Risk of second primary cancers after Hodgkin's disease by type of treatment. *BMJ* 1992; 304:1137–1143.

122. Glanzmann C, Veraguth A, Lutolf UM. Incidence of second solid cancer in patients after treatment of Hodgkin's disease. *Strahlentherapie und Onkologie* 1994; 170(3):140–146.
123. Klaase JM, Kroon BB, van Geel AN, Eggermont AM, Franklin HR, van Dongen JA. A retrospective comparative study evaluating the results of a single-perfusion versus double-perfusion schedule with melphalan in patients with recurrent melanoma of the lower limb. *Cancer* 1993; 71(10):2990–2994.
124. Friedman HS, Archer GE, McLendon RE, Schuster JM, Colvin OM, Guaspari A, Blum R, Savina PA, Fuchs HE, Bigner DD. Intrathecal melphalan therapy of human neoplastic meningitis in athymic nude rats. *Cancer Res* 1994; 54(17):4710–4714.
125. Silber R, Degar B, Costin D, Newcomb EW, Mani M, Rosenberg CR, Morse L, Drygas JC, Canellakis ZN, Potmesil MM. Chemosensitivity of lymphocytes from patients with B-cell chronic lymphocytic leukemia to chlorambucil, fludarabine, and camptothecin analogs. *Blood* 1994; 84(10):3440–3446.
126. Begleiter A, Lee K, Israels LG, Mowat MR, Johnston JB. Chlorambucil induced apoptosis in chronic lymphocytic leukemia (CLL) and its relationship to clinical efficacy. *Leukemia* 8 suppl 1994; 1:S103–S106.
127. Walton MI, Whysong D, O'Connor PM, Hockenbery D, Korsmeyer SJ, Kohn KW. Constitutive expression of human Bcl-2 modulates nitrogen mustard and camptothecin induced apoptosis. *Cancer Res* 1993; 53(8):1853–1861.
128. Demur C, Chiron M, Saivin S, Attal M, Dastugue N, Boussquet C, Galinier JL, Colombies P, Laurent G. Effect of melphalan against self-renewal capacity of leukemic progenitors in acute myeloblastic leukemia. *Leukemia* 1992; 6:204–208.
129. Lazarus HM, Gray R, Ciobanu N, Winter J, Weiner RS. Phase I trial of high-dose melphalan, high-dose etoposide and autologous bone marrow re-infusion in solid tumors: an Eastern Cooperative Oncology Group (ECOG) study. *Bone Marrow Transplantation* 1994; 14(3):443–448.
130. Kergueris MF, Milpied N, Moreau P, Harousseau JL, Larousse C. Pharmacokinetics of high-dose melphalan in adults: influence of renal function. *Anticancer Res* 1994; 14(6A):2379–2382.
131. Alexanian R, Dimpoulos MA, Hester J, Delasalle K, Champlin R. Early myeloablative therapy for multiple myeloma. *Blood* 1994; 84:4278–4282.

2 Phosphoramide and Oxazaphosphorine Mustards

Joel E. Wright, PhD

CONTENTS

1. ANTICANCER ACTIVITY OF MUSTARDS

Phosphoramide mustards and their metabolic precursors, the oxazaphosphorine mustards, belong to the general class of alkylating agents. Some related compounds, the aryl- and alkyl-bis(2-chloroethyl)amines, were covered in the previous chapter. Two oxazaphosphorines, cyclophosphamide and ifosfamide (Fig. 1), are the only phosphorylated mustard compounds ordinarily prescribed for cancer treatment today (*1*). The developmental work that led to their prominent role in medicine and the outgrowth of prospective agents based on their structures and activities will be presented in this chapter.

1.1. Sulfur Mustard

Cyclophosphamide and ifosfamide grew from the original development of sulfur and nitrogen mustards. An early lead compound was 2-chloroethyl sulfide, commonly known as sulfur mustard, a vesicant used as a chemical weapon during World War I. Victims of this notorious aerosol agent suffered rapid reddening and blistering of the skin, blinding eye irritation, violent coughing, and projectile vomiting. Later, some surviving casualties developed bone marrow depression and lymphoid aplasia (*2*).

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

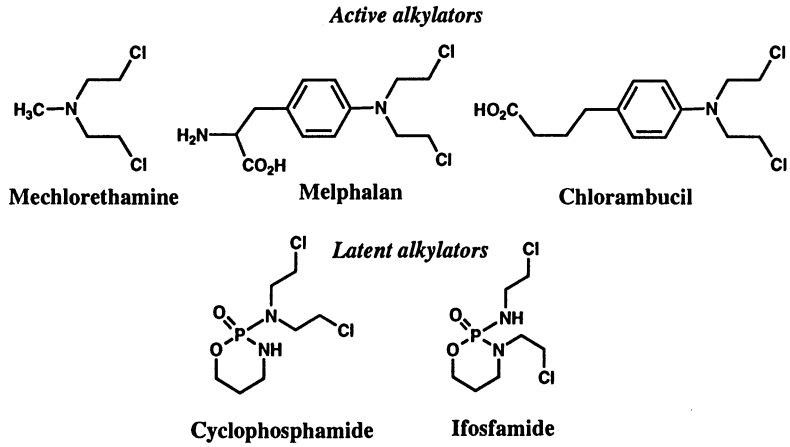


Fig. 1. Clinically useful mustards (*1*).

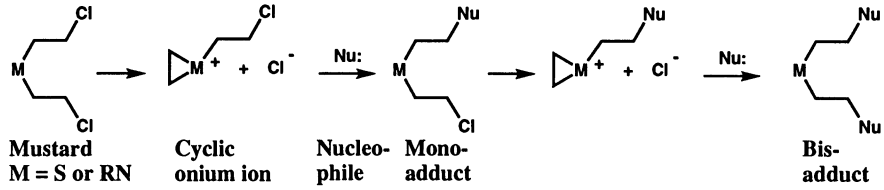


Fig. 2. Mechanism of action of bifunctional mustards (*5*).

The delayed bone marrow depression caused by sulfur mustard suggested its possible usefulness in low doses for treatment of cancer. Preclinical studies showed that the compound did possess anticancer activity and a clinical trial was conducted, but the therapeutic activity of sulfur mustard proved to be insubstantial and of short duration (*3*).

Seeking to overcome the deficiencies of sulfur mustard, investigators applied the lead drug concept and tested a number of sulfur mustard analogs in the hope that a structurally related compound would succeed where the parent drug had failed (*4*). However, none of the structural variants proved fruitful. Because of the lack of persistent benefit and perhaps the onus of its horrendous history as a chemical warfare agent, interest in sulfur mustard rapidly waned.

1.2. Nitrogen Mustards

As World War II began, research on vesicant mustards resumed. The focus was redirected toward a series of 2-haloethyl amine compounds that had been synthesized almost a half-century earlier. Biological activities superior to those of sulfur mustard were found with bis- and tris(2-chloroethyl)amine, and the mechanism of action was determined (*5*). The delayed systemic effects of sulfur and nitrogen mustards were tied to a chemical reaction that both classes of mustards have in common (Fig. 2), the anchimerically assisted formation of a cyclic onium ion capable of alkylating "a vital cellular constituent." Convincing evidence indicated that the vital constituent was nuclear chromatin. For example, alkylation by mechlorethamine produced inheritable

abnormalities in the chromosomes of fruit flies (*6*). DNA was not specifically mentioned, although its central biological role as the carrier of genetic information had been reported two years earlier (*7*). Wartime secrecy had delayed publication of the nitrogen mustard data by three years and more. Thus, the two lines of work were approximately concurrent. Prior to 1944, it was widely believed that chromosomal proteins transmitted genetic information (*8*). When the results of wartime investigations were finally revealed in 1946, the report did not specify which macromolecular species in the nucleus were targeted (*6*).

Despite severe hematopoietic side effects (*9*), preclinical results with tris(2-chloroethyl)amine and *N*-methylbis(2-chloroethyl)amine, now known as mechlorethanime led to a clinical trial (*10*). Substantial activity was demonstrated against Hodgkin's disease, thus establishing the first beachhead for cancer chemotherapy. Lymphosarcoma and leukemia patients were also treated, but the outcome was less favorable.

Several hundred patients were treated prior to the end of the war in an extensive investigation that uncovered the salient biological characteristics of bi- and trifunctional alkylators (*10–14*). An early observation was the susceptibility of renewal cell populations, including lymphatics, bone marrow, and gastrointestinal epithelia. This model, the cytokinetic mechanism of selectivity, describes the general behavior of mustards whose alkylating activity is rapid and direct. It is a prodominant mechanism for the three aryl and alkyl mustards commonly used in the clinic today, mechlorethamine, chlorambucil, and melphalan (Fig. 1) (*15*).

It was soberly concluded that sensitivity to nitrogen mustard treatment varied greatly from tumor to tumor. Furthermore, the clinicians observed a gradual loss of drug sensitivity during retreatment of recurrent disease. This phenomenon was identified as acquired resistance (*16*). Host toxicity did not vary as much as tumor response, allowing establishment of a maximum tolerated dose (MTD) (*17*). However, the frequent occurrence of tumors resistant to all reasonable doses was recognized as an inherent problem for drugs whose sole mechanism is cytokinetic selection. This understanding prompted renewed efforts to find exploitable differences between tumor and normal cells that did not depend on their proliferative state.

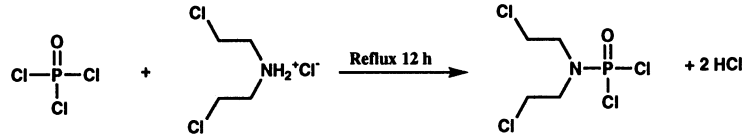
2. THE RATIONAL ROAD TO SERENDIP

The search for differences between tumor and normal tissues was motivated by the hope of improving selectivity through rational drug development. Historically, this approach has afforded a lower probability of success than other drug development methods, such as random screening of natural or synthetic products (*18*). The development of oxazaphosphorine anticancer agents was no exception, owing more to large-scale programmatic screening than to *ab initio* reasoning.

2.1. Tumor Phosphoamidase

Arnold Seligman and coworkers in Boston were interested in finding ways to improve selectivity based on differences in hydrolytic enzyme levels of tumor vs normal tissues (*19*). A method for measuring hydrolysis rates of phosphorus amides had been reported in the 1930s (*20*) using *N*-(4-chlorophenyl)diamidophosphoric acid as the substrate (*21*). Histochemical staining methods had shown apparent phosphoamidase (EC 3.9.1.1) overproduction in 24 tumors of the gastrointestinal tract and in a variety of breast, lung, cervical, testicular, and other carcinomas (*22*).

Friedman's Precursor [24]



Phosphoryl chloride | *Nor*-nitrogen mustard | N,N-*Bis*-(2-chloroethyl) phosphoramide dichloride

Latent Phosphorylated Mustards [24, 63]

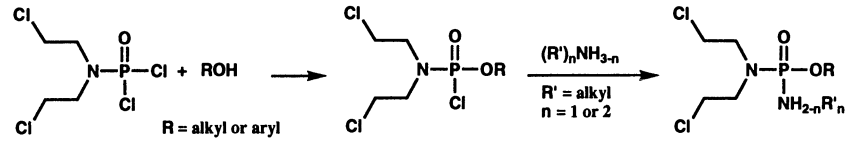
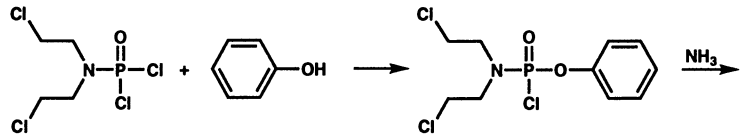
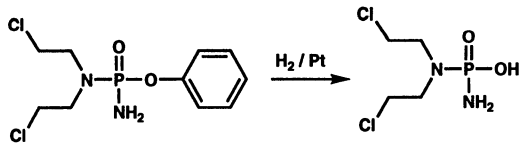


Fig. 3. Synthesis of phosphorylated mustards.



N,N-*Bis*-(2-chloroethyl) phosphoramide dichloride



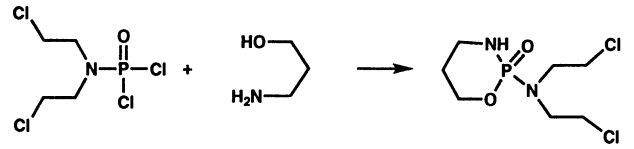
Phosphoramide mustard

Fig. 4. Synthesis of phosphoramide mustard (*24*).

2.2. Phosphorylated Mustards

In order to exploit differential phosphoamidase levels, Seligman and his colleagues envisioned the synthesis of water-soluble toxagenic substrates whose intracellular hydrolysis would release a cytotoxic product (*19*). Postwar interest in mustards was still high, and this undoubtedly influenced their choice of bis(2-chloroethyl)amine and related compounds as candidate precursors of the hypothetical prodrug (*23*). Since the alkylating activity of a nitrogen mustard depends on the basicity of its β-nitrogen, covalent attachment of an electron-withdrawing phosphoryl group to the nitrogen provided a logical deactivation step (Fig. 3).

Several *N*-phosphorylated secondary nitrogen mustards were synthesized. One of these was phosphoramide mustard, later identified as an important metabolite of cyclophosphamide (Fig. 4) (*24*). Unfortunately, the small academic research group in



N,N-*Bis*-(2-chloroethyl) phosphoramide dichloride

3-Amino-propanol

Cyclophosphamide

Fig. 5. Synthesis of cyclophosphamide (*27*).

Boston was not able to find the selective, latent agent they were seeking. A very effective phosphorylated mustard compound did soon surface, however, as a result of efforts on a larger scale in Germany.

3. CYCLOPHOSPHAMIDE

Under the leadership of Norbert Brock in Brackwede, Westphalia, the pharmacology department of ASTA-Werke AG had embarked on an anticancer drug discovery program based on biophysical, biochemical, pharmacological, and clinical investigations. The information garnered by these efforts was used to develop a rational approach to the synthesis and testing of new anticancer agents (*25*). In parallel with the ideas of Seligman and coworkers, Hermann Druckrey in Freiberg had proposed that a labile, toxic anticancer agent should be administered in a stable "transport form" to be activated within tumor cells (*26*). Brock's goal was to bring Druckrey's philosophical concept to fruition.

3.1. Discovery

Orrie Friedman, a chemist on Seligman's team, had phosphorylated bis(2-chloroethyl)amine with phosphoryl chloride, yielding *N,N*-bis(chloroethyl)phosphoramide dichloride, a versatile precursor from which straight- and branched-chain alkyl as well as aromatic mono- and diamidophosphate esters could be prepared (Fig. 3) (*24*). ASTA chemists Herbert Arnold and Friedrich Bourseaux recognized that such compounds fulfilled the stability and solubility criteria, but failed the crucial activation requirement (*25,26*). They sought to overcome this defect by a slight modification of the earlier design, incorporating a saturated heterocyclic ring in place of the open-chain substituents. Thus, they prepared Friedman's precursor and condensed it with α,ω-alkanolamines. With 3-aminopropanol, their efficient two-step synthesis (Fig. 5) gave cyclophosphamide (*27*).

3.2. Preclinical Testing

In vivo screening showed that cyclophosphamide had excellent anticancer activity against transplanted rat tumors (*28–31*) and murine leukemia (*32*). Researchers were especially encouraged by the lack of in vivo crossresistance against L1210 cell lines with acquired resistance to the antimetabolites methotrexate, 8-azaguanine, and 6-mercaptopurine (*33,34*).

3.3. Clinical Trials

Immediately after publication of the preclinical studies came a report from Rudolf Gross and Klaus Lambers of the University of Marburg/Lahn on cyclophosphamide's

first clinical trial (*35*). Results from 45 patients with a wide variety of tumors indicated that cyclophosphamide had a spectrum of activity similar to those of mechlorethamine and mannitol mustard, but with less severe toxicity. This result was confirmed by worldwide studies (*36–44*) that established the usefulness of cyclophosphamide for treatment of chronic lymphocytic and granulocytic leukemias, acute myelogenous, monocytic and childhood lymphoblastic leukemia, Hodgkin's Burkitt's, and follicular lymphoma, lymphocytic and lymphoblastic lymphosarcoma, reticulum cell sarcoma, multiple myeloma, mycosis fungoides, breast, lung, and cervical carcinoma, neuroblastoma, retinoblastoma, Wilm's tumor, rhabdomyosarcoma, and ovarian adenocarcinoma (*45*). More prescriptions are written today for cyclophosphamide than any other alkylating agent. Indeed, its use may exceed that of any other chemotherapeutic anticancer agent (*46*).

3.4. Tissue Distribution

Why was cyclophosphamide less toxic than mechlorethamine and mannitol mustard in the clinic? One explanation came from the Medical University of Cologne, where investigators were able to demonstrate that cyclophosphamide and/or some of its metabolites selectively accumulated in neoplastic tissues. They prepared tritiated cyclophosphamide and administered it to three moribund patients a few hours before death from metastatic lung carcinoma. Autopsy showed that in all three cases, the highest concentrations of tritiated drug and metabolites (cpm/gm dry wt) had selectively partitioned into the primary tumors and metastases of the liver (2/3 patients), kidneys (2/3), adrenal glands (1/3), and diaphragm (1/3). Lower concentrations were found in normal tissues of the lungs, kidneys, brain, adrenal glands, spleen, lymph nodes, skin, pancreas, skeletal muscle, heart muscle, and testicles (*47*).

These data could be interpreted in various ways. Selective influx into tumor cells or selective efflux from the cells of normal host tissues are two possibilities. Cellular transport studies of cyclophosphamide and a metabolite precursor, 4-hydroperoxycyclophosphamide, were performed with several murine tumor and L929 fibroblast cell lines, but no significant differences in influx or accumulation were seen (*48*). In contrast, the study of cyclophosphamide's metabolism has been an extremely fruitful area of endeavor. The next section is a rough chronology of the effort to uncover the basic features of cyclophosphamide metabolism.

3.5. Metabolism of Cyclophosphamide

Soon after the synthesis of cyclophosphamide, a simple biological experiment overturned the notion of its hydrolysis within tumor cells, as a mechanism of selectivity. Prior to transplantation into rats, Yoshida, Jensen, or Walker tumor cells were incubated with high concentrations (up to 1 $mg \cdot mL^{-1}$) of cyclophosphamide. Their engraftment and growth did not differ from untreated controls. Since these tumors were sensitive to cyclophosphamide administered directly to the animal, it was obvious that normal cells somewhere in the host must be responsible for the activation (*49*).

3.5.1. Role of the Liver

In 1961, the in vivo site of cyclophosphamide activation was established by Foley et al. (*50*). They showed that cyclophosphamide *per se* was inactive against serial cultures of several tumor cell lines, but became cytotoxic on incubation with

homogenized mouse liver. Homogenates from other normal tissues or tumors failed to activate cyclophosphamide.

Two years later, the pivotal role of liver in the activation of cyclophosphamide was confirmed in much greater detail by Norbert Brock and Hans-Jürgen Hohorst. They perfused a cyclophosphamide solution through intact rat liver in organ culture and tested the outflow against tumor cells in tissue culture. They also administered cyclophosphamide to hepatectomized rats, harvested their blood serum, and tested that against cultured tumor cells. The serum's cytotoxicity was only one-fourth to one-tenth that of unhepatectomized controls. Using tissue slices, they showed that the remainder of the activity derived from renal cortex and lung. They established that the process did not involve hydrolysis and that bis(chloroethyl)amine was not the primary product of cyclophosphamide activation. Most significantly, they demonstrated that cyclophosphamide activation was an oxidative process requiring NADPH and molecular oxygen. From their results they concluded that the primary activation product was not the effective form, but an intermediate from which the ultimate cytotoxic species was spontaneously generated. They were then unable to characterize the structures of the labile transport form or the subsequent alkylator. However, when these were finally determined, the descriptive conclusions of the ASTA scientists were fully confirmed (*51*).

The finding that cyclophosphamide is activated in the liver by mixed-function oxidases, rather than by tumor phosphoamidases, appears to undermine the rational hypothesis that predicated its development. A degree of serendipity was surely involved. In spite of this, the rational approach played an essential role in motivating and guiding the inventors of cyclophosphamide (*25*). As is often the case, discovery resulted from fact and fortune working hand in hand.

3.5.2. Molecular Mechanism

Well before any firm data had appeared on the molecular structure of cyclophosphamide metabolites, Klaus Norpoth had hypothesized that the mechanism of hepatic oxidation of cyclophosphamide should parallel that of nicotine, as shown in Fig. 6. According to this scheme, the initial step involved hydroxylation of the ring methylene group adjacent to nitrogen. Furthermore, he maintained that subsequent transformations should also resemble those of nicotine (*52*). Unfortunately, the inability to confirm this hypothesis experimentally using a synthetic standard for the carbonyl derivative, 4-oxocyclophosphamide, led him to an erroneous revision in which cleavage of the cyclophosphamide ring by *O*-dealkylation was suggested (*53*).

Although the proposed *O*-dealkylation of cyclophosphamide later proved incorrect, it did lead to an interesting finding. The structure of Norpoth's putative "primary metabolite" contained a 3-substituted propionaldehyde group. This made it a likely metabolic precursor of acrolein, according to R.A. Alarcon and Johannes Meienhofer of the Children's Cancer Research Foundation. Although its immediate precursor had to be determined later, their experimental evidence for the in vitro production of acrolein from cyclophosphamide with liver microsomes, NADPH, and oxygen (*54*) received strong confirmation (*55*).

Evidence that Norpoth's initial proposal had been the correct one came in 1970 from the Southern Research Institute in Birmingham, AL. A metabolite from the urine of a dog treated with [6-^{14}C]cyclophosphamide was isolated and purified by

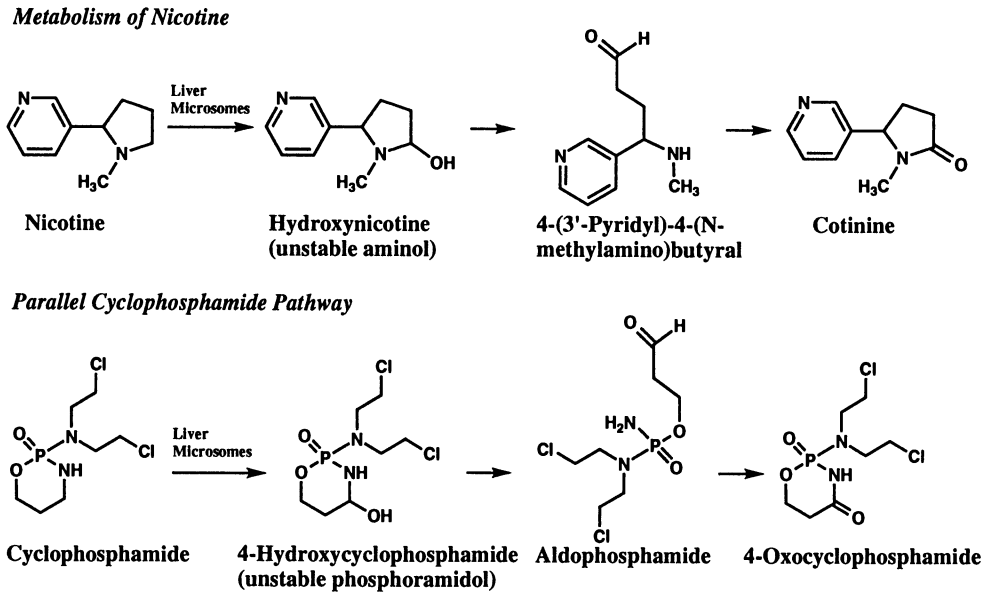


Fig. 6. Norpoth's hypothesis (*52,53*).

chromatography and crystallization. Oxidation adjacent to the ring nitrogen of cyclophosphamide was shown by infrared spectrophotometry (IR) and mass spectrometry (MS) to give 4-oxocyclophosphamide. The structure was confirmed by definitive synthesis (*56*). A few months later, 4-oxocyclophosphamide was also identified as a cyclophosphamide metabolite in the urine of sheep who received the drug as part of an experimental defleecing procedure (*57*).

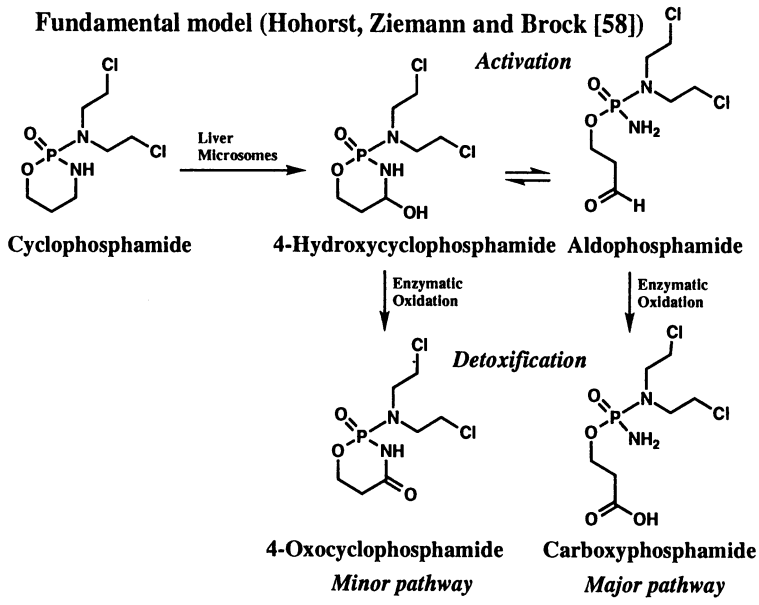
As a consequence of the above reports that empirically defined the position of oxidation of the cyclophosphamide ring and of its *N*-dealkylation, ASTA scientists were able to deduce the basic molecular features of cyclophosphamide activation and detoxification (Fig. 7). As Norpoth had originally suggested, the initial hepatic metabolite was 4-hydroxycyclophosphamide, which was enzymatically detoxified to 4-oxocyclophosphamide and eliminated in urine.

The pathway to 4-oxocyclophosphamide was not, however the major metabolic route. A larger portion of the primary 4-hydroxy metabolite was spontaneously converted to its ring-opened tautomer, commonly called aldophosphamide. The most abundant renally excreted metabolite of cyclophosphamide is a product of aldophosphamide oxidation by aldehyde dehydrogenase, commonly known as carboxyphosphamide (*58*).

An additional minor pathway of aldophosphamide metabolism is reduction (*57*), catalyzed by aldose reductase or an unidentified aldo-keto reductase. Consistent with the trivial nomenclature noted above, the product was named alcophosphamide. Formation of alcophosphamide from aldophosphamide is a detoxification pathway, since alcophosphamide possesses little antitumor activity in vivo (*45*).

3.5.3. Metabolite Characterization

The fundamental model of cyclophosphamide metabolism, outlined in Fig. 7, was firmly grounded in basic science, but still required confirmation by synthetic and



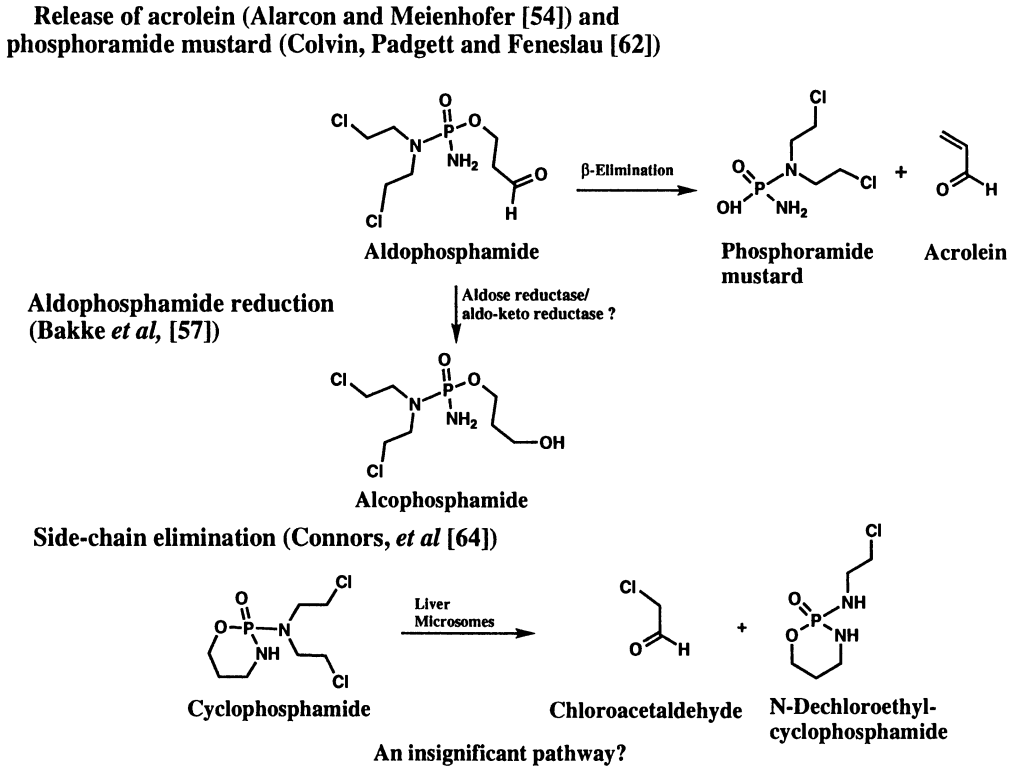


Fig. 7. Cyclophosphamide metabolism.

analytical methods. 4-Oxocyclophosphamide was well established, but more structural evidence was needed for the other three metabolites, 4-hydroxycyclophosphamide, aldophosphamide, and carboxyphosphamide.

A report from the Southern Research Institute provided the needed data for the latter compound. The labile acyclic carboxylic acid metabolite from dog and human urine was stabilized by the reaction with diazomethane and purified by column and thin-layer chromatography. The resulting methyl ester was characterized by IR, MS, and definitive synthesis. The Birmingham group showed that in humans, more than 25% of the cyclophosphamide dose was converted to the open-chain metabolite, carboxyphosphamide, whereas only 1–2% ended up as the ring-carbonyl metabolite, 4-oxocyclophosphamide. Finally, they showed that carboxyphosphamide and 4-oxocyclophosphamide had little or no anticancer activity, and concluded that they were "radically detoxified derivatives of cyclophosphamide" (*59*).

The expected aldehyde precursor of cyclophosphamide, commonly known as aldophosphamide, was converted *in situ* to a semicarbazone derivative by Norman Sladek of the University of Minnesota in 1973 (*60*). Sladek's semicarbazone was purified by thin-layer chromatography, and isolated and characterized by IR and ^{1}H nuclear magnetic resonance spectroscopy (NMR) by Robert Struck in Birmingham (*61*).

Aldophosphamide was shown by Colvin et al. of Johns Hopkins School of Medicine to be the true intermediate from which acrolein derived. Also of great interest was the coproduct of acrolein release, phosphoramide mustard, which they derivatized with diazomethane in order to obtain a mass spectrum (*62*). The synthesis of phosphoramide mustard had been reported 20 years earlier by Orrie Friedman and Arnold Seligman (*24*) and the compound was known to be a potent alkylating agent with good antitumor activity (*63*).

The first metabolite in the framework model was the last to yield the secrets of its physical properties. At the Chester Beatty Research Institute in London, Connors and coworkers tried to characterize the primary metabolite, 4-hydroxycyclophosphamide, but like many before them, found that it was too unstable for direct identification. By their efforts, however, they were able to derivatize it with ethanol and HCl to form 4-ethoxycyclophosphamide, which was then isolated and analyzed (*64*).

3.5.4. Current Perspective

The discussion above has only covered the bare essentials of cyclophosphamide metabolism. In the years since the framework model was pieced together, many new features have been fleshed out. New chemotherapeutic agents are now being developed that owe much to prior metabolism studies (*65*). Further progress will undoubtedly derive from knowledge of oxazaphosphorine metabolites and the enzymes that catalyze their formation and degradation. Toward this goal, recent studies have significantly deepened our understanding of the enzymes that activate and deactivate cyclophosphamide. Other investigations conducted during the last two decades have uncovered additional biochemical pathways and previously unknown metabolites.

The specific cytochrome P450 isozymes responsible for the primary activation of cyclophosphamide have been determined in rats and humans. The IIC6 and IIC11 isozymes are apparently the constitutive forms of importance in rats, but the phenobarbital-inducible IIB1 isozyme appears to be more efficient in response to appropriate pretreatment (*66*). The corresponding human isozymes will be identified in Section 7.1.

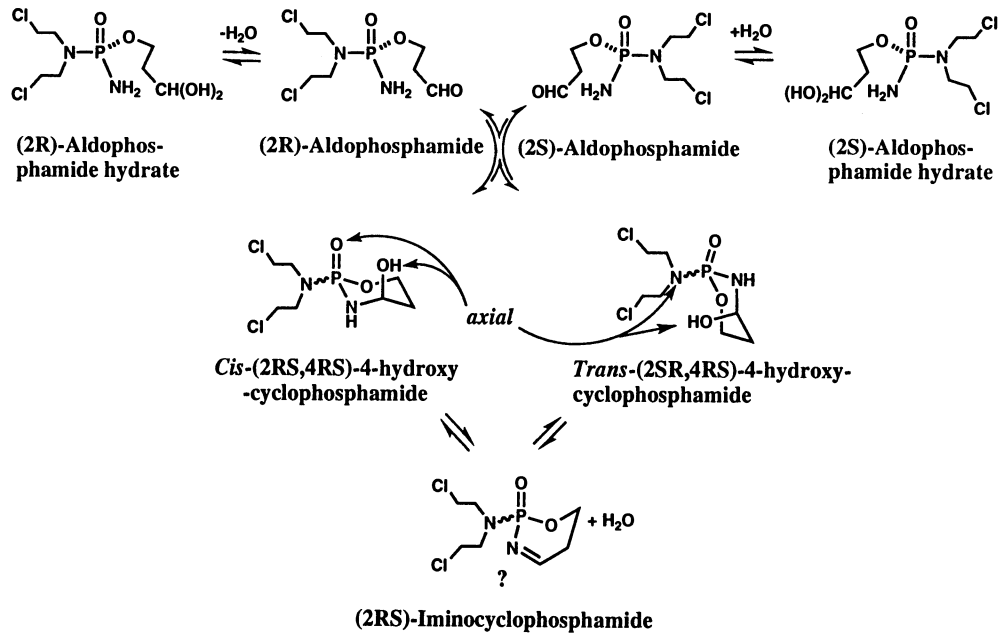


Fig. 8. Mechanisms of 4-hydroxycyclophosphamide trans-isomerization [*67,68*]).

The determination of active metabolites and exploration of their interconnecting and branching biochemical pathways have continued. The cis- and trans-isomers of 4-hydroxycyclophosphamide were analyzed by ^{31}P, ^{13}C, ^{2}H, and ^{1}H NMR spectroscopy and their equilibrium ratio, approx 2:1, was determined under physiological conditions of pH and temperature (*67*). Interconversion of the two isomers is mediated by ring opening to give aldophosphamide, which may reclose to give either cis- or trans-4-hydroxycyclophosphamide, as shown in Fig. 8. Another possible pathway has also been proposed, the dehydration of 4-hydroxycyclophosphamide to give iminocyclophosphamide. Unselective rehydration of iminocyclophosphamide may given either cis- or trans-4-hydroxycyclophosphamide (*68*).

Branching pathways lead from 4-hydroxycyclophosphamide to a series of thio-conjugates in which the mercaptide groups of endogenous compounds, such as reduced glutathione (GSH), replace the 4-hydroxyl group. The reaction is reversible and the thio-conjugates are more stable than 4-hydroxycyclophosphamide. By virtue of these properties, the reversible thio-conjugate family functions as a depot supply of 4-hydroxycyclophosphamide, which would quickly decompose in the absence of a stabilizing mechanism (*67*). Reversible thiol conjugation is illustrated in Fig. 9. Irreversible coupling with GSH is also possible, leading to oxazaphosphorine and phosphoramide mustard detoxification. Reversible and irreversible coupling with thiols will be discussed later, in conjunction with mafosfamide, a useful synthetic analog of the thio-conjugates.

The detailed enzymology of carboxyphosphamide formation has now been elucidated. Aldehyde dehydrogenases in mice (AHD) and humans (ALDH) catalyze the conversion of aldophosphamide to carboxyphosphamide. In the mouse, AHD 2, 8a and 8b are active against pharmacologic concentrations of aldophosphamide (*69*). In humans, ALDH 1 is the isozyme responsible for >80% of the liver's capacity to

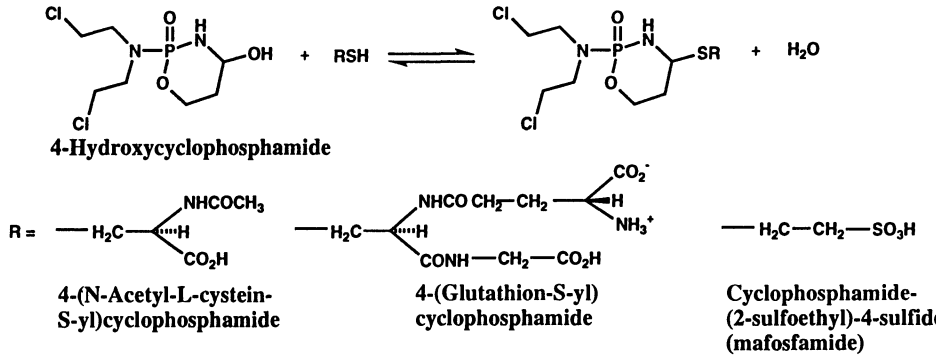


Fig. 9. Thiol conjugates of 4-hydroxycyclophosphamide [*67,68*]).

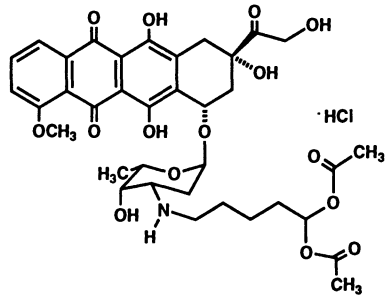


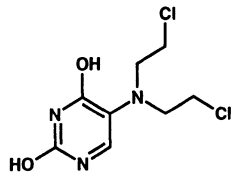
Fig. 10. *N*-(5,5-Diacetoxypent-1-yl)doxorubicin (*71*).

detoxify aldophosphamide (*70*). However, liver may not be the most important site of aldophosphamide detoxification.

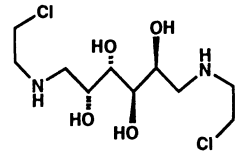
Aldehyde dehydrogenase is present in nearly every nonmalignant tissue, but little or none is found in cyclophosphamide-sensitive tumors. The ability of proliferative normal tissues to detoxify aldophosphamide is curtailed in or absent from many tumors. Although other proposed mechanisms may eventually be borne out, at the present time, this the most firmly established explanation for the noncytokinetic selectivity of cyclophosphamide (*45*). Sladek has suggested the design of aldehydes or aldehyde precursors with cytotoxic pharmacophores other than phosphorylated mustard groups, in order to make further use of selective detoxification by aldehyde dehydrogenases. He cites the example of *N*-(5,5-diacetoxypentyl)doxorubicin (Fig. 10), a promising new agent that may operate on this principle (*71*).

4. STRUCTURE–ACTIVITY RELATIONSHIPS

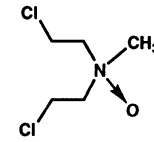
In 1961, three of the principal figures in the development of cyclophosphamide, Arnold, Bourseaux, and Brock published the results of a large study correlating the structures and biological activities of 15 nonphosphorylated mustards and 109 mono-, di-, and triamides of phosphoric and thiophosphoric acid. The nonphosphorylated mustard group included, "the last decade's most important experimentally and clinically established nitrogen-mustard compounds": mechlorethamine, chlorambucil, and melphalan (Fig. 1); uracil mustard, mannitol mustard, and nitromin (Fig. 11).



Uracil mustard



Mannitol mustard



Nitrogen mustard-N-oxide (nitromin)

Fig. 11. Tertiary mustards used clinically in the 1950s (*72*).

The phosphorylated and thiophosphorylated nitrogen mustards were studied, "as representative compounds selected from nearly 500 synthetic products" (*72*).

4.1. In Vivo Testing

In keeping with their goal of finding anticancer agents with wider margins of safety, the ASTA scientists evaluated prospective agents against a direct measure of selectivity, the therapeutic index (TI = lethal dose/effective dose in 50% of test animals [*73*]). For TI determination, they used the Yoshida ascites sarcoma, transplanted in outbred F49 rats (*74*). They defined the effective dose as that which achieved complete tumor remission lasting 90 d in 50% of the rats. Intraperitoneal treatment was begun 2 h after transplantation and continued for a total of 4 consecutive days. In consideration of the four-injection regimen and the end point, they used the term $DC_{50}(4)$, or *dosus curativa,* to denote effective dose.

All of the mustards from the clinic and many of the new phosphorylated nitrogen mustards "cured" the Yoshida sarcoma, by the 90-d criterion. For speed and economy, the lethal dose was evaluated by a single ip injection followed by 21 d of observation and denoted *dosus lethalis* or $DL_{50}(1)$. The ratio $DL_{50}(1)/DC_{50}(4)$ was calculated as a modified TI and used as the biological end point for evaluation of structure–activity relationships (SAR) (*72*).

This approach differed from procedures employed elsewhere at the time. For example, the massive US government-sponsored screening program used T/C, a ratio of life-spans for treated vs control animals with transplanted tumors, such as L1210 lymphocytic leukemia grown as an ascites in BDF1, DCBA, or D2BC mice. Several other transplanted murine and a few rat tumor systems were also used (*75*). Doses were escalated until the maximal increase in median survival time (ILS = [T/C] − 1) was reached. This MTD and associated ILS were reported as indicators of potency and therapeutic effect, but the margin of safety (TI) was not routinely determined (*76*).

4.2. Congeneric Groups

Because a single change in a lead drug molecule will frequently affect interactions with multiple biological systems, such as cellular transport, metabolism, and target binding, SAR studies of large unrelated groups of compounds have little chance of success (*77*). Thus, the ASTA compounds were divided into 14 congeneric groups, as shown in Table 1. Group assignments were made on the basis of unifying structural features, e.g., fragments of the molecular skeleton that all members of a group possessed in common. The 14 congeneric groups also included a group of "clinically established" tertiary mustards, eight groups of phosphorylated ring compounds, four

Table 1
Congeneric Groups

Group no.	*No. of members*	*Unifying structural features*
1	6	Unphosphorylated tertiary mustards
2	4	Cyclophosphamide analogs with contracted or expanded rings
3	7	Substituted pentacyclic phosphoric diamides
4	14	Oxazaphosphorines, most with alkyl substituents at positions 4, 5, or 6
5	12	*N*-substituted oxazaphosphorines
6	8	Thiophosphoric diamides
7	8	*O*-*n*-alkyl phosphoramide mustards
8	16	*O*-ω-halo-*n*-alkyl phosphoramide mustards
9	12	Cyclic phosphoric or thiophosphoric monoamides
10	7	Ayclic phosphoric monoamides
11	3	Cyclic phosphoric triamides
12	7	Acyclic phosphoric triamides
13	9	Unphosphorylated secondary mustards
14	9	Oxazaphosphorines with one or both chloroethyl side chains altered

groups of phosphorylated acyclic-mustards, and one group of secondary nor-nitrogen mustard analogs with one or two modified side chains.

The unphosphorylated mustards in group 1 were included to show the range of selectivity of the best compounds available prior to cyclophosphamide. Their TI values ranged from 1.8 for uracil mustard to 6.4 for nitromin. The authors maintained that selectivity within this narrow range was inversely related to the rate of ionization of their chloro-substituents in 26 m*M* bicarbonate buffer, pH 7.5, at 37 °C (incubation assay). Melphalan and nitromin had slower ionization rates than mechlorethamine, chlorambucil, uracil mustard, and mannitol mustard. This hydrolytic stability was seen as an element of latency, a property believed to confer selectivity. In proper perspective, however, the selectivities of melphalan and nitromin are sorely inadequate. The TI values of cyclophosphamide and some of its congeners are more than threefold greater than those of melphalan and nitromin, yet their margins of safety are also far from ideal.

As an ultimate example of acidic functionalization, the basicities of a series of *N*-acyl derivatives of bis(2-chloroethyl)amine prepared by Preussmann (*78*) were said to have, "practically disappeared" (*72*). They showed nominally no chloride hydrolysis and, as a consequence, were thoroughly inactive pharmacologically. However, between the extremes represented by the labile tertiary alkyl nitrogen mustards and the inert *N*-acylated secondary mustards, a continuum was envisioned from which it might be possible to pick an ideal functional type.

4.3. Limiting the Scope

A numbing range of structural possibilities were presented, even within the particular domain of phosphorylated mustards, owing to the high connectivity of the phosphorus nucleus. However, many of the groups were formed around a common

structural feature that conferred decreased selectivity on all members. For example, a ≥ 50% reduction in selectivity, *vis-a-vis*cyclophosphamide, resulted when phosphorus was absent, when any atom geminal to phosphorus was replaced, when the ring size was altered, when N^3 was alkylated, or when either chloroethyl group was extended, branched, or replaced by ethyl. Thus, negative SAR findings characterized all members of the following groups: 1–3, 5, 6, and 9–14.

In the next section, SAR data for all compounds in groups 4, 7, and 8 are presented. This should not imply that that excluded groups represent structural types that hold no promise. Indeed, the authors of the original study did prudently reinvestigate some of them and made a major find, described in Section 5.

4.4. Structural Correlates of Activity and Toxicity

Three of the congeneric groups, a total of 40 compounds, included 11 compounds with TI > 10. The oxazaphosphorines in group 4, with a total of 14 compounds, had 5 members whose TI values ranged from 11.3 (B690)–20 (B618; cyclophosphamide). Group 7, with a total of eight *O*-*n*-alkyl phosphoramide mustards, and group 8, which contained 16 *O*-ω-halo-*n*-alkyl phosphoramide mustards each contained three highly selective compounds with TI values ranging from 12.8–17.9.

The 11 compounds with the highest activities and selectivities had several structural features in common. They were all phosphorylated monoester-diamides derived from bis(2-chloroethyl)amine. Furthermore, as the original authors of the data observed, "The most favorable derivatives are those with *n*-propylene groups in amide-like linkages with oxygen in the end position. They may be arranged either cyclically... (e.g. B 518, B699, B717 and B576) or aliphatically (e.g. B633, B636, B637, B612, B700, B701) " (*72*).

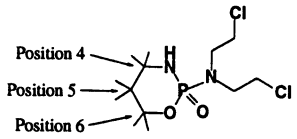
Among the oxazaphosphoramide group, shown in Table 2, substitution of any of the ring methylenes diminished selectivity. Thus, monomethylation of cyclophosphamide at positions 4, 5, or 6 caused a 28–40% decrease and vicinal 4,5-dimethylation caused a 43.5% decrease in TI. Mono substitutions by alkyl groups larger than methyl or multisubstitution beyond 4,5-dimethylation produced 50–100% reduction in TI (*72*).

Six of the active, selective compounds in the 1961 SAR study were phosphoramide mustard analogs, shown in Table 3. All possessed an *N*-(3-hydroxy-*n*-propyl) group. In group 7, the ethyl and *n*-propyl esters B633 and B636 were 2.3- to 2.6-fold less potent than cyclophosphamide, but their respective TI values were only 11 and 14% less.

In group 8 (Table 4), the 2-(chloroethyl) ester, B612 was also 2.6-fold less active than cyclophosphamide, but its TI was only 16% less.

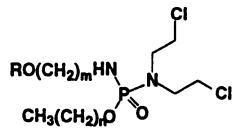
These active and selective *N*-(3-hydroxy-*n*-propyl)phosphoramide mustard esters showed little frank alkylating activity and good hydrolytic stability. Four of the compounds, B633, B636, B612, and B702, released only about 15–20 mol% of their available chloride equivalents during the 24-h in vitro incubation assay with 26 m*M* bicarbonate buffer, pH 7.5. In vivo formation of an ionizable phosphoramide mustard from these compounds requires *N*-dealkylation and thus may involve a different kinetic mechanism than the base-catalyzed *O*-dealkylation of aldophosphamide. With such interesting properties, one wonders why they have been left quietly on the shelf for the past 25 years.

Table 2
Structural Correlates of Activity and Selectivity:
Congeneric Group 4: Oxazaphosphorines



ASTA Code Number	*Position 4*	*Position 5*	*Position 6*	*DC_{50} (4) mmol/kg*	*Therapeutic index*
Unsubstituted					
B518 (cyclo-phosphamide)	H, H	H, H	H, H	0.03	20.0
Mono-substituted					
B699	H_3C, H	H, H	H, H	0.04	12.5
B717	H, H	H_3C, H	H, H	0.09	14.4
B576	H, H	H, H	H_3C, H	0.04	12.0
Di-substituted					
B690	H_3C, H	H_3C, H	H, H	0.05	11.3
B708	H, H	H_3C, H	H_3C, H	0.28	3.8
B707	H, H	H_3C, H_3C	H, H	inactive	nil
B709	H, H	H_3C, H	C_2H_5, H	0.33	6.0
B783	H_3C, H	H, H	n-C_3H_7, H	0.25	3.8
B784	H_3C, H	H, H	i-C_3H_7, H	0.50	2.5
B785	H_3C, H	H, H	n-C_6H_{13}, H	0.67	1.7
Tri-substituted					
B714	H_3C, H	H, H	H_3C, H_3C	0.10	10.0
B693	H, H	H_3C, H_3C	H_3C, H	1.99	1.0
B617	H_3C, H_3C	H, H	H_3C, H	2.64	0.5
B792	H_3C, H	H_3C, H	n-C_3H_7, H	0.91	0.7
B787	H_3C, H	H_3C, H	n-C_6H_{13}, H	inactive	nil

Table 3
Structural Correlates of Activity and Selectivity:
Congeneric Group 7: *N*-(ω-hydroxy/methoxy-*n*-alkyl)-*O*-*n*-alkyl Phosphoramide Mustards



ASTA code number	*R* =	*m* =	*n* =	*DC50 (4) mmol/kg*	*Therapeutic index*
B547	H	2	1	1.02	2.7
B635	H	2	2	0.49	4.0
B649	H	2	7	0.55	3.0
B633	H	3	1	0.08	17.2
B636	H	3	2	0.07	17.8
B637	H	3	7	0.05	12.8
B874	CH_3	3	1	0.86	1.3
B634	H	4	1	0.93	2.0

4.5. Stereoisomers

Although stereoisomerism was not discussed in the original paper, in 1982, Gerald Zon, of Washington, DC, reviewed the data for the compounds in group 4. Recognizing the chirality of cyclophosphamide at phosphorus and the additional asymmetric centers of the ring monosubstituted analogs, he pointed out that nongeminal substitution of 1-, 2-, or 3-ring carbon atoms would generate 4, 8, or 16, respective diasteriomers. Figure 12 shows the four stereoisomers of compound(s) B783.

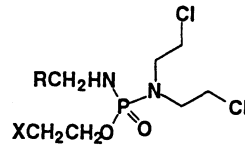
Zon noted that the in vivo environment is chiral; therefore, each asymmetric center could influence the outcome of transport, enzyme metabolism, and perhaps even purely chemical reactions, which may be subject to asymmetric induction. Certainly, he argued, such isomers should possess different therapeutic indices (*79*).

Differential metabolism of stereospecifically deuterated cyclophosphamide had been demonstrated in nontumor-bearing, female Balb/c mice. Analysis of the urine metabolites by MS showed that more of the excreted 4-oxocyclophosphamide was derived from (*R*)-cyclophosphamide than from its antipode (*80*). However in humans, neither enantiomer was therapeutically superior to racemic cyclophosphamide (*81,82*).

5. DEVELOPMENT OF IFOSFAMIDE

In the SAR studies with phosphorylated mustards, a number of congeneric structural types seemed to offer no productive leads. Two examples were analogs of cyclophosphamide in which either of the *N*-(2-chloroethyl) groups was altered or the hydrogen on N^3 was substituted. These modifications caused a dramatic loss of cytotoxicity and selectivity, but when both transformations were embodied in a single substance, an important new molecule resulted (*83*). In the design of this molecule, one of the 2-chloroethyl groups was taken from the bifunctional mustard side chain of cyclophosphamide and moved to the ring nitrogen (*84*).

Table 4
Structural Correlates of Activity and Selectivity:
Congeneric Group 8: *N*-Substituted-*O*-(ω-halo-*n*-alkyl) Phosphoramide Mustards



ASTA code number	*R* =	*X* =	*DC_{50} (4) mmol/kg*	*Therapeutic index*
B711	CH_2CH_3	Cl	0.49	1.0
B643	CH_2NH_2	Cl	0.92	2.0
B640	CH_2OH	Cl	0.61	4.0
B718	CH_2Cl	Cl	0.48	1.3
B612	CH_2CH_2OH	Cl	0.08	16.9
B700	CH_2CH_2OH	Br	0.05	15.0
B701	CH_2CH_2OH	CH_2Cl	0.06	15.0
B698	$CH(OH)CH_3$	Cl	0.47	3.8
B719	$CH_2CH_2OCH_3$	Cl	0.84	1.0
B861	$CH_2CH_2O_2CCH_3$	Cl	0.52	4.0
B720	$CH_2CH_2N(C_2H_5)_2$	Cl	inactive	nil
B865	$CH_2CH_2O_2C(CH_2)_3Br$	Cl	0.81	2.0
B870	$CH_2CH_2O_2C$-C6H5	Cl	inactive	nil
B864	$CH_2CH_2O_2C$, Cl, CH-C6H5	Cl	0.32	3.8
B664	$CH_2CH_2CH_2OH$	Cl	1.69	0.7
B696	$CH_2CH(OH)CH_3$	Cl	0.11	5.0

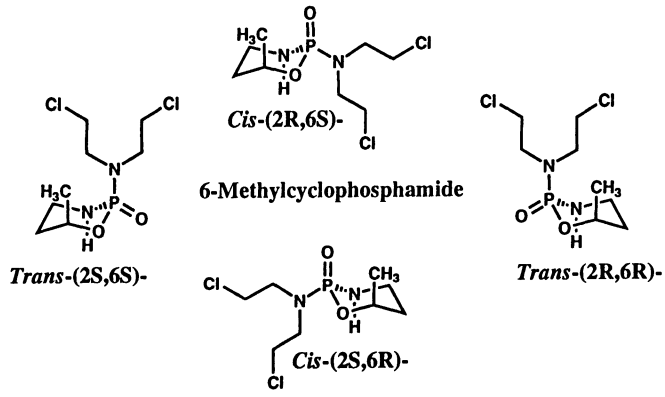
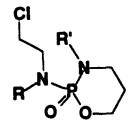


Fig. 12. Stereoisomers of cyclophosphamide analog B576: one substituent (*79*).

Table 5
Ifosfamide and Trofosfamide—Initial Preclinical Results



Common name	*R =*	*R' =*	*24-h Hydrolysis Cl^- equiv/mol*	*DC_{50} (4) mmol/kg*	*Therapeutic index*
Cyclophosphamide	CH_2CH_2Cl	H	0.19	0.032	20
Ifosfamide	H	CH_2CH_2Cl	0.06	0.024	24
Trofosfamide	CH_2CH_2Cl	CH_2CH_2Cl	0.04	0.025	9

The new compound, an isomer of cyclophosphamide, was given the common name ifosfamide. An isostere, trofosfamide, with 2-chloroethyl groups at all available nitrogen valences was also synthesized and received considerable developmental attention, but has subsequently been overshadowed by ifosfamide.

5.1. Preclinical Studies

The techniques used to determine the activity of cyclophosphamide were resurrected, with a slight modification, for characterization of ifosfamide and trofosfamide. The toxicity end point was shortened to 14 d, apparently without affecting the result, since the TI of cyclophosphamide did not differ from the earlier value. Ifosfamide and trofosfamide were shown to be hydrolytically stable prodrugs by incubation at 37°C in 26 m*M* bicarbonate, pH 7.5 (*85*).

5.1.1. In Vivo Screening

Against the Yoshida rat sarcoma (Table 5) a single injection of 7.7 $mg \cdot kg^{-1}$ of ifosfamide or 3.9 $mg \cdot kg^{-1}$ of cyclophosphamide gave a 50% cure rate. However, when divided doses were given daily ×4, 50% cures required only 1.58 $mg \cdot kg^{-1} \cdot d^{-1}$ of ifosfamide vs 2.25 $mg \cdot kg^{-1} \cdot d^{-1}$ of cyclophosphamide. This indicated that for ifosfamide, the cumulative therapeutic effect (concentration × time) was particularly important, whereas for cyclophosphamide, achieving a high peak concentration appeared to be more efficacious (*86*).

A variety of routes and schedules were tested by Abraham Goldin and John Venditti against "early" L1210 leukemic ascites transplant in BDF1 mice (*87,88*). Three of these regimens were especially informative and will be described here. Single doses of cyclophosphamide and ifosfamide given ip were both highly active (ILS > 300%). As in prior studies, the optimal single dose of ifosfamide, 300 $mg \cdot kg^{-1}$ exceeded that of cyclophosphamide, 180 $mg \cdot kg^{-1}$. When daily ip doses were divided over 9 d, the optimum dose of ifosfamide, 65 $mg \cdot kg^{-1} \cdot d^{-1}$ still exceeded that of cyclophosphamide, 39 $mg \cdot kg^{-1} \cdot d^{-1}$. Furthermore, this schedule was inferior to the single dose, since for both drugs, optimal total doses were almost twofold greater and the response much poorer (ILS = 50%). In a third regimen, the doses were divided into 3 ip injections given on days 1, 5, and 9. The optimal dose of ifosfamide was 39 $mg \cdot kg^{-1} \cdot d^{-1}$, and it gave an ILS of 85%, but that of cyclophosphamide was 108 $mg \cdot kg^{-1}$ giving an ILS of

70%. Therefore, ifosfamide was more potent and therapeutic than cyclophosphamide on this schedule, but was yet more effective as a single dose, contrary to the Yoshida sarcoma data.

Taken together, the rat sarcoma and mouse leukemia data were inconclusive. Interspecies data did not reveal an unequivocally optimal schedule and probably cannot. For humans, however, a clearer answer to this question did surface, as described below in Section 5.2.

Two additional models studied by Goldin (*88*) were an advanced L1210 leukemia that was naturally resistant to cyclophosphamide and an L1210 with acquired cyclophosphamide resistance developed by treatment over a series of transplant generations. In the naturally resistant system, the optimal single dose of cyclophosphamide, 180 $mg \cdot kg^{-1}$ gave an ILS of 144%. The optimal single dose of ifosfamide, 500 $mg \cdot kg^{-1}$, gave an ILS of 178%. Thus, ifosfamide was tolerated at a higher dose and was moderately more effective than cyclophosphamide. Goldin did note, however, that the advanced L1210, unlike the early strain, was metastatic at the time treatment began. The subline of L1210 with acquired cyclophosphamide resistance was completely crossresistant to ifosfamide.

Against murine Lewis lung carcinoma, C3H mammary adenocarcinoma, and TA nephroblastoma, ifosfamide was slightly more effective than cyclophosphamide. Against Ridgway osteogenic sarcoma and DS carcinosarcoma, ifosfamide was much more active than cyclophosphamide.

In combination with cisplatin, 5-fluorouracil, cytarabine, camptothecin, or cyclocytidine, the activity of ifosfamide against L1210 was greater than additive. It was only additive with cyclophosphamide, lomustine, or semustine (*88*). Against Yoshida sarcoma, ifosfamide was synergistic with vincristine (*89*).

5.1.2. Preclinical Toxicology

Acute, subacute, and chronic toxicity, carcinogenicity, and genotoxicity were monitored in mice, rats, dogs, or rabbits. In mice and rats, acute toxicity was determined using oral, iv, and ip administration, and in rats, the sc route was also tested. The dogs and rabbits were given iv injections. Ifosfamide was well tolerated parenterally, and toxicities were milder than expected for a nitrogen mustard.

Acute toxicities included reversible leucopenia, lymphoid depression, cystitis, and alopecia. Acute and subacute urine bladder irritation also occurred. The delayed toxicities were marrow depletion cistitis, enteritis, pneumonia, and testicular atrophy. Chronic lethality was owing to enteritis and pneumonitis.

Rat carcinogenicity assays revealed significant leiomyosarcoma and mammary fibroadenoma in females. In mice, carcinogenicity testing showed a dose-related increase in malignant lymphomas.

The reproductive toxicology of ifosfamide was evaluated in pregnant mice, rats, and rabbits. Lethal and sublethal embryotoxic effects, e.g., diminished survival, stunting, and craniofacial abnormalities, were seen in all three species (*90*).

5.2. Early Clinical Trials

Any analog of a highly active agent must be shown to possess distinct advantages over those of the lead drug before it can be introduced into the clinic. One would expect this criterion to be especially stringent in the case of an isomer of the parent com-

pound. In spite of this, a multi-institutional clinical trial in which 49 patients were treated with ifosfamide and 244 with trofosfamide was begun in 1967 (*83*) and results were published early in 1970 (*91*). The latter publication came only three years after the two compounds were first announced to the world at the Fifth International Chemotherapy Congress (*84*). Favorable preclinical screening results with the Yoshida sarcoma apparently accelerated the transition from bench to bedside (*83*). Although some of the initial enthusiasm dampened after reports of severe urothelial toxicity (*92*), development of an effective uroprotector rekindled worldwide interest (*93*).

The early clinical development of ifosfamide went through three stages. The first clinical trial, begun in 1967, used low doses, with disappointing results. For solid tumors, the average dose and duration of treatment were 75 $mg \cdot m^{-2} \cdot d^{-1} \times 98$ d and for systemic disease, 180 $mg \cdot m^{-2} \cdot d^{-1} \times 63$ d (*91*). Next, single doses of 2.9–10 $g \cdot m^{-2}$ were given, resulting in a high remission rate against metastasized solid tumors, but leucopenia, CNS toxicity, and urotoxicity were dose-limiting above 5 $g \cdot m^{-2}$(*94*). Animal experiments had shown that fractionated doses of ifosfamide had a cumulative therapeutic effect, but toxicity was less cumulative. These findings were incorporated into plans for the third stage of clinical testing.

A large cooperative trial, with 390 patients, were performed with ifosfamide as a single agent. The study compared single vs divided daily doses. A broad spectrum of malignancies were treated, including ovarian, mammary, gastric, pancreatic, colonic, cervical, uterine, and bronchial carcinomas, germ-cell tumors, malignant melanoma, and various sarcomas. The best results were obtained with daily injections of 2.0–2.4 $g \cdot m^{-2} \cdot d^{-1} \times 5$ d (*95*). This finding was confirmed in another cooperative trial with 360 patients, also with a wide variety of solid tumors. Optimum daily doses of 2.4 $g \cdot m^{-2} \cdot d^{-1} \times 5$ d were determined, except for treatment of osteo- or chondrosarcoma, where the schedule was 1.2 $g \cdot m^{-2} \cdot d^{-1} \times 10$ d (*96*).

The dose-limiting urotoxicity that occurred in these early clinical trials had been seen previously with cyclophosphamide, but with an average frequency of only 7%. The incidence of urotoxicity with ifosfamide ranged between 20 and 40% (*97*). Development of an effective uroprotector was sorely needed.

5.3. Supportive Care: MESNA and Beyond

Acrolein, formed by β-elimination from aldophosphamide, was identified as the principal etiologic agent of oxazaphosphorine urotoxicity (*93*). Chloroacetaldehyde, a minor metabolite of cyclophosphamide, but a major neurotoxic metabolite of ifosfamide, also contributes to oxazaphosphorine urotoxicity (*98*). The ability of thiol compounds, such as *N*-acetylcysteine, to detoxify acrolein and chloroacetaldehyde was recognized, but when given systemically, *N*-acetylcysteine also conjugated therapeutivally active metabolites. Local instillation of *N*-acetylcysteine into the bladder failed to protect the renal pelvis or ureter (*93*).

A detoxicant was sought for systemic administration that would act only in the urine to protect the whole urinary tract, without compromising therapeutic activity. In 1979, Scheef and associates published preliminary data indicating that 2-mercaptoethanesulfonate, or MESNA, met these criteria (*99*). Subsequent trials have demonstrated that MESNA is the agent of choice for uroprotection because of its effectiveness, ease of administration, and lack of systemic toxicity (*100*).

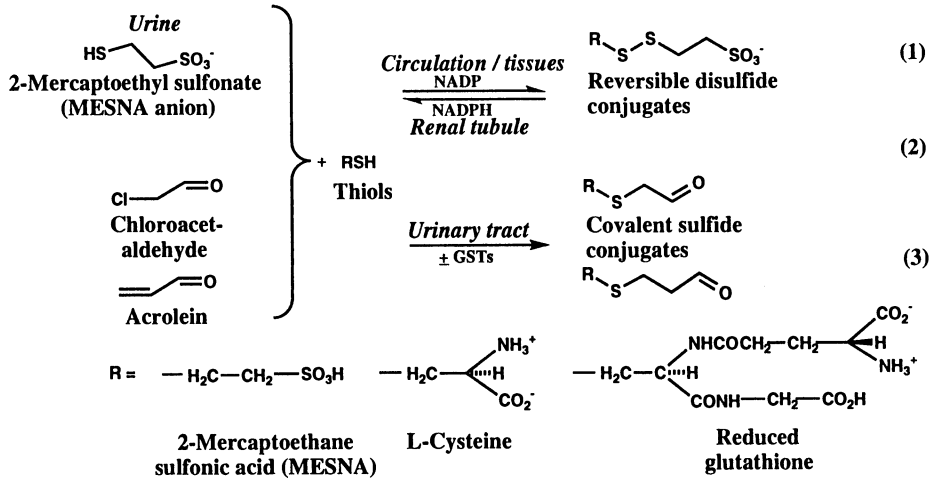


Fig. 13. Chemistry of MESNA and endogenous thiols in vivo (*93,102*).

The MESNA molecule has a negatively charged, water-solubilizing sulfo-group linked by an ethylene spacer to a strongly nucleophilic thiol group. MESNA is capable of inactivating alkylating agents, such as chloroacetaldehyde, or its oxidation product, chloroacetic acid, by covalent replacement of their halogen leaving groups (*101*). When given with cyclophosphamide, MESNA can reversibly sequester 4-hydroxycyclophosphamide by substituting the 2-sulfoethanesulfide moiety in place of the hydroxyl. Most importantly, it can detoxify an α, β-unsaturated ketone or aldehyde, such as acrolein, by conjugate addition to the olefinic double bond (*102*). The structure and in vivo chemistry of MESNA are shown in Fig. 13.

After entering the circulation, MESNA is rapidly oxidized to a nonnucleophilic symmetrical disulfide known as diMESNA. More than 90% of the circulating drug is in this form. Unsymmetrical disulfides are also formed by conjugation of MESNA with endogenous thiol compounds, such as cysteine. The disulfide products are incapable of systemic detoxification of acrolein, alkylators, or activated oxazaphosphorines, nor do they affect the activity of other electrophilic anticancer agents, such as cisplatin, nitrosoureas, or doxorubicin (*93*).

In the renal tubule, a large proportion of the disulfides are reconverted to thiol compounds by reduction. The reducing agent is NADPH, and the reaction is catalyzed by GSH. The proposed catalytic cycle is shown in Fig. 14. The reaction takes place during glomerular filtration, and the thiols are released into the urine. Their protective action is present in the renal pelvis, ureter, and bladder (*102*).

The discovery of MESNA was a pivotal step in the development of supportive care. The field is growing, as demonstrated by the development of hematopoietic growth factor (*103*) and stem cell support (*104*).

6. RECENT CLINICAL TRIALS WITH IFOSFAMIDE

Clinical trials of new anticancer agents are ordinarily conducted in sequential phases denoted phase I, II, and III (*105*). Occasionally, phases I and II are combined into a single sequence. Phase IV trials are conducted infrequently, when the need arises to monitor uncommon, idiosyncratic, or delayed complications of drug treatment.

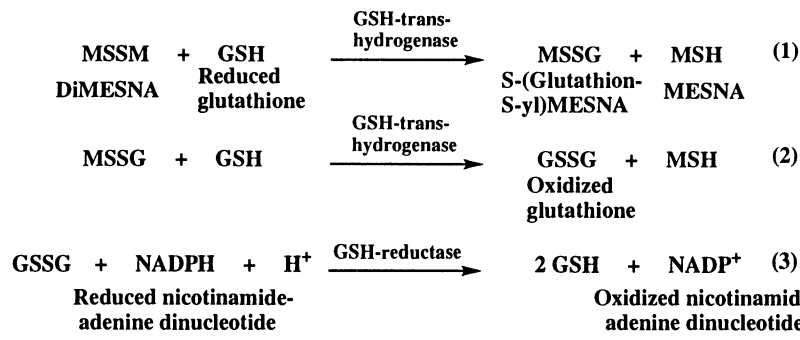


Fig. 14. MESNA/diMESNA catalytic cycle (*102*).

Phase I clinical trials of anticancer agents have two objectives: providing the best available treatment and determining the optimal treatment dose. The method involves escalation of doses (one drug at a time in combination studies) to a level at which either the desired biologic effect is observed or the limit of tolerability is reached. In the latter case, the penultimate dose is then usually considered the MTD. Since patients with advanced cancer are usually chosen for phase I studies, the toxic end point is most often encountered, rather than the therapeutic. Nonetheless, the therapeutic intent is implicit; studies are not undertaken to see how sick a drug will make someone.

Phase II trials are undertaken to provide a population of patients the most effective diagnosis-specific treatment available, to assess the degree of response of each evaluable patient, and to determine diagnosis-specific response rates and their statistical significance. Although Phase I trials may include diverse and sometimes unusual types of cancer, Phase II trials are more homogeneous and are often specific to one tumor type.

Phase III investigations are comparative trials undertaken to determine whether a new treatment is better than standard therapy or other competitive therapies. Comparative statistical inference requires randomized groups and a large sample. Therefore, Phase III trials are often cooperative studies requiring the joint effort of many hospitals (*106*).

Single-agent clinical regimens were once the rule, but are now the exception. Most current ifosfamide studies include one or more other drugs. Structures of some of the drugs used in combination chemotherapy with ifosfamide are shown in Fig. 15. Several combinations currently under investigation are defined in Table 6.

6.1. Phase I: Defining Doses and Toxicity Limits

The toxicities of cyclophosphamide and ifosfamide are steeply dose-dependent, but for ifosfamide, schedule and route are also important determinants of certain specific toxicities (*89*). Dose-related renal failure was documented in early clinical trials with ifosfamide possibly because of pre-existing renal dysfunction and/or inadequate hydration (*92*). The problem persists, but its incidence is declining as a result of closer monitoring of renal function and improved support. Nonetheless, high-dose continuous infusion of 5.0 or 8.0 $g \cdot m^{-2}$ of ifosfamide produced renal deterioration in 7/40 evaluable patients in a phase II single-agent study. The toxicities occurred despite administration of 400 or 600 $mg \cdot m^{-2}$ of MESNA, before and during chemotherapy. Two of these patients died, and two others suffered renal tubular damage (*107*).

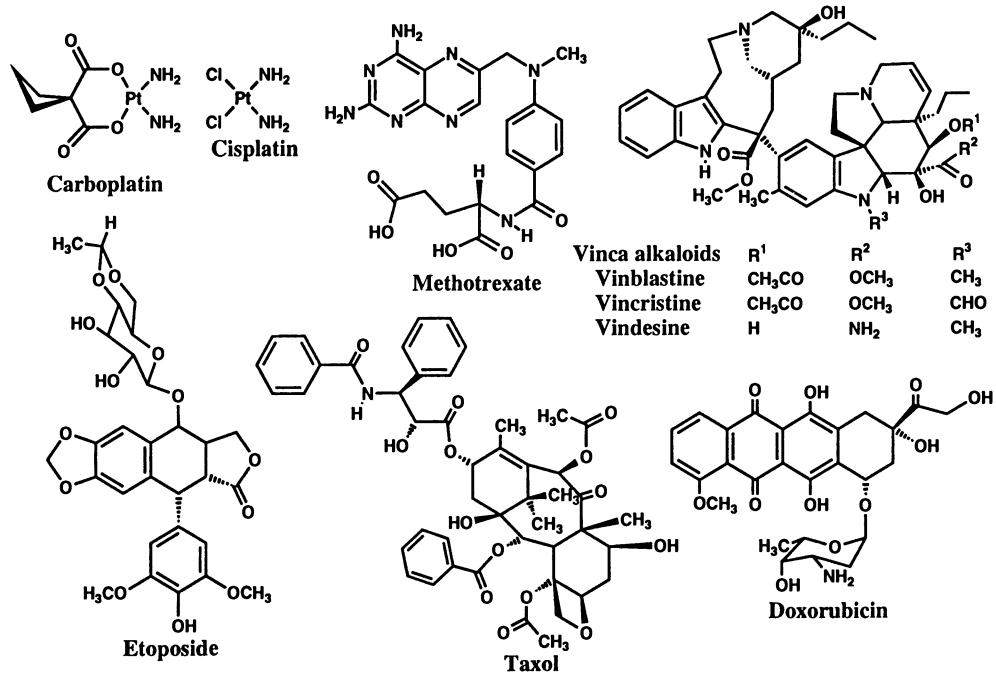


Fig. 15. Anticancer agents used in combination with ifosfamide.

The schedule dependency of ifosfamide toxicity found in preclinical studies and confirmed in early clinical trials has not been ignored in phase I trials. Fractionated single-agent ifosfamide doses of 2.4 $g \cdot m^{-2} \cdot d^{-1} \times 5$ d with MESNA uroprotection have appeared in several reports (*105*).

Intravenous fractionated dosing is labor-intensive, costly, and prone to accidents. These and other motivations have prompted the use of continuous infusions. In a recently published study, marathon 240-h continuous infusions of single-agent ifosfamide were given without stem cell support. The MTD was 1.2–1.3 $g \cdot m^{-2} \cdot d^{-1} \times 10$ d (*108*).

The most important route-dependent side effect of ifosfamide is neurotoxicity associated with oral administration. Symptoms of neurotoxicity occurred in 5/10 patients who received ifosfamide po 1.5 $g \cdot m^{-2} \cdot d^{-1} \times 5$ d and in 4/6 who received ifosfamide po 2.0 $g \cdot m^{-2} \cdot d^{-1} \times 5$ d (*109*).

The neurotoxic side effects of ifosfamide are attributed to overproduction of chloroacetaldehyde. Chloroacetaldehyde is a minor metabolite of cyclophosphamide, but is formed in significant amounts from ifosfamide. In sporadic cases, it is the most abundant ifosfamide metabolite. In two instances, patients who developed encephalopathy during ifosfamide treatment were relieved of their symptoms by treatment with methylene blue (*110,111*), a redox regulator that has been used in the treatment of cyanide poisoning (*112*).

It is frequently noted that, "the optimal doses and schedule of administration of ifosfamide still remain to be defined" (*105*). This may be a repercussion of the attractive array of new treatment options that are now under development, with very specific disease states as targets. Some of these, which utilize ifosfamide-based chemotherapeutic combinations, may require dosage reduction in order to facilitate an overall increase in dose intensity. Other designs may employ up-to-date support measures, such as

Table 6
Ifosfamide-Based Chemotherapeutic Drug Combinations

Acronym	*Ifosfamide/MESNA combined with*	*Application*
DI	Doxorubicin	Induction of SCLC, extensive NSCLC, relapsed ALL, advanced soft tissue sarcoma
IF/DDP	Cisplatin	Salvage of Ewing's spindle-cell, osteogenic, and synovial sarcomas, liposarcoma, fibrosarcoma testicular carcinomas
VI	Etoposide	Salvage of Hodgkin's and non-Hodgkin's lymplomas, salvage of testicular cancers
VIP	Etoposide (VP-16) and cisplatin	Induction of salvage of germ-cell tumors, salvage of SCLC, induction of NSCLC
IFF/MTX	Methotrexate	Intensification of SCLC
IFF/VDS	Vindesine	Salvage of SCLC
VIPE	Vincristine, etoposide, and carboplatin	Salvage of germ-cell tumors
VeIP	Vinblastine and cisplatin	Salvage of germ-cell tumors, salvage of SCLC, state IV head and neck carcinoma
ICE	Carboplatin and etoposide	Alternated weekly with DI for induction of SCLC; recurring pediatric solid tumors
VICE	Vincristine, carboplatin and etoposide	Salvage of SCLC
ICE-T	Carboplatin, etoposide and taxol	Phase I lung, breast cancer, sarcoma, adenoid cystic carcinoma
IME	Methotrexate and etoposide	Salvage of non-Hodgkin's lymploma
IMEP	Methotrexate, etoposide, and prednisone	Salvage of non-Hodgkin's lymploma; induction treatment of Hodgkin's disease
MIME	Mitoguazone, methotrexate, and etoposide	Salvage of non-Hodgkin's lymploma
IMV	Methotrexate and vincristine	Advanced malignant lymphoma
MAID	Mesna, doxorubicin, and dacarbazine	Advanced soft-tissue sarcoma
No acronym	Epirubicin and fluorouracil	Advanced breast cancer

administration of autologous hematopoietic progenitor cells, to counterbalance those adjustments (*113,114*). The establishment of "standard" doses, schedules, or routes of ifosfamide administration may be delayed indefinitely because its wide activity spectrum opens up so many avenues of clinical exploration.

Two recent protocols demonstrate the broad range of scheduling that may be applied to a single drug combination. One of these, a 96-h concurrent, continuous, iv infusion of the three-drug ICE combination, with autologous hematopoietic stem cell reinfusion, had an ifosfamide MTD of 4.0 $g \cdot m^{-2} \cdot d^{-1} \times 4$ d (*104*). For another ICE/transplant protocol, divided daily doses of ifosfamide were given by 1-h iv infusion with an MTD of 3.35 $g \cdot m^{-2} \cdot d^{-1} \times 6$ d. In the latter version of ICE, the carboplatin was given 11 h after each ifosfamide dose, by 1-h infusion. Etoposide was administered by 11-h continuous infusion twice daily when the other two drugs were off (*113*). These two studies span the range of possibilities from continuous, concurrent to discontinuous, countercurrent scheduling.

6.2. Phases II and III: Evaluation and Comparison

Depending on the site and type of disease, ifosfamide treatment may be given in any of the following settings: induction, or primary therapy, where the patient is previously untreated and the intent is cure or at least remission; consolidation, treatment given to maintain a complete remission or convert a partial response after induction into a complete remission (also termed intensification); and salvage, treatment given in the hope of re-establishing remission after relapse or to overcome the resistance of a refractory tumor. Neoadjuvant chemotherapy may be given prior to surgery to facilitate complete resection of bulky or otherwise inaccessible disease (*105*).

End point data from different clinical trials need to be determined in a uniform manner, allowing comparison of intertrial results. Therefore, standard response descriptions have been established by the World Health Organization and the International Union Against Cancer. According to their criteria, the number of objective responses in a given clinical trial is the sum of patients exhibiting partial and complete responses. Complete response is the absence of clinical, histological, and biochemical evidence of disease for 2 mo or more. For assessment of partial response, pretreatment measurements are taken in all patients of the longest diameter (D) of each lesion and the widest diameter (d) perpendicular to this. The two measurements of each lesion are multiplied together, and all of these products are summed ($\Sigma = D_1d_1 + D_2d_2 + D_3d_3 + \ldots + D_nd_n$). The measurements are repeated posttreatment for patients who do not have a complete response. If Σ(posttreatment)/Σ(pretreatment) $\leq$ 0.5 and no lesion increases in size and no new lesions are found, this constitutes a partial response (*106*).

Among the malignancies against which ifosfamide/MESNA have been tested, alone or in combination with other agents, we must include pancreatic, head and neck, esophageal, gastrointestinal, colorectal, and renal cell cancers. Data are sparse, but for some of these diagnoses, the outlook is grim (*105*). Against this, there is guarded optimism for the development of ifosfamide/MESNA regimens, with or without other drugs, to combat lung, breast, gynecological and testicular cancers, lymphomas, sarcomas, and pediatric solid tumors.

Within these seven disease categories are found tumors that differ widely in their sensitivity to chemotherapeutic agents. They are therefore subclassified according to

prognosis. For example, non-Hodgkin's lymphoma and nonsmall-cell lung cancer (NSCLC) have, on average, poorer treatment outcomes than Hodgkin's disease and small-cell lung cancer (SCLC). Comparative and noncomparative trials usually cover one, and rarely more than two diagnostic subtypes, since the data for each must be analyzed individually (*106*).

6.2.1. Lung Cancers

Lung cancers are the most common malignancies and their incidence is increasing (*115*). Although cisplatin or carboplatin in combination with etoposide are the more frequenly used regimens for SCLC (*116*), ifosfamide is highly active against this disease (*117*). Against extensive SCLC, single-agent ifosfamide gave an overall response rate of 48% (*118*).

Combination chemotherapy is superior to single-agent treatment for SCLC (*119*). In a randomized trial with 166 patients with previously untreated, extensive SCLC, ifosfamide, etoposide, and cisplatin (VIP), given daily for 4 d, were compared with the standard etoposide/cisplatin regimen. Overall response rates were not significantly different (70% for VIP vs 66% for etoposide/cisplatin), but 1-, 2-, and 3-yr survival rates and toxicity were significantly greater with the three-drug combination (*120*).

For initial chemotherapy of NSCLC, ifosfamide has provided objective response rates of $\geq 20\%$ in single-agent trials with MESNA uroprotection (*121*). However, its inclusion into standard induction therapy (*105*) or high-dose triple or quadruple regimens (*122*), with autologous bone marrow reinfusion, has not improved them significantly. In the neoadjuvant setting, ifosfamide and MESNA have been combined with etoposide and cisplatin (VIP), allowing a 55% complete resection rate (*123*).

6.2.2. Breast Cancers

Breast cancers remain the most prevalent malignancies of women, and their incidence appears to be increasing (*124*). Metastatic disease is considered incurable, although high-dose cyclophosphamide-based combination regimens with stem-cell support are now being evaluated in the hope of curing at least some of these patients (*125*). Ifosfamide/MESNA also has substantial activity as a single agent against advanced breast cancer (*126*), and this has prompted its integration into combination protocols (*105*). A trial of ifosfamide/epirubicin/fluorouracil gave a 79% objective response rate and 25% complete responses with 28 evaluable patients (*127*).

6.2.3. Gynecological Cancers

Primary therapy of advanced ovarian carcinoma with ifosfamide gave a 33% objective response rate. This was viewed as similar to the response rate with conventional alkylating agents. In a Phase II Gynecologic Oncology Group (GOG) trial of ifosfamide for salvage treatment of ovarian carcinoma, there were 3 complete and 5 partial clinical responses among 41 evaluable patients who were refractory to, or had relapsed after cisplatin treatment. In two other salvage trials against advanced ovarian carcinoma, lack of complete crossresistance was observed toward cyclophosphamide (3 objective responses/49 evaluable patients) and chlorambucil (3 objective responses/12 chlorambucil refractory patients) (*128*).

Another GOG trial of ifosfamide for salvage treatment of advanced squamous carcinoma of the cervix gave 3 responses, duration 1.8, 2.2, and 3.1 mo in 27 evaluable patients. Two responders had previously relapsed after cisplatin and the third after carboplatin chemotherapy (*129*).

Salvage treatment of advanced or metastatic uterine sarcoma with ifosfamide gave 5 complete and 3 partial responses among 26 patients with about equal numbers of homogeneous and heterogeneous müllerian tumors. Among 28 patients with advanced uterine leiomyosarcomas, 4 partial responses to ifosfamide were seen (*129*).

6.2.4. Testicular Cancers

About 95% of testicular cancers are seminomatous and nonsemanomitous germ-cell tumors. Approximately 25% of nonseminomatous tumors relapse after standard treatment. Salvage chemotherapy with ICE has achieved a complete remission rate of 25% in a 42 patient study, with 40 evaluable (*130*).

Standard treatment for early seminoma is radiotherapy, but for bulky disease, chemotherapy is the primary treatment modality. In these cases, cisplatin, bleomycin, and either vinblastine or etoposide are used for induction (*131*). Substitution of ifosfamide for bleomycin has given an 87% complete response rate in 24 patients with bulky disease (*132*).

6.2.5. Lymphomas

Induction treatment of Hodgkin's disease by rapidly alternating cycles of two noncrossresistant regimens, such as cyclophosphamide/vincristine/procarbazine/prednisone (COPP) and doxorubicin/bleomycin/vinblastine/dacarbazine (ABVD), has been supplemented with a third combination, ifosfamide/methotrexate/etoposide/prednisone (IMEP). In the first group of 38 patients to receive IMEP, the complete response rate was 95% (*133*).

A small group of six patients treated with IMV as induction treatment for non-Hodgkin's lymphoma had 67% complete responses. This is about as good as cyclophosphamide/doxorubicin/vincristine/prednisone (CHOP), on the best standard induction protocol (*134*).

For late consolidation and salvage treatment of Hodgkin's and non-Hodgkin's lymphoma, the related combinations IME and MIME are promising additions to the armamemtarium (*134,135*). For salvage and late intensification treatment of non-Hodgkin's lymphoma, MIME has achieved a 60% objective response rate, 24% of which were complete responses in a 208 patient study (*134*).

6.2.6. Sarcomas

For a group of 105 patients with advanced soft tissue sarcomas, the MAID combination achieved a 45% objective response rate. Median survival was 15.5 mo (*136*).

High-dose ifosfamide (4 g $\cdot m^{-2} \cdot d^{-1} \times 3$ d) without stem-cell support was able to overcome resistance to standard-dose ifosfamide (SDI) in a French study. Among 36 evaluable patients, 12 achieved partial responses. Among the responders, five had been refractory to SDI, two were SDI resistant, four were listed as indeterminate SDI sensitive, and only one had not received SDI previously (*110*).

Responding to Phase III results, The Soft Tissue and Bone Sarcoma Group of the European Organization for Research on Treatment of Cancer (EORTC) has determined that ifosfamide is the latent alkylator of choice for soft tissue sarcoma (*105*). This decision was based on a comparative study of ifosfamide/MESNA vs cyclophosphamide/MESNA that found a statistically significant difference in overall responses of 18 vs 8%, respectively (*136*).

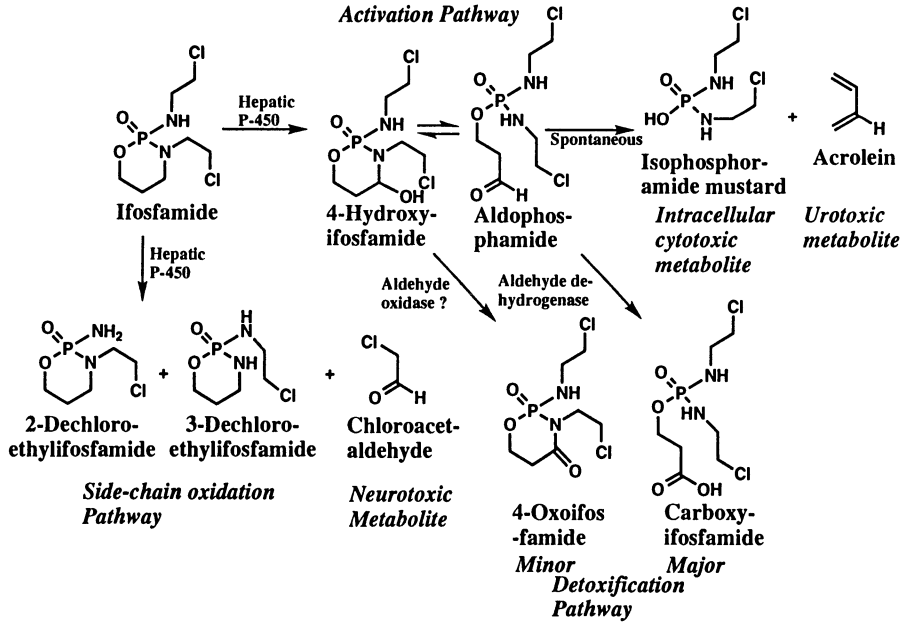


Fig. 16. Ifosfamide metabolism.

6.2.7. Pediatric Solid Tumors

Single-agent ifosfamide with MESNA uroprotection has demonstrated activity as salvage treatment against pediatric solid tumors resistant to cyclophosphamide (*105*).

The ICE combination has produced >50% objective response rates against persistent or recurring pediatric solid tumors of various diagnostic types. Investigations of new modalities of supportive care are under way, seeking to enhance recovery and allow greater dose intensification (*137*).

7. OXAZAPHOSPHORINE DISPOSITION

The in vivo dispositions of cyclophosphamide and ifosfamide are qualitatively similar, but quantitatively very different. What structural differences influence their comparative metabolism and pharmacokinetics? In cyclophosphamide, the ring phosphoramide group is secondary and the sidechain phosphoramide group is tertiary. This situation is reversed in ifosfamide. As a consequence, the relative rates of initial activation are not the same, nor are the active/toxic metabolite ratios. These quantitative differences in metabolism lead to dose- and schedule-dependent differences in pharmacokinetic half-life, whole-body clearance, and apparent volume of distribution.

The in vivo activation and degradation of cyclophosphamide and the methods used to characterize its metabolites were described previously. Corresponding studies of ifosfamide are presented here.

7.1. Metabolism of Ifosfamide

Hepatic microsomal hydroxylation of the ifosfamide ring carbon adjacent to N^3 generates the primary activation products (Fig. 16). The C^4-hydroxyl group may

occupy axial or equitorial positions on the chair conformers of the four diasteriomers. Because they are too unstable in aqueous solution for direct isolation, cis- and trans-4-hydroxyifosfamide were derivatized by addition of ethanol during the in vitro microsomal oxidation of the ^{32}P-labeled ifosfamide. The resulting derivatives, cis-and trans-4-ethoxyifosfamide, were separated by thin-layer radiochromatography and characterized by low-temperature ($\leq 100\,°C$) direct insertion MS (*64*).

Akira Takamizawa and coworkers in Osaka, Japan described the synthesis of cis-4-hydroxyifosfamide (mp 74–75 °C) by reduction of cis-4-hydroperoxyifosfamide with triethylphosphite (*138*). Subsequently, they discovered that cis-4-hydroperoxyifosfamide could be trans-isomerized in chloroform solution by a catalytic amount of 4-toluenesulfonic acid. They separated the isomers by column chromatography and reduced the trans-4-hydroperoxyifosfamide with triethylphosphite to obtain trans-4-hydroxyifosfamide (mp 49–50 °C) (*139*).

In aqueous solution, the underivatized conformers of 4-hydroxyifosfamide are in rapid pseudo-equilibrium. Their interconversion is mediated by hydrolytic cleavage of N^3—C^4 bond, which generates aldoisophosphamide hydrate. Reannulation may proceed by nonspecific syn- or anti-addition of N^3—H to the carbonyl group, leading to a mixture of isomers. The isomers and their equilibria were characterized by Jila Boal and associates in Washington, DC and Bethesda, MD, using ^{31}P NMR spectroscopy (*140*).

The individual steps involved in ifosfamide metabolism are analogous to those of cyclophosphamide (*45*), but there are important kinetic and thermodynamic differences that must not be overlooked. Although cyclophosphamide and ifosfamide are converted to their primary activation products by cytochrome P-450-catalyzed oxidation at positionally and stereochemically analogous ring sites, the human isozymes responsible are not the same. The major constitutive activator of cyclophosphamide is CYP2C9, but that of ifosfamide is the less catalytically efficient CYP3A4 isozyme (*141*).

Interpatient differences in the extent of cyclophosphamide activation have been ascribed to phenotypic polymorphism in the CYP2C cluster (*142*). However, the K_m of ifosfamide for these isozymes is so large that this polymorphism cannot be the reason for the observed variability of ifosfamide activation.

Cyclophosphamide activation rates are enhanced by phenobarbital induction of CYP2B6. Cyclophosphamide metabolism by this inducible form of cytochrome P-450 is more efficient than that of ifosfamide by eightfold, and it makes little (*141*) or no contribution to ifosfamide metabolism (*142*).

Aldehyde dehydrogenases, found in most normal human cells, detoxify aldoisophosphamide (*45*). The product, carboxyisophosphamide, is eliminated in the urine. Further oxidation of 4-hydroxyifosfamide probably takes place in the liver, giving 4-oxoifosfamide as a minor detoxification product, which also appears in the urine.

The ability of normal cells to detoxify aldophosphamide is absent or weakly expressed in oxazaphosphorine-sensitive tumor cells. This is a well-defined mechanism of selectivity for cyclophosphamide. It probably works for ifosfamide as well, although the definitive experiments have not yet been performed.

Both carboxyisophosphamide and 4-oxoifosfamide have been found in human urine by Alan Boddy and Jeffrey Idle, of the University of Newcastle upon Tyne. They isolated ifosfamide, carboxyisophosphamide, 4-oxoifosfamide, 2-dechloroethylifosfamide, and 3-dechloroethylifosfamide by thin-layer chromatography and identified them by cochromatography of authentic standards (*143*).

T. A. Connors and coworkers originally identified 2- and 3-dechloroethylifosfamide during their characterization of cis- and trans-4-hydroxyifosfamide (*64*). The two dechloroethylated species, account for as much as 48 (*144*) or 50% (*145*) of an administered dose of ifosfamide in some patients. Their in vivo abundance and variability are consequences of the 2-chloroethyl group on the ring nitrogen. Owing to steric hindrance, inductive effects, or both, the N^3-substituted molecule is a poor substrate for the efficient, constitutive CYP2C9 or inducible 2B6 isozymes that rapidly activate cyclophosphamide (*141*). The less-efficient CYP3A isozymes that oxidize ifosfamide are apparently also less regioselective. The hydroxylation responsible for *N*-dechloroethylation of oxazaphosphorine mustards occurs vicinal to chlorine, producing an unstable phosphoramidol that spontaneously eliminates chloroacetaldehyde, a neurotoxic metabolite (*146*) that can also be harmful to kidney function (*147,148*).

Most of an administered dose of ifosfamide is eliminated by the toxification and detoxification reactions described above. Its therapeutic power resides in the small remnant of ring-activated molecules that are converted to aldoisophosphamide, and then avoid further oxidation by entering sites of low aldehyde dehydrogenase activity. These sites lie specifically within tumor tissues. Here the cytotoxic isophosphoramide mustard is released together with acrolein. The *O*-dealkylation of aldophosphamide and aldoisophosphamide is catalyzed by general base (*149*). Acrolein elimination from aldophosphamide may also be catalyzed by enzymes, possibly DNA polymerase-associated 3′,5′-exonucleases (*150*). If so, aldoisophosphamide could, likewise, be a substrate.

Alarcon et al. first demonstrated the release of acrolein from ifosfamide after its microsomal conversion to aldoisophosphamide (*151*). The DNA-binding coproduct, isophosphoramide mustard, was identified by ^{31}P-NMR (*140*).

Cyclophosphamide is an analog of mechloroethamine, but ifosfamide is not. This distinction impacts their metabolism from beginning to end, but particularly at the level of DNA binding. Because cyclophosphamide and mechlorethamine have the same bis(2-chloroethyl)amino pharmacophore, their respective phosphoramide mustard and aziridinium metabolites span similar interhelical DNA crosslinking distances. In contrast, the covalent binding arms of isophosphoramide mustard can connect more widely separated DNA nucleobases (Fig. 17).

In addition to their structural dimensions, there are important kinetic properties that distinguish phosphoramide and isophosphoramide mustards. The 2-chloroethyl groups of isophosphoramide mustard, each occupying its own secondary nitrogen, are capable of cyclizing independently. However, phosphoramide mustard's chloroethyl groups share a single nitrogen and must cyclize in order. In view of these differences, one might expect the first alkylation step of isophosphoramide mustard to be twice as rapid as that of phosphoramide mustard, but it is not. Kinetic experiments have shown that step one is fourfold faster for phosphoramide mustard ($t_{1/2}$ = 20 min) than for isophosphoramide mustard ($t_{1/2}$ = 80 min). The apparent twofold statistical edge is heavily counterbalanced by the weaker nucleophilicity of isophosphoramide mustard's nitrogens.

The rate difference is even greater in the second alkylation step, since, at this point, both compounds have a single cyclizing group. The $t_{1/2}$ for cyclization of the remaining chloroethyl group of the phosphoramide mustard monoadduct is unchanged, 20 min. For the isophosphoramide mustard monoadduct, it is extended to 170 min. The biological consequences of these rate disparities are not entirely clear, but it has been

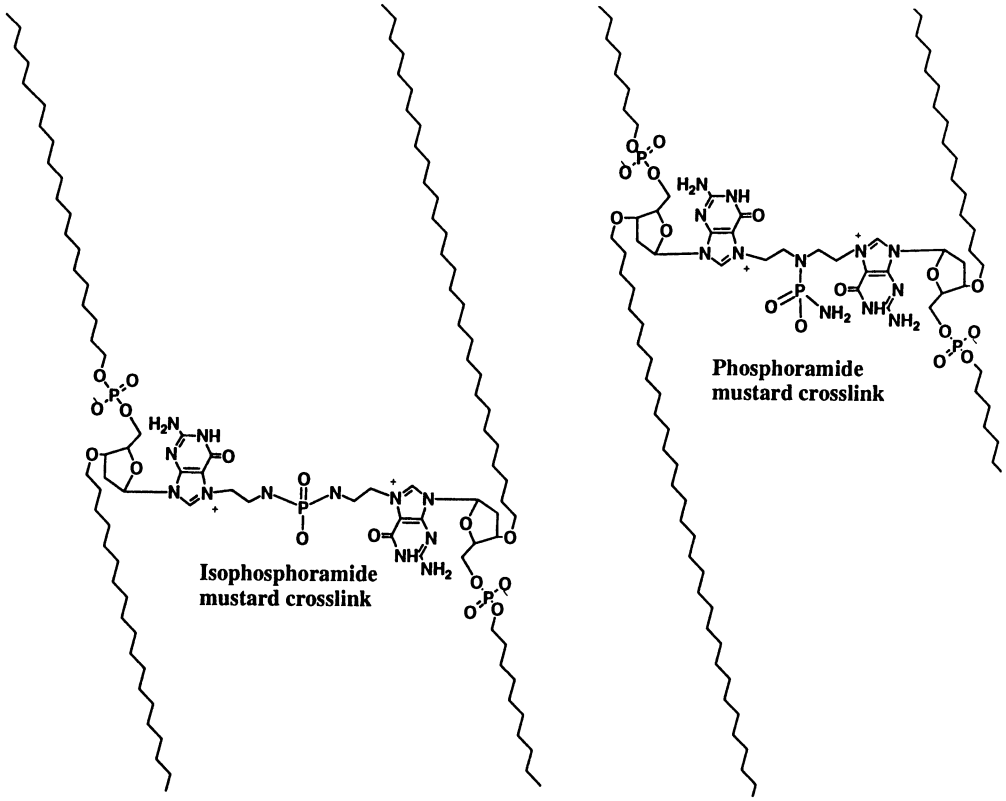


Fig. 17. Arm lengths of phosphoramide and isophosphoramide mustards.

speculated that within the cell, proportionately more isophosphoramide mustard may endure the commute to the nucleus and end up in a DNA crosslink (*140*).

7.2. Pharmacokinetics of Cyclophosphamide and Ifosfamide

In vivo rates of absorption, distribution, and elimination of drugs and their conversion to metabolites are the concern of pharmacokinetics. Three fundamental pharmacokinetic parameters are: elimination half-life ($t_{1/2}$), apparent distribution volume (V_{ap}), and whole-body clearance (Cl_t). They are interrelated according to the expression: $t_{1/2} = (V_{ap}/Cl_t)(\ln 2)$.

Elimination half-life is the time required for clearance of one-half of a bolus dose from the body fluid, blood, or plasma in which it is uniformly distributed. Apparent distribution volume is the volume of the phase in which the drug initially distributes to give a peak concentration, C_{max}. Thus C_{max} is inversely proportional to V_{ap}. Whole-body clearance ($mL \cdot min^{-1}$) is the fractional relationship between the delivered dose, D (millimoles), and the total exposure, AUC ($mmol \cdot mL^{-1} \cdot min$) (*152*).

7.2.1. Analytical Methods for Pharmacokinetic Monitoring

Cyclophosphamide and ifosfamide are rich in nitrogen and phosphorus, and sufficiently volatile for sensitive and rapid gas chromatographic (GC) analysis with thermionic nitrogen-phosphorus (NP) detection. This is an effective method, with one caveat: relative sensitivity to analyte and internal standard can change with detector

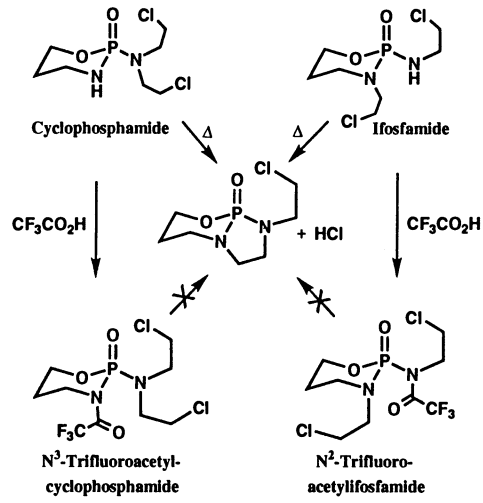


Fig. 18. Thermal cyclization of cyclophosphamide and ifosfamide.

usage. This does not happen if the two compounds have the same percentage content of nitrogen and phosphorus. Therefore, cyclophosphoramide and ifosfamide are ideal internal standards for one another.

Cyclophosphamide exhibits a slight thermal instability at GC injector port temperatures owing to intramolecular alkylation of N^3 by one of its chloroethyl groups (Fig. 18) (*153*). Acylation of N^3 with trifluoroacetic acid prior to GC analysis solves the problem (*154*). Intramolecular alkylation of ifosfamide was also formerly troublesome, requiring protection of the nucleophilic nitrogen. This difficulty was overcome by modifying the GC operating conditions (*155*). However, the new method is not suitable for analysis of underivatized cyclophosphamide. Therefore, trofosfamide is used as the internal standard.

Analytical resolution and quantitation of the stereoisomers of cyclophosphamide, ifosfamide, and their chiral metabolites are important, underutilized capabilities. Efficient separations have been achieved with cellulose-column HPLC (*156*) and capillary GC (*157*) with a bonded cyclodextrin stationary phase.

The indirect analysis of 4-hydroxyoxazaphosphorines/aldo(iso)phosphamide by quantitative acrolein release, derivatization with 3-aminophenol (Fig. 19) and spectrofluorometric quantitation (*54,151*), is subject to approximately twofold enhancement by other fluorogenic substances in blood. Resolution of these fluorochromes by high-performance liquid chromatography (HPLC) appears to be a reliable method for the cyclophosphamide metabolite, assuming subsequent hepatic ring hydroxylation of dechloroethylcyclophosphamide is insignificant (*158*).

Dechloroethylcyclophosphamide and 3-dechloroethylifosfamide are the same substance, although the ratio of enantiomers formed in vivo may differ. 3-Dechloroethylifosfamide is unsubstituted at the N^3 position. Therefore, it may be a good ring hydroxylation substrate for CYP2C9 or CYP2B6, enzymes that have no role in hepatic ifosfamide metabolism (*142*). Because of its greater catalytic efficiency *vis-à-vis* CYP3A4, for which ifosfamide is a substrate, conceivably more acrolein could be released from this inactive metabolite than from the parent drug. If so, 4-hydroxy-

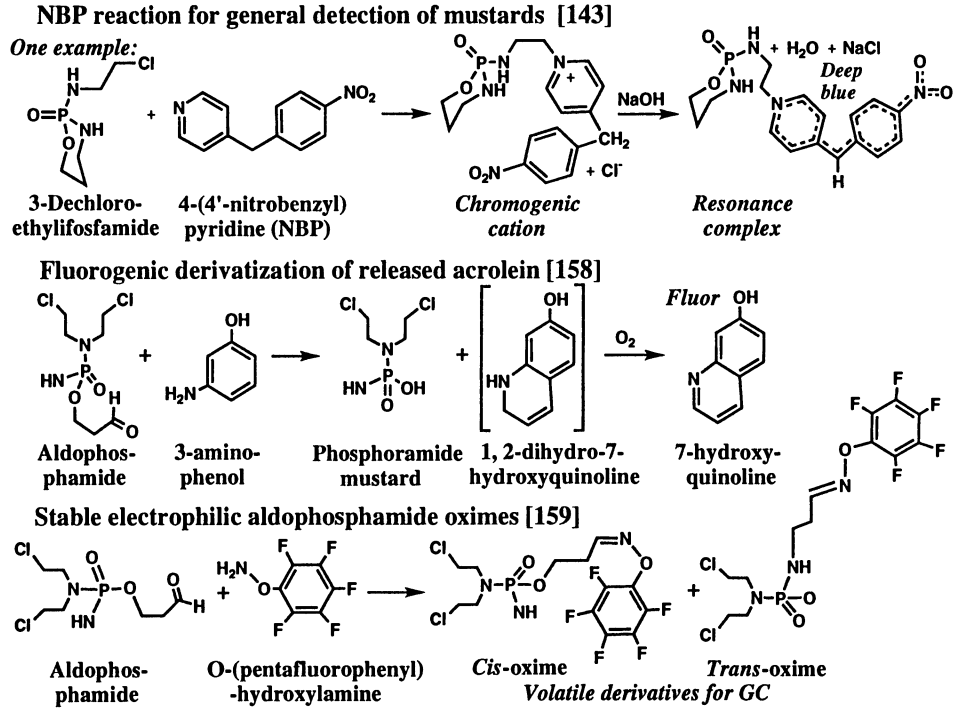


Fig. 19. Derivatization for analytical detection of oxazaphosphorine metabolites.

ifosfamide levels determined by acrolein release assay would be overestimated. Rate and fate determinations in appropriate hepatic or microsomal systems have not been performed with this metabolite, but ought to be done.

The foregoing considerations are a mandate for analysis of intact aldophosphamide/aldoisophosphamide. A number of aldehyde derivatization reagents have been tried, but elimination of phosphoramide/isophosphoramide mustard during workup or analysis has been a persistent problem. This has been solved recently with the discovery of stable derivatives of aldophosphamide and aldoisophosphamide. The oximes formed by coupling these labile metabolites with pentafluorophenyl hydroxylamine are amenable to GC-MS analysis (Fig. 19). The method is specific, sensitive, and accurate (*159*).

Assays have been developed that are suitable for pharmacokinetic monitoring of phosphoramides and isophosphoramide mustards in human plasma by GC with MS (*160*) and NP detection (*161*), respectively.

For simultaneous determination of cyclophosphamide, carboxyphosphamide, 4-oxocyclophosphamide, phosphoramide mustard, and nor-nitrogen mustard in urine, high-performance thin-layer chromatography with 4-(4'-nitrobenzyl)pyridine staining (Fig. 19) and photodensitometric detection appears to be efficient and reliable. The system has also been used extensively for analysis of 2- and 3-dechloroethylifosfamide, isocarboxyphosphamide, 4-oxoifosfamide, and isophosphoramide mustard in plasma and urine (*143*).

Efficient GC methods for analysis of *N*-dechloroethylated ifosfamide metabolites by GC with NP detection have been reported that allow their determination in the same run with the parent drugs (*155,162*).

Chloroacetaldehyde in human blood has been derivatized with thiourea, isolated by solid-phase extraction, and analyzed by HPLC. The detection limit was 0.5 μM (*163*). Another method uses direct GC with electron capture detection (*155*). The analyte is protected from reactive components in blood or plasma by addition of excess of formaldehyde.

7.2.2. Oral Absorption

Peak concentrations of cyclophosphamide occur 1 h, and of ifosfamide, 1–2 h after ingestion (*164, 165*). Bioavailability of oral cyclophosphamide and ifosfamide is slightly under 100%. This is owing to first-pass metabolism. Therefore, C_{max} values for the 4-hydroxy metabolites are higher than after iv administration. In view of this, the effective bioavailability is probably total (*45*). Oral cyclophosphamide is well tolerated, but po administration of ifosfamide is avoided, because toxicity is more frequent than by the iv route. The side effects are associated with elevated chloroacetaldehyde levels (*105*).

7.2.3. Apparent Distribution Volume

Oxazaphosphorines are polar, water-soluble, nonlipophilic drugs. As a consequence, they distribute uniformly into total body water in nonobese patients. Apparent distribution volumes of cyclophosphamide and ifosfamide (L) are about 2/3 × body mass (kg) of the patient (*166*).

7.2.4. Clearance

Whole-body clearance of cyclophosphamide is approx 82 mL/min, but extremely variable from patient to patient, range 35–200 $mL \cdot min^{-1}$. For a divided dose of ifosfamide, whole-body clearance on day one is about 70 $mL \cdot min^{-1}$ increasing by day 5 to about 120. Interpatient variability of ifosfamide clearance is similar to that of cyclophosphamide. Renal clearance of cyclophosphamide is approx 10 $mL \cdot min^{-1}$ and that of ifosfamide, 16 $mL \cdot min^{-1}$ (*166–168*).

7.2.5. Hepatic Elimination

Elimination of cyclophosphamide and ifosfamide are predominantly by hepatic oxidation. Both drugs give large, interpatient differences in elimination half-life. Standard single or divided doses of cyclophosphamide or ifosfamide are eliminated according to first-order kinetics, consistent with a one-compartment, open model. The average elimination half-life of cyclophosphamide is approx 6 h; that of ifosfamide is 7 h (*168*).

Data indicating biexponential elimination of ifosfamide, with $t_{1/2(\beta)} = 16$ h (*169*), have been cited repeatedly over the years. These data should be re-examined with a view to the method of ifosfamide analysis. [^{14}C]Ifosfamide was administered, and plasma and urine levels assayed as the radioactivity that partitioned into chloroform. Today we can presume that much of the β-phase elimination would represent dechloroethylated species, whose partition coefficients are similar to that of ifosfamide (*77*). At the time the work was performed, little was known of the side chain elimination pathway, and the need for high-resolution separation was not recognized.

The 4.8-fold slower hepatic elimination of ifosfamide vs cyclophosphamide was attributed to its lower affinity for a rat P-450 preparation, in vitro. However, the reported K_m values for ifosfamide and cyclophosphamide, 19.4 and 4.0 mM, respec-

tively, far exceed blood levels attainable in vivo. Identical V_{max} values were seen for the two drugs, 5.4 μmol·g protein^{-1}·h^{-1}. The data need to be reconsidered, in view of the miniscule catalytic efficiencies implied by these quantities (*169*).

From the standpoint of hepatic elimination, the most consequential difference between cyclophosphamide and ifosfamide is the extent of dechloroethylation. For ifosfamide, this is normally 15–23% of an administered dose. However, some patients may eliminate as much as one-half of their ifosfamide dose by side chain metabolism. Ordinarily, 3-dechloroethylifosfamide is found in 2.5-fold greater abundance than 2-dechloroethylifosfamide (*170*). Only about 10% of cyclophosphamide is eliminated by dechloroethylation (*145*).

7.2.6. Renal Elimination

Urine recovery of unchanged cyclophosphamide is about 13% of dose administered. That of ifosfamide varies considerably (14–34% in children [*171*]) and averages 18% (*170*).

7.2.7. Recent Reviews

Additional details are available in recent reviews on pharmacokinetics and metabolism of cyclophosphamide (*45*) and ifosfamide (*105,166*). Despite its title, *Ifosfamide Metabolism and Pharmacokinetics (Review)* includes cyclophosphamide in a comparative discussion (*165*). Lind and Ardiet have described the pharmacokinetics of oxazaphosphorines, chlorambucil, melphalan, BCNU, CCNU, fotemustine, thiotepa, alkylsulfonates, dacarbazine, and procarbazine in a unified presentation (*168*).

In 1982, Gerald Zon prefaced a critical survey of cyclophosphamide analog development, with the observation, "it is rather ironic that the unmodified cyclophosphamide molecule has not yet been displaced from its position of superior therapeutic value." (*79*) In this chapter, we have seen data indicating that ifosfamide has a broader activity spectrum than its prototype, and can induce some cyclophosphamide-resistant sistant tumors to respond. Yet we cannot properly say that ifosfamide is truly superior. Its nonhematopoietic toxicity and 8.5-fold greater cost per dose have raised stern questions about its continued use in the high-dose setting (*145*). Nonetheless, there are instances in which it does afford an appropriate replacement for cyclophosphamide.

Zon's review concluded hopefully, "Assuming that cyclophosphamide continues to attract widespead attention, it seems inevitable that analogues having superior anticancer properties will eventually be realized." (*79*) The search goes on.

8. IDENTIFICATION OF NEW AGENTS

In the US, new agent screening protocols developed at the National Cancer Institute (NCI) are designed to uncover patterns of activity against a wide variety of human tumor cell lines. The panel consists of 60 cell lines, including NSCLC, SCLC, breast, central nervous system, ovarian and renal cancers, leukemia, and melanoma (*172*).

Although the NCI's primary screen should be capable of selecting useful preactivated metabolite precursors, it would not be suitable for direct testing of latent prodrugs, such as cyclophosphamide and ifosfamide, which require microsomal activation. However, agents of this class may be properly evaluated by pretreatment with appropriate microsomal preparations prior to dilution and addition to cultures (*173*).

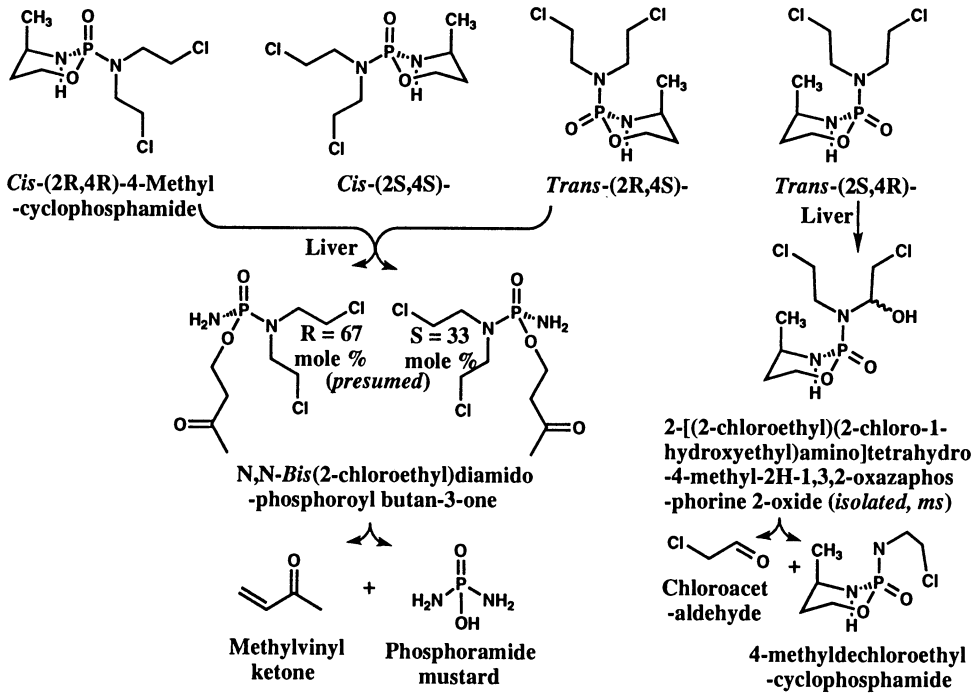


Fig. 20. Stereochemistry of rat cytochrome P450 catalyzed oxidation of 4-methylcyclophosphamide (*175*).

Another possible approach would use recently developed tumor cell lines transfected with oxazaphosphorine-metabolizing CYP genes capable of expressing the required hydroxylating activity (*174*).

8.1. Latent Oxazaphosphorine Analogs

In the aftermath of ifosfamide, most of the new latent phosphorylated mustards have been synthesized strategically, with the goal of identifying substituents, positions of substitution, or stereochemical arrangements that confer desired properties, the foremost being efficient hepatic activation (*79*).

8.1.1. 4-Methyl Analogs

As part of a study of the stereochemistry of CYP hydroxylation, each of the four diasteriomers of 4-methylcyclophosphamide were prepared. They were incubated in vitro with liver microsomes from mice, rats, and rabbits. Rates of disappearance of the four diasteriomers were all similar, but with rat microsomes, only three gave activated products (Fig. 20). The (2*S*,4*R*)-diasteriomer gave chloroacetaldehyde and dechloroethylcyclophosphamide. The phosphorylated metabolites were identified by TLC and MS. Chloroacetaldehyde and the coproduct of 4-hydroxylation, methyl vinyl ketone, were trapped as 2,4-dinitrophenylhydrazones and identified by TLC against authentic standards. The metabolic intermediate prior to dechloroethylation was also isolated and identified as 1′-hydroxy-4-methylcyclophosphamide by chemical ionization MS with deuteroammonia (*175*).

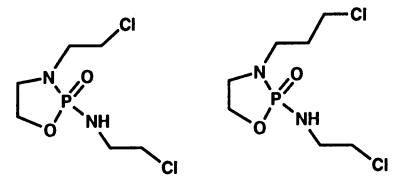


Fig. 21. Inactive ifosfamide pentagonal analog and isomer (*176*).

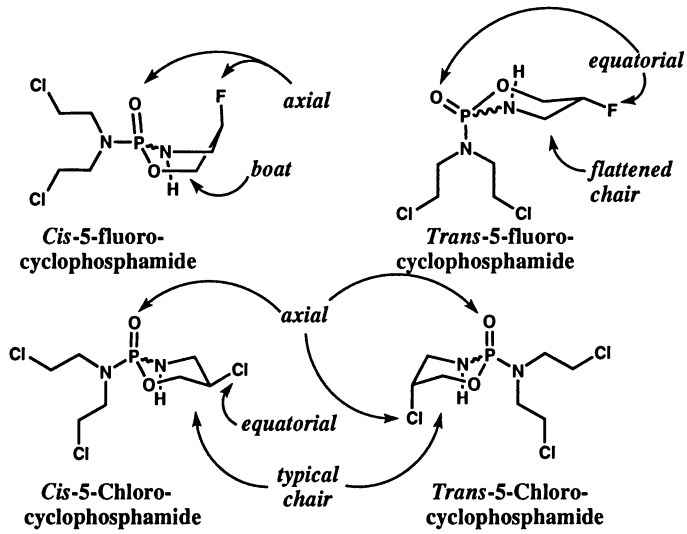


Fig. 22. Conformations of 5-fluoro- and 5-chlorocyclophosphamide epimers.

8.1.2. Ring Size

Negative results can be revealing, one example being a recent description of two ifosfamide analogs with five-membered rings (Fig. 21). One of these simply had one less methylene in the ring than the parent. The other was an isomer with a ring of methylene transferred to the N^3 side chain, giving a 3-chloropropyl group. Like their elder cousins prepared as cyclophosphamide analogs (*72*), these compounds were inactive against cultured cells and in vivo. The authors concluded that latent precursors of isophosphoramide mustard also require a six-membered ring for initial hepatic activation (*176*).

8.1.3. Steric and Electronic Effects

Other strategic studies have probed steric and electronic effects on the metabolism of cyclophosphamide. Individual cis- and trans-isomers of 5-fluoro- and 5-chlorocyclophosphamide have been prepared for conformational analysis by ^{13}C and ^{19}F NMR and tested for biological activity in vitro and in vivo (*177*).

The preferred solution conformations of both isomers of 5-fluorocyclophosphamide were unlike that of cyclophosphamide, for which the chair form with an axial phosphoryl oxygen predominates (Fig. 22). Cis-5-fluorocyclophosphamide appears to be unique among cyclophosphamide analogs, its most stable conformer being the boat form with axial fluorine and phosphoryl oxygen atoms. The corresponding trans-

isomer is a flattened chair with an axial fluorine and equatorial phosphoryl oxygen. In contrast, both isomers of 5-chlorocyclophosphamide are conformationally similar to cyclophosphamide.

In vitro rat microsomal activations of coincubated cis- and trans-5-fluorocyclophosphamide were 4 and 46%, respectively, complete after 50 min at 37°C. The corresponding values for cis- and trans-5-chlorocyclophosphamide were 9 and 41%. The assays measured disappearance of the parent compounds by GC of their trifluoroacetylated derivatives.

In vivo, cis-5-fluorocyclophosphamide was inactive against transplanted murine ADJ/PCY plasma cell tumors. Trans-5-fluorocyclophosphamide, cis-5-chlorocyclophosphamide and trans-5-chlorocyclophosphamide gave 90% reductions in tumor weight (ED_{90}), at 37, 9.4, and 6.6 $mg \cdot kg^{-1}$, respectively. The ED_{90} of cyclophosphamide was 2.25 $mg \cdot kg^{-1}$ in this assay. The activity of cis-5-chlorocyclophosphamide, in vivo, was considered surprising by the authors because of its slow activation rate. However, there may be no discrepancy if exposures (AUC) to the active transport metabolite derived from the two isomers are the same. This pharmacokinetic parameter is probably the major determinant of activity (*178*). AUC should be independent of activation rate if nonhepatic clearance is slow, since the open-chain metabolite enantiomers derived from the two unresolved diasteriomeric pairs are identical.

Several similar strategic studies have been reported, with trifluoromethyl (*179*), phenyl (*180*), and other electron withdrawing (and donating) substituents. Some of them are mechanistically informative, but regretably, no latent analog other than ifosfamide has been as active or selective as the lead compound. Reviews by Zon (*79*) and Wojciech Stec (*181*) provide ample coverage of these analogs.

8.2. Active Metabolite Analogs

When the site of 4-hydroxylation of cyclophosphamide was found to be the liver, it became apparent that this step was not directly involved in tumor selectivity. This understanding spawned considerable interest in the development of new precursors of 4-hydroxycyclophosphamide, aldophosphamide, and phosphoramide mustard that did not require hepatic activation (*139*). John Montgomery and Robert Struck have surveyed the synthesis of 80 potential precursors representing all three of these classes and some preactivated analogs (*182*).

8.2.1. Analogs of 4-Hydroxycyclophosphamide

Efficient syntheses of 4-hydroperoxycyclophosphamide (*183*) and 4-hydroperoxyifosfamide (*184*) by Takamizawa and coworkers provided abundant supplies for direct pharmacological study and as convenient precursors of 4-hydroxycyclophosphamide and 4-hydroxyifosfamide. They also developed simple, reliable routes to the individual cis- and trans-isomers of the primary activation products of cyclophosphamide and ifosfamide (Fig. 23) (*139*).

Preclinical studies of 4-hydroperoxycyclophosphamide given 1 wk after allogeneic transplantation of human MX-1 breast carcinoma in nude mice showed superior activity compared with cyclophosphamide. Furthermore, the difference was greater when treatment was delayed to 3 wk after transplant. Activation studies showed that as the tumor advanced, the ability of the murine liver to activate cyclophosphamide decreased (*185*).

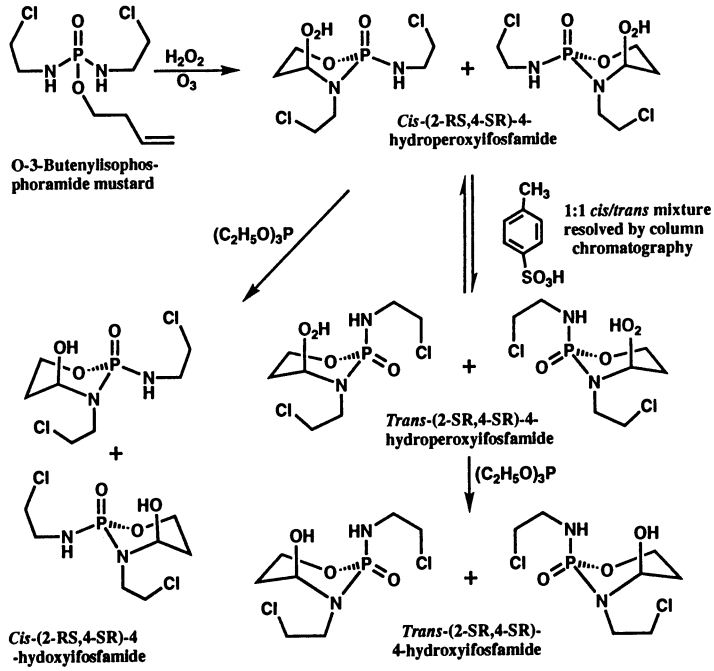


Fig. 23. Preparation of cis- and trans-4-hydroxyifosfamide (*139*).

Clinical studies of iv administration of 4-hydroperoxycyclophosphamide or 4-hydroperoxyifosfamide have not been reported to media accessible by Cancerlit of Medline information retrieval services. Trials of these compounds for purging, aimed at reducing residual disease from re-engrafted marrow or other autologous hematopoietic precursor tissues, appear often and prominently in the literature (*186*). The implication may be that these compounds have gone the way of other rapid metabolite generators, described below.

In addition to their direct use in the clinic and laboratory, 4-hydroperoxycyclophosphamide and 4-hydroperoxyifosfamide are also efficiently reduced to 4-hydroxycyclophosphamide and 4-hydroxyifosfamide, respectively. These in turn may be converted *in situ* to other useful analogs, such as the 4-alkylsulfides (*187*), modeled after the reversible glutathione and cysteine conjugates (*67*). This chemistry, with MESNA as the thiol component, launched the next significant achievement in the field, the synthesis of mafosfamide (*188*).

Mafosfamide is not formed directly from 4-hydroxycyclophosphamide, but as a consequence of its tautomerization to aldophosphamide. Subsequent hydration of the aldehyde group to the gem-diol, its protonation and displacement of water from the oxonium intermediate by MESNA, forms the hemithioacetal. Recyclization, via another round of hydroxyl protonation and displacement of water by the primary phosphoramide give mafosfamide (Fig. 24) (*189*).

In preclinical testing against L1210 ascites in mice, the TI of mafosfamide was comparable to that of cyclophosphamide. Bladder inflammation was examined and the mafosfamide-treated mice showed none, in contrast with the cyclophosphamide-treated animals. Intraperitoneal administration of mafosfamide did cause pericap-

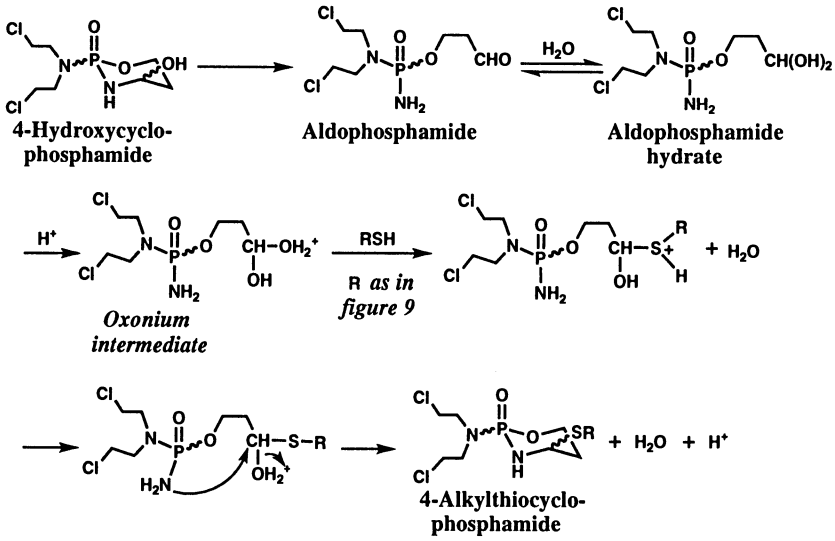


Fig. 24. Mechanism of reversible thiol conjugation (e.g., MESNA) with 4-hydroxycyclophosphamide (*189*).

sular hepatic fibrosis. This was an early warning that local toxicity near the site of injection could present a serious problem for administration of mafosfamide (*190*).

When clinical trials began, mafosfamide was administered intravenously as a single dose given every 3 wk. It had to be administered as a 2–3 h infusion because of severe pain along the injected vein. This side effect limited the dosage to 700 (*191*) or 1000 $mg \cdot m^{-2}$ (*192*). The drug was reformulated as a lysine salt, but this did not ameliorate the pain. The authors concluded that rapid release of active oxazaphosphorine metabolites prior to systemic distribution was clinically untenable. They suggested that derivatives giving low concentrations of active metabolites within the injection vein may be more promising (*191,192*). As in the case of 4-hydroperoxycyclophosphamide, mafosfamide is now widely used for purging (*193*).

A strategic study has pointed the way to regulating the formation of aldophosphamide analogs and release of acrolein and phosphoramide mustard derivatives from 4-hydroperoxy- and 4-(2-sulfoethyl)thiooxazaphosphoranes. Chul-Hoon Kwon and Richard Borch measured the associated rates and equilibria at 37 °C, pH 7.4, in 100 m*M* phosphate. 4-Hydroperoxy and 4-(2-sulfoethyl)thio derivatives of:

1. Cyclophosphamide;
2. 3-Methyl cyclophosphamide;
3. Ifosfamide; and
4. Trofosfamide

were investigated (Fig. 25). Ring opening of the 4-hydroxylated derivatives of these precursors was acid-catalyzed for derivatives of 1 and 2, but base-catalyzed for those of 3 and 4. In contrast with the 4-hydroxy derivatives of 1, 3, and 4, ring opening and subsequent generation of *N*-methyl phosphoramide mustard and acrolein from the 4-hydroxy derivative of 2 were markedly slower. Half-lives for these ring-opening reactions were: 1. 9.9; 2. 23; 3. 6.9; and 4. 3.3 min (*194*).

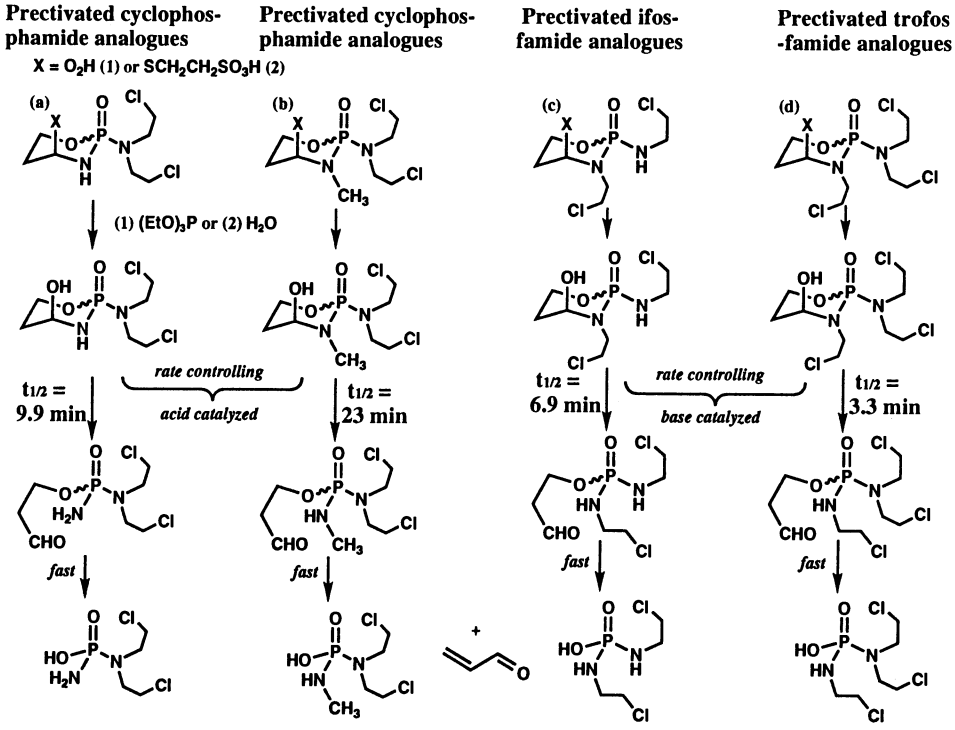


Fig. 25. Kinetics and mechanism: *N*-dealkylation of cis-4-hydroxy analogs (*194*).

8.2.2. Aldophosphamide Analogs

The prototype aldophosphamide precursor was its diethyl acetal (Fig. 26). It was originally prepared as a synthetic precursor from which aldophosphamide would be generated by mineral acid or Lewis acid treatment. However, the reaction did not yield the desired product (*183*). It was later tested against L1210 ascites in mice and was devoid of activity (*182*).

Recognizing that hydrolysis of acyl acetals is spontaneous at pH 7.4, Yu-Qiang Wang and David Farquhar of the M. D. Anderson Cancer Center in Houston, TX, prepared diacetylacetal derivatives of aldophosphamide, four analogs with *N*-substituents (monomethyl, dimethyl, monoethyl, and diethyl), and two with methoxy and ethoxy groups in place of the primary amino group (Fig. 26).

Aldophosphamide diacetylacetal was highly cytotoxic against cultured murine L1210 lymphoblastic leukemia cells with an IC_{50} of 0.9 μM (1-h exposure). The IC_{50} values of the more active analogs were as follows: monomethyl, 39; monoethyl, 28; and diethyl, 78 μM. These values were similar to those of mafosfamide, 42 and 4-hydroperoxycyclophosphamide, 90 μM. The other derivatives were less active, with IC_{50} values ranging from 150 to >300 μM. Half-lives of hydrolysis to form the aldehyde in 0.05*M* phosphate buffer at 37 °C, pH 7.4 ranged between 29 and 42 h. Thus, the compounds were sufficiently stable for preparation of injectable solutions. When they were catalytically hydrolyzed with pig liver carboxylate esterase, half-lives ranged from 36–42 s (*195*). In the case of mafosfamide, such rapid release of cytotoxic products was associated with local toxicity. Available publications on clinical applica-

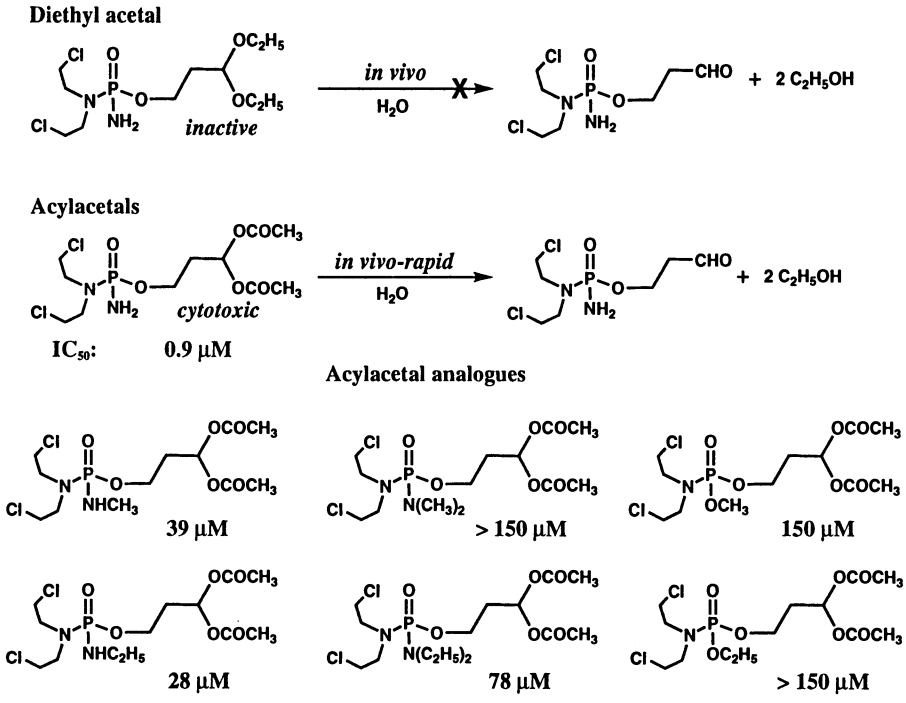


Fig. 26. Aldophosphamide analogs: acetals and acylacetals (*183,195*).

tions describe the suitability of acetaldophosphamide (*196*) and its isomer, acetaldoifosfamide, as the compounds are commonly called, for bone marrow purging (*197*).

Eight perhydrooxazine analogs of aldophosphamide were synthesized and characterized by Richard Borch and Ronald Valente (*198*). They invoked an alternative mechanism of intracellular phosphoramide release, initiated by acid-catalyzed opening of the perhydrooxazine ring to form an enamine, shown in Fig. 27. Two of the compounds had pairs of 2-chloroethyl groups on both phosphoramide nitrogens. Sensitivity of wild-type L1210 and P388 cells on tissue culture to 1-h exposure to either of these tetrakis(2-chloroethyl)amidophosphorane oxides was greater than to 4-hydroperoxycyclophosphamide. Against cyclophosphamide-resistant L1210 and P388 cells, they were 6- to 70-fold more effective than 4-hydroxycyclophosphamide.

The presence of mustard groups at all four phosphoramide valences appeared to be an important factor contributing to potency. This aspect of the molecular design was intended to avoid detoxification by ethylene bridging between the two nitrogens, which may occur when a primary or secondary phosphorylated amino group is present.

The perhydrooxazine analogs' low level of cyclophosphamide crossresistance may be owing to absence of substrate activity for aldehyde dehydrogenases. Aldehyde dehydrogenase overproduction is a common mechanism of oxazaphosphorine resistance development (*16*). On the other hand, the in vivo selectivity of oxazaphosphoranes used in the clinic today may depend on their efficient detoxification by aldehyde dehydrogenases in the host's nonneoplastic regenerating cells (*199*), such as those of blood and bone marrow (*200*). The anticancer activity of the perhydrooxazinophosphoramidates against transplanted L1210 ascites cells in mice (T/C $\leq$ 170%) was substantially lower than that of 4-hydroperoxycyclophosphamide (T/C = 240%) (*198*).

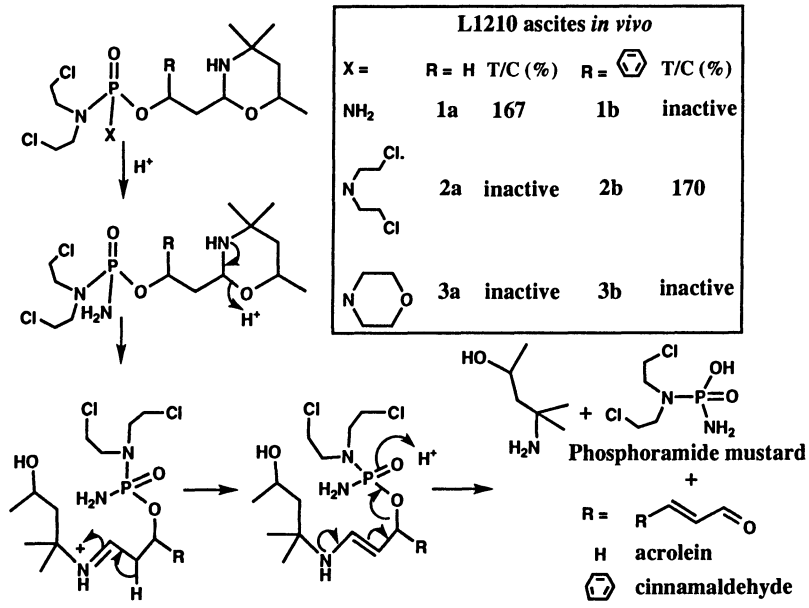


Fig. 27. Perhydrooxazinophosphorodiamidate mustards: activation mechanism and activity (*198*).

These strategic studies emphasize the difficulty of maintaining selectivity while overcoming resistance. Drug resistance development by tumor cells is also a strategic attack against the drug's selectivity mechanisms.

8.2.3. Phosphoramide Mustard Analogs

Phosphoramide mustard, the first active member of its class, was synthesized by Orrie Friedman before the advent of cyclophosphamide. In view of its precedence, phosphoramide mustard is the true prototype, and cyclophosphamide, the second-generation analog. In its preclinical activity spectrum, phosphoramide mustard was better than several clinically approved nitrogen mustards of the time, including some in use today (Fig. 1) (*201*).

Phosphoramide mustard is less active than cyclophosphamide against most transplanted tumors, and less cytotoxic than 4-hydroperoxycyclophosphamide or mafosfamide against most cultured tumor cell lines. Clinically responsive cell types, such as acute lymphocytic pre-B-lymphoblast and T blast II leukemias, Burkitt's B blast I, and histiocytic monoblast lymphoma cell lines are 14- to 26-fold more sensitive in culture to 4-hydroperoxycyclophosphamide than to phosphoramide mustard. Acute and chronic myelocytic leukemia and normal hematopoietic progenitor cells are 6.5-to 7.2-fold more sensitive to 4-hydroperoxycyclophosphamide than to phosphoramide mustard (*45*).

The differences in activity between the aldophosphamide precursors and phosphoramide mustard may be owing, in part, to transport. 4-Hydroxycyclophosphamide/aldophosphamide readily penetrates perfused U937 human histiocytic leukemia cells. The U937 cell membrane is relatively impermeable to phosphoramide mustard influx or efflux. Phosphoramide mustard generated within U937 cells after perfusion of 4-hydroxycyclophosphamide/aldophosphamide is retained after the cells are transferred to drug-free medium. 4-Hydroxycyclophosphamide/aldophosphamide effluxes

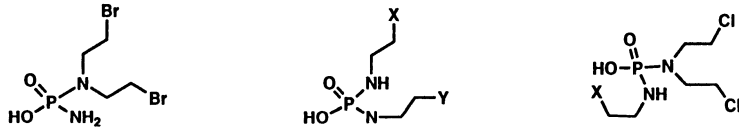


Fig. 28. Halogen-substituted phosphoramide mustard analogs (*205*).

from the cells into drug-free medium (*202*). Similar results have been obtained with Ehrlich ascites (*203*) and P388 cells (*204*).

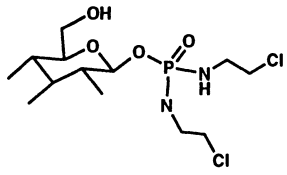
With another active, phosphorylated mustard, Struck et al. showed that cytotoxicity differences between active and latent phosphorylated mustards involve more than just transport (*205*). The compound in question was synthesized as part of a study of isophosphoramide and triphosphoramide mustards, the cytotoxic metabolites of ifosfamide and trofosfamide, respectively. The parental mustards and eight derivatives, with one or both chlorogroups replaced by bromine or fluorine atoms, were screened against cultured cells and transplanted murine tumors in vivo. Of particular interest was CB-IPM, the chlorobromo derivative of isophosphoramide mustard (Fig. 28). Tested against human ACHN renal and NCI-H23 lung tumor cell lines in tissue culture, CB-IPM was 5- and 50-fold, respectively, more potent than 4-hydroperoxycyclophosphamide. Against SK-MEL28 melanoma and SNB-7 CNS tumor cells, it was 5- and 2.5-fold, respectively, less sensitive than 4-hydroperoxycyclophosphamide. Against DLD-1 colon cells, CB-IPM appeared to be inactive.

Halogen substitution on one mustard group is unlikely to alter the steric or electronic properties of isophosphoramide mustard enough to overcome a major transport problem. The panel of five human tumor cell lines described above exhibit a range of sensitivities to other latent and active oxazaphosphorine metabolites. As such, they afford an excellent representation of nature's variety, pertinent to cellular uptake and other cytotoxicity determinants.

A number of in vivo experiments were performed with BC-IPM. Against IP L1210 leukemia in mice, BC-IPM produced 4/6 long-term survivors, as did isophosphamide mustard, vs 2/6 for cyclophosphamide. For BC-IPM, ILS was 388% (2/6 dying mice), compared with 133% for cyclophosphamide (4/6 dying mice). Against subcutaneous, cyclophosphamide-resistant L1210 cells, 2/6 BC-IPM-treated mice were long-term survivors, but CPA was inactive. However, against subcutaneous, wild-type L1210 cells, cyclophosphamide gave 6/6 long-term survivors, compared with 2/6 for BC-IPM. Against subcutaneous murine 16/C mammary tumor, BC-IPM gave a 60% longer tumor growth delay than cyclophosphamide. However, response of the subcutaneous B16 melanoma transplant favored cyclophosphamide by 2.6-fold over BC-IPM, in a tumor growth delay assay (*205*).

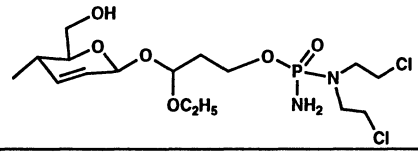
Although comparison with ifosfamide would have made the interpretation more definite, the in vivo data for BC-IPM and cyclophosphamide suggest that the selectivities of these model systems may not be based entirely on aldehyde dehydrogenase detoxification. Some of the total cellular burden of 4-hydroxycyclophosphamide is conjugated with GSH (*206*), for which the GSTM1 system is catalytically active. The effect of GSTM1 is not significant for phosphoramide mustard (*207*). GSTM1 is lacking in certain lung and breast cancers (*208*). This system may play a role in determining whether cells are more sensitive to BC-IPM or cyclophosphamide. The

ASTA D-19575: transmembrane carrier strategy



Survival against IP murine P388 leukemia (60-day survivors)	
Compound	Survival
Ifosfamide	6/6
D-19575	6/6

HEX-ALD: Lactate acidosis activation



Rat M1R mammary carcinoma cell line: clonogenic assay (plating efficiency % of untreated controls)			
Compound	pH 7.4, 24 h	pH6.2, 24 h	pH6.2, 48 h
Ifosfamide	0.001	no data	no data
HEX-ALD	nil	5	0.01

Fig. 29. Hexose-linked phosphoramide mustards (*212,224*).

deleterious effect of acrolein (*209*) and chloroacetaldehyde (*210*) on glutathione and GST (*211*) levels may limit the tolerated dose of cyclophosphamide or ifosfamide.

In view of evidence that ionized phosphoramide mustards are poorly transported into some tumors, scientists at ASTA Medica AG, Frankfurt am Main, have taken a two-pronged approach to overcome the deficiency. They have masked the ionizing diamidophosphate oxygen of isophosphoramide mustard by esterification with the 1-hydroxyl group of glucose (Fig. 29). In doing so, they hoped to gain the additional benefit of glucose transmembrane transport, since the derivative (D-19575) is an analog of glucose-1-phosphate. The IC_{90} of D-19575 against L1210 and KB cells was 6.3 and 5.2 μM, respectively, in a colony-forming assay. Corresponding values for isophosphoramide mustard were 1.4 and 8.1 μM. Both ifosfamide and D-19575 were curative (6/6 mice) against P388 ascites transplants. The best dose of ifosfamide given QD × 5 was 0.57 $mmol \cdot kg^{-1} \cdot d^{-1}$ × 5 d. That of D-19575 was 0.65 $mmol \cdot kg^{-1} \cdot d^{-1}$ × 5 d. Phlorizin, an inhibitor of the sodium-dependent glucose transporter, decreased cytotoxicity of D-19575 and increased renal recovery of the unchanged drug (*212*).

8.3. Overcoming Drug Resistance with Bioreductive Alkylators

Insensitivity of the hypoxic fraction of neoplastic cells within a tumor to conventional anticancer agents has been recognized as a serious impediment to curative treatment (*213*). There may be a bright side, however, because the disorganized vasculature associated with hypoxia distinguishes tumor cells from their normal counterparts. This distinction may provide a more specific basis for cell killing than proliferation rate, the predominant mechanism of current therapies (*214*). The development of *N*-oxides (*215*), nitroheterocycles (*216*), and quinones (*217*) as bioreducible precursors of toxic metabolites has been widely explored.

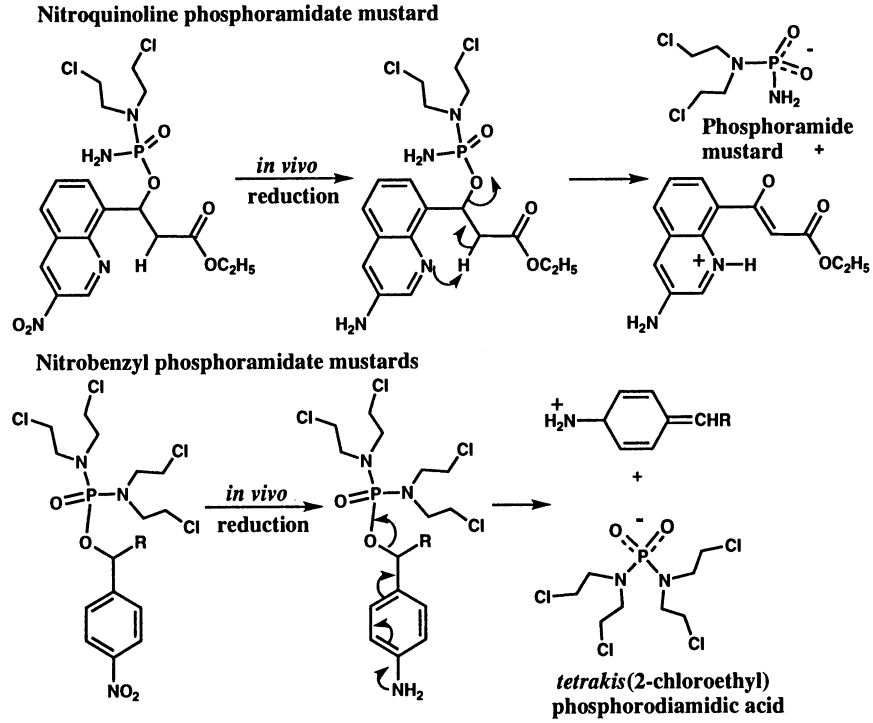


Fig. 30. Bioreductive alkylators.

The cytotoxicity of activated cyclophosphamide was greater against hypoxic than aerobic Chinese hamster V79 cells (*218*) and murine C3H mammary carcinoma (*219*) in tissue culture, but the effect was not reproducible in murine 16/C (*220*) or B16 melanoma (*221*) in vivo.

Juxtaposition of latent drug activation and selective nitro group reduction by hypoxic cells has led to the synthesis of a compound that releases phosphoramide mustard when its nitro group is reduced (Fig. 30). Four hours of exposure to the compound at concentrations between 50 and 300 μM gave >4.5 logs of cell kill against an hypoxic HT-29 human colon carcinoma cell line, but <1 log of cell kill against the same cells in aerobic culture (*222*).

The bioreductive activation of phosphorylated mustards was further elaborated in the synthesis of a series of (4-nitrophenyl)alkanol derivatives esterified with tetrakis(2-chloroethy)diamidophosphoryl chloride. Nitro-reduction was intended to release the tetrakis mustard and various iminoquinone methides. One of these compounds (Fig. 30) was selectively cytotoxic to hypoxic HT-29 cells, giving a 2-log cell kill from a 4-h exposure to 0–25 μM concentrations. Four hours of exposure between 0 and 100 μM killed about 1/2 log–1 log of aerobic HT-29 cells and 1/2 log of aerobic murine bone marrow reticulocyte/macrophage progenitor cells. Four other compounds were cytotoxic toward hypoxic and aerobic cells, indicating that they are activated by processes other than hypoxia-dependent nitro reduction (*223*).

An ancillary cellular consequence of hypoxia is lactate acidosis in response to glucose feeding. Glucose feeding of hypoxic tumor cells readily generates acidosis down to pH 6.2. In vivo blood glucose levels of 20–25 mM are needed to maintain

this pH. This effect has been exploited in the design of a selective precursor of aldophosphamide. The compound, denoted HEX-ALD, is the acetalglycoside of aldophosphamide and 2,3-dideoxy-D-erythro-2-hexenopyranoside (Fig. 29).

Aerobic M1R rat mammary carcinoma cells were exposed to 100 μg/mL HEX-ALD for 72 h at pH 7.4, giving < 1 log of cell kill. After 48 h at pH 6.2, a cell kill of > 4 logs was seen. Although the required exposure time was excessive, the authors indicated that they were preparing new analogs that will hopefully be more acid-labile (*224*).

9. CONCLUSION

The studies reviewed above show that the selectivity mechanisms of phosphorylated mustards are multifactorial, with governing determinants that vary from tumor to tumor. In terms of drug design and development for the future, a variety of agents with differing transport, cytotoxicity, and selectivity mechanisms are needed. Our vision of the future should include optimizing the administration of these agents on a per-patient basis. Regimen selection based on tumor isozyme expression (GST mRNAs, for instance [*225*]), and pharmacokinetic dosing, are two steps in this direction.

All anticancer agents, old and new, must be utilized in the safest, most effective ways possible. This calls for an unrelenting commitment to discovery of broader and better supportive care, focused on end-organ protection. The search for new end-organ support modalities is as important as drug development in the overall treatment picture, especially because supportive care impacts on tolerable dose intensity.

Following each defeat of an end-organ toxicity comes a new round of dose reintensification (*17*). Current manifestations of this progress are the high-dose regimens developed in the wake of hematopoietic growth factor (*226*) and progenitor cell support (*227*). Every advance across the log-linear dose–response curve leads inevitably toward the one cell limit (*228*). High hopes are pinned on the reiteration of this cycle to the point of curative treatment with minimal side effects (*229*).

REFERENCES

1. Colvin M. Alkylating agents and platinum antitumor compounds. In: Holland JF, Frei E III, Bast RC Jr, Kufe DW, Morton DL, Weichselbaum RR, eds. *Cancer Medicine,* 3rd ed. Philadelphia: Lea & Febiger. 1993:733–754.
2. Krumbhaar EB. Role of the blood and the bone marrow in certain forms of blood poisoning. 1. Peripheral blood changes and their significance. *JAMA* 1919; 72:39–45.
3. Adair FE, Bagg HJ. Experimental and clinical studies on the treatment of cancer by dichloroethylsulphide (mustard gas). *Ann Surg* 1931; 93:190–199.
4. Berenblum I. Experimental inhibition of tumor induction by mustard gas and other compounds. *J Pathol Bacteriol* 1935; 40:549–558.
5. Salomon G. Kinetics of ring-formation and polymerisation in solution. *Trans Faraday Soc* 1936; 32:153–178.
6. Gilman A, Phillips FS. The biological actions and therapeutic applications of the β-chloroethyl amines and sulfides. *Science* 1946; 103:409–415, 436.
7. Avery OT, MacLeod CM, McCarty M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a deoxyribonucleic acid fraction isolated from Pneumococcus Type III. *J Exp Med* 1944; 79:137–158.
8. Stryer I. *Biochemistry,* 2nd ed. New York: W. H. Freeman and Company. 1981:562.
9. Anslow WP, Karnovsky DA, Val Jager B, Smith HW. The toxicity and pharmacological action of the nitrogen mustards and certain related compounds. *J Pharmacol Exp Ther* 1947; 91: 224–235.

10. Gilman A. The initial clinical trial of nitrogen mustard. *Am J Surg* 1963; 105:574–578.
11. Rhoads CP. Nitrogen mustards in the treatment of neoplastic disease. *JAMA* 1946; 131: 656–659.
12. Goodman LS, Wintrobe MM, Dameshek W, Goodman MJ, Gilman A, McLennan MT. Nitrogen mustard therapy. *JAMA* 1946; 132:126–131.
13. Jacobson LO, Spurr CL, Guzman Barron ES, Smith T, Lushbaugh C, Dick GF. Nitrogen mustard therapy. *JAMA* 1946; 132:263–271.
14. Gilman A. Therapeutic applications of chemical warfare agents. *Fed Proc* 1946; 5:285–292.
15. Golomb FM. Agents used in cancer chemotherapy. *Am J Surg* 1963; 105:579–590.
16. Sladek NE. Oxazaphosphorine-specific acquired cellular resistance. In: Teicher BA, ed. *Drug Resistance in Oncology*. New York: Marcel Dekker. 1993:375–410.
17. Frei III E, Canellos GP. Dose: a critical factor in cancer chemotherapy. *Am J Med* 1980; 69:585.
18. Narayanan V. Development of new chemotherapeutic agents. In: Magrath I, ed. *New Directions in Cancer Treatment*. Berlin: Springer-Verlag. 1987:227–242.
19. Seligman AM, Nachlas MM, Manheimer LH, Friedman OM, Wolf G. Development of new methods for the histochemical demonstration of hydrolytic intracellular enzymes in a program of cancer research. *Ann Surg* 1949; 130:333–341.
20. Ichihara M. Über die phosphamidase. *J Biochem* 1933; 18:87–106.
21. Rorig K. *N*-(*p*-chlorophenyl-)diamidophosphoric acid. *J Am Chem Soc* 1949; 71:3561,3562.
22. Gomori G. Histochemical demonstration of sites of phosphamidase activity. *Proc Soc Exp Biol Med* 1948; 69:407–409.
23. Seligman AM, Milden M, Friedman OM. A study of the inhibition of tumor growth in mice and rats with 10-methyl-1,2-benzanthracene and derivatives related to the nitrogen and sulfur beta-chloroethyl vesicants. *Cancer* 1949; 2:701–706.
24. Friedman OM, Seligman AM. Preparation of *N*-phosphorylated derivatives of bis β-chloroethylamine. *J Am Chem Soc* 1954; 76:655–661.
25. Brock N. Oxazaphosphorine cytostatics: past-present-future. *Cancer Res* 1989; 49:1–7.
26. Druckrey H. Esperimentelle Grundlagen des Krebses. *Dtsch Med Wochenschr* 1952; 77: 1534–1537.
27. Arnold H, Bourseaux F. Synthese und abbau zytostatisch wirksamer zyklischer *N*-phosphamidester des bis-(chloroethyl)-amins. *Angew Chem* 1958; 70:539–544.
28. Arnold H, Bourseaux F, Brock N. Neuartige krebs-chemotherapeutika aus der gruppe der zyklischen N-lost phosphoramidester. *Naturwiss* 1958; 45:64–66.
29. Brock N, Wilmanns H. Wirkung eines zyklisches N-lost-phosphoramidesters auf experimentell erzeugte tumoren der ratte. *Deutsch Med Wschr* 1958; 83:453–458.
30. Arnold H, Bourseaux F, Brock N. Chemotherapeutic action of a cyclic nitrogen mustard phosphamide ester (B518-ASTA) in experimental tumors of the rat. *Nature* 1958; 181:931.
31. Brock N. Zur pharmakologischen characterisierung zyklischer N-lost-phosphamid-ester als krebs-chemotherapeutica. *Arzneim-Forsch* 1958; 8:1–9.
32. Lane M. Some effects of cyclophosphamide (cytoxan) on normal mice and mice with L1210 leukemia. *JNCI* 1959; 23:1347–1357.
33. Venditti JM, Humphreys SR, Goldin A. The effectiveness of cytoxan against mouse leukemia L1210 and resistant sublines. *Cancer Chemother Rep* 1959; 3:6–8.
34. Venditti JM, Humphreys SR, Goldin A. Investigation of the activity of cytoxan against leukemia L1210 in mice. *Cancer Res* 1959; 19:986–995.
35. Gross R, Lambers K. Erste erfahrungen in der behandlung maligner tumoren mit einem neuen N-lost-phosphamidester. *Dtsch Med Wochenschrft* 1958; 83:458–462.
36. Coggins PR, Ravdin RG, Eisman SH. Clinical pharmacology and preliminary evaluation of cytoxan (cyclophosphamide). *Cancer Chemother Rep* 1959; 3:9–11.
37. Foye LV Jr, Chapman CG, Willet FM, Adams WS. Cyclophosphamide a preliminary study of a new alkylating agent. *Cancer Chemother Rep* 1960; 6:39,40.
38. Haar H, Marshall GJ, Bierman H, Steinfeld JL. The influence of cyclophosphamide upon neoplastic diseases in man. *Cancer Chemother Rep* 1960; 6:41–51.
39. Bethall FH, Louis J, Robbins A, et al. Phase II evaluation of cyclophosphamide a study by the midwest cooperative chemotherapy group. *Cancer Chemother Rep* 1960; 6:112–115.

40. Bergsagel DE, Levin WC. A prelusive clinical trial of cyclophosphamide. *Cancer Chemother Rep* 1960; 6:120–134.
41. Korst DR, Johnson FD, Frenkel EP, Challener III WL. Preliminary evaluation of the effect of cyclophosphamide on the course of human neoplasms. *Cancer Chemother Rep* 1960; 7:1–12.
42. Nissen-Meyer R, Hoest H. A comparison between the hematologic side effects of cyclophosphamide and nitrogen mustard. *Cancer Chemother Rep* 1960; 9:51–55.
43. Hoest H, Nissen-Meyer R. A preliminary clinical study of cyclophosphamide. *Cancer Chemother Rep* 1960; 9:47–50.
44. Papac R, Petrakis NL, Amini F, Wood DA. Comparative clinical evaluation of two alkylating agents mannitol mustard and cyclophosphamide (cytoxan). *JAMA* 1960; 172:1387–1391.
45. Sladek NE. Metabolism and pharmacokinetic behaviour of cyclophosphamide and related oxazaphosphorines. In: Powis G, ed. *Anticancer Drugs, Reactive Metabolism and Drug Interactions.* Newcastle upon Tyne: Pergamon. 1955:79–156.
46. Colvin M. Cyclophosphamide and analogues. In: Crooke ST, Prestayko AW, eds. *Cancer and Chemotherapy,* vol. III. New York: Academic. 1981:25–36.
47. Bolt W, Ritzl F, Toussaint R, Nahrmann H. Verteilung und ausscheidung eines cytostatisch wirkenden, mit tritium markierted N-lost derivatives beim krebskranken menschen. *Arzneim-Forsch* 1961; 11:170–175.
48. Draeger U, Hohorst H-J. Permeation of cyclophosphamide (NSC-26271) metabolites into tumor cells. *Cancer Chemother Rep* 1976; 60:423–427.
49. Arnold H, Bourseaux F, Brock N. Chemotherapeutic action of a cyclic nitrogen mustard phosphoramide ester (B 518-ASTA) in experimental tumors of the rat. *Nature* 1958; 181:931.
50. Foley GE, Friedman OM, Drolet BP. Studies on the mechanism of action of cytoxan. Evidence of activation in vivo and in vitro. *Cancer Res* 1961; 21:57–63.
51. Brock N, Hohorst H-J. Über die aktivierung von cyclophosphamide in vivo und in vitro. *Arzneim-Forsch* 1963; 13:1021–1031.
52. Norpoth K. Untersuchungen zur oxidativen umzetzungen von endoxan® *in vivo* und *in vitro* [Habilitationsschrift]. Münster (Westf.):Fach Physiologische Chemie, Westfälischen Wilhelms Universität, 1969.
53. Norpoth K, Golovinsky E, Rauen HM. Untersuchungen an hypothetischen metaboliten des 2-[bis(2-chloroäthyl)amino]tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxids (cyclophosphamid). *Z Physiol Chem* 1970; 351:377–383.
54. Alarcon RA, Meienhofer J. Formation of the cytotoxic aldehyde acrolein during in vitro degradation of cyclophosphamide. *Nature New Biol* 1971; 233:250–252.
55. Sladek NE. Therapeutic efficacy of cyclophosphamide as a function of its metabolism. *Cancer Res* 1972; 32:535–542.
56. Hill DL, Kirk MC, Struck RF. Isolation and identification of 4-ketocyclophosphamide, a possible active form of the antitumor agent cyclophosphamide. *J Am Chem Soc* 1970; 92: 3207,3208.
57. Rakke JE, Feil VJ, Zaylskie RG. Characterization of the major sheep urinary metabolites of cyclophosphamide, a defleecing chemical. *J Agric Food Chem* 1971; 19:788–790.
58. Hohorst H-J, Ziemann A, Brock N. 4-Ketocyclophosphamide, a metabolite of cyclophosphamide. *Artzneim-Forsch* 1971; 21:1254–1257.
59. Struck RF, Kirk MC, Mellett LB, El Dareer S, Hill DL. Urinary metabolites of the antitumor agent cyclophosphamide. *Mol Pharmacol* 1971; 7:519–529.
60. Sladek N. Evidence for an aldehyde possessing alkylating activity as the primary metabolite of cyclophosphamide. *Cancer Res* 1973; 33:651–658.
61. Struck RF. Isolation and identification of a stabilized derivative of aldophosphamide, a major metabolite of cyclophosphamide. *Cancer Res* 1974; 34:2933–2935.
62. Colvin M, Padgett CA, Fenselau C. A biologically active metabolite of cyclophosphamide. *Cancer Res* 1973; 33:915–918.
63. Friedman OM, Boger E, Grubliauskas V, Somer H. Synthesis of *N*-phosphorylated derivatives of nitrogen mustards with latent cytotoxicity. *J Med Chem* 1963; 6:50–58.
64. Connors TA, Cox PJ, Farmer PB, Foster AB, Jarman M. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide.

Biochem Pharmacol 1974; 23:115–129.
65. Borch RF, Valente RR. Synthesis, activation and cytotoxicity of aldophosphamide analogues. *J Med Chem* 1991; 34:3052–3058.
66. Clarke L, Waxman DJ. Oxidative metabolism of cyclophosphamide: identification of the hepatic monooxegenase catalysts of drug activation. *Cancer Res* 1989; 49:2344–2350.
67. Zon G, Ludeman SM, Brandt JA, et al. Nmr spectroscopic studies of intermediary metabolites of cyclophosphamide. A comprehensive kinetic analysis of the interconversion of *cis*- and *trans*-4-hydroxycyclophosphamide with aldophosphamide and the concomitant partitioning of aldophosphamide between irreversible fragmentation and reversible conjugation pathways. *J Med Chem* 1984; 27:466–485.
68. Feneslau C, Lehman JP, Myles A, et al. Iminocyclophosphamide as a chemically reactive metabolite of cyclophosphamide. *Drug Metab Dispos* 1982; 10:636–640.
70. Dockham PA, Lee M-O, Sladek NE. Identification of the human liver aldehyde dehydrogenases that catalyze the oxidation of aldophosphamide and retinaldehyde. *Biochem Pharmacol* 1992; 43:2453–2469.
71. Cherif A, Farquhar D. *N*-(5,5-diacetoxypent-1-yl)doxorubicin: a new intensely potent doxorubicine analogue. *J Med Chem* 1992; 35:3208–3214.
72. Arnodl H, Bourseaux F, Brock N. Über beziehungen zwischen chemischer konstitution und cancertoxischer wirkungen in der reihe der phosphamidester des bis-(β-chloräthyl)-amins. *Arzneim-Forsch* 1961; 11:143–158.
73. Goldstein A, Arownow L, Kalman SM. *Principles of Drug Action.* New York: Harper & Row. 1969:351.
74. Ishidate M, Kobayashi K, Sakurai Y, Sato H, Yoshida T. *Proc Japan Acad* 1951; 27:493–500.
75. Skipper H E, Schmidt LH. A manual on quantitative drug evaluation in experimental tumor systems. Part I. Background, description of criteria, and presentation of quantitative therapeutic data on various classes on drugs obtained in diverse experimental tumor systems. *Cancer Chemother Rep* 1962; 17:1–143.
76. Goldin A, Venditti JM. A manual on quantitative drug evaluation in experimental tumor systems. Part II. Quantitative assessment of various classes of agents employing advanced leukemia L1210 in mice. *Cancer Chemother Rep* 1962; 17:145–178.
77. Reinhoudt DN, Connors TA, Pinedo HM, van den Poll KW, eds. *Structure-Activity Relationships of Anti-tumor Agents.* The Hague: Martinius Nijhoff Publishers, 1983.
78. Preussmann R. Chlor-abspaltbarkeit und wirksamkeit von N-lost-verbindungen. *Arzneim-Forsch* 1958; 8:9,10.
79. Zon G. Cyclophosphamide analogues. In: Ellis GP, West GB, eds. *Progress in Medicinal Chemistry.* Amsterdam: Elsevier Biochemical. 1982:205–246.
80. Cox PJ, Farmer PB, Foster AB, et al. Application of deuterium labelling mass spectrometry in a study of the metabolism of the enantiomers of cyclophosphamide. *Biomed Mass Spectrom* 1977; 4:371–375.
81. Farmer PB. Enantiomers of cyclophosphamide and ifosfamide. *Biochem Pharmacol* 1988; 37:145–148.
82. Holm KA, Kindberg CG, Stobaugh JF, Slavik M, Riley CM. Stereoselective pharmacokinetics and metabolism of the enantiomers of cyclophosphamide. *Biochem Pharmacol* 1990; 39: 1375–1384.
83. Burkert H. Ifosfamide: European perspective. *Semin Oncol* 1982; 9(suppl 1):28–35.
84. Arnold H. Über die chemie neuer zytostatisch wirksamer N-chlor-aethyl-phosphorsäuresterdiamide. Vortrag auf dem V. Internat. Kongreb für Chemotherapie; 1967 Jun 26–Jul 1; Wien. Verlag der Wiener Medizinische Akademie Abstr A IV-1a/5.
85. Brock N. Nouveau esters phosphamidés de moutarde azotée et leur activité cytostatique. *Laval Médical* 1968; 39:696–701.
86. Brock N. The oxazaphosphorines. *Cancer Treatment Rev* 1983; 10:3–15.
87. Venditti JM. Treatment schedule dependency of experimentally active antileukemic (L1210) drugs. *Cancer Chemother Rep* (Part 3) 1971; 2:35–59.
88. Goldin A. Ifosfamide in experimental tumor systems. *Semin Oncol* 1982; 9(suppl 1):14–23.
89. Zalupski M, Baker LH. Ifosfamide. *JNCI* 1988; 80:556–566.

90. Barnett D. Preclinical toxicology of ifosfamide. *Semin Oncol* 1982; 9(suppl 1):8-13.
91. Drings P, Allner R, Brock N, et al. Erfahrungen mit neuartigen N-lost-phosphoramidestern. *Dtsch Med Wochenschrft* 1970; 95:491-497.
92. van Dyk JJ, Falkson HC, van der Merwe. Unexpected toxicity in patients treated with iphosphamide. *Cancer Res* 1972; 32:921-924.
93. Munshi NC, Loehrer PJ, Williams SD. Uroprotection in patients receiving cyclophosphamide and ifosfamide. In: Muggia FM, ed. *New Drugs, Concepts and results in Cancer Chemotherapy.* Boston: Kluwer Academic Publishers. 1992:119-126.
94. Drings P, Fritsch H. Ifosfamide in higher single doses for treatment of metastasized solid tumors. *Veh Deut Ges Inn Med* 1972; 78:166-169.
95. Schnitker J., Brock N, Burkert H, Fichter E. Evaluation of a cooperative clincal study of the cytostatic agent ifosfamide. *Arzneim-Forsch* 1976; 26:1783-1793.
96. Hoefer-Janker H, Scheef W, Gunther W, Hüls W. Erfahrungen mit der fraktionierten ifosfamide-stoβtherapie bei generalisierten malignen tumoren. *Med Welt* 1975; 26:972-979.
97. Brade WP, Herdrich K, Varini M. Ifosfamide, pharmacology, safety and therapeutic potential. *Cancer Treatment Rev* 1985; 12:1-47.
98. Pohl J. Stekar J, Hilgard P. Chloroacetaldehyde and its contribution to urotoxicity during treatment with cyclophosphamide or ifosfamide. *Arzneim-Forsch* 1989; 39:704,705.
99. Scheef W, Klein HO, Burkert H, et al. Controlled clinical studies with an anitidote against the urotoxicity of oxazaphosphorines: preliminary results. *Cancer Treatment Rep* 1979; 63: 501-505.
100. Brock N, Pohl J, Stekar J. Studies on the urotoxicity of oxazaphosphorine cytostatics and its prevention. 2. Comparative study on the uroprotective efficacy of thiols and other sulfur compounds. *Eur J Cancer* 1981; 17:1155-1163.
101. Colvin M. The comparative pharmacology of cyclophosphamide and ifosfamide. *Semin Oncol* 1982; 9(suppl 1):2-7.
102. Brock N, Pohl J. The development of mesna for regional detoxification. *Cancer Treatment Rev* 1983:33-43.
103. Crawford J, George M. The role of hematopoietic growth factors in support of ifosfamide/carboplatin/etoposide chemotherapy. *Semin Oncol* 1995; 22(suppl 7):18-22.
104. Elias AD, Ayash LJ, Wheeler C, et al. High-dose ifosfamide/carboplatin/etoposide with autologous hematopoietic stem cell support: safety and future directions. *Semin Oncol* 1994; 21 (suppl 12):83-85.
105. Dechant KL, Brogden RN, Pilkington T, Faulds D, Ifosfamide/Mesna. A review of its antineoplastic activity, pharmacokinetic properties and therapeutic efficacy in cancer. *Drugs* 1991; 42:428-467.
106. Freireich EJ, Bodey GP. Evaluating new agents: planning, execution and evaluation of clinical studies. In: Kuemmerle HP, ed. Berkarda B, Karrer K, Mathé G, coeds. *Clinical Chemotherapy,* vol. III, *Antineoplastic Chemotherapy.* New York: Thieme-Stratton. 1984:115-138.
107. Stuart-Harris R, Harper PG, Kaye SB, Wiltshaw E. High-dose ifosfamide by infusion with mesna in advanced soft tissue sarcoma. *Cancer Treatment Rev* 1983; 10(suppl A):163,164.
108. Keizer HJ, Ouwerkerk J, Welvaart K, van der Velde CJH, Cleton FJ. Ifosfamide treatment as a 10-day continuous intravenous infusion. *J Cancer Res Clin Oncol* 1995; 121:297-302.
109. Lewis LD. Ifosfamide pharmacokinetics. *Invest New Drugs* 1991; 9:305-311.
110. Le Cesne A, Antoine E, Spielmann M, et al. High-dose ifosfamide: circumvention of resistance to standard dose ifosfamide in advanced soft tissue sarcomas. *J Clin Oncol* 1995; 13: 1600-1608.
111. Küpfer A, Aeschlimann C, Wermuth B, Cerny T. Prophylaxis and reversal of ifosfamide encencepalopathy with methylene blue. *Lancet* 1994; 343:763,764.
112. Zullian GB, Tullen E, Maton B. Methylene blue for ifosfamide associated encepalopathy. *New Eng J. Med* 1995; 332: 1239,1240.
113. Fields KF, Elfenbein GJ, Lazarus HM, et al. Maximum tolerated doses of ifosfamide, carboplatin, and etoposide given over 6 days followed by autologous stem-cell rescue: toxicity profile. *J Clin Oncol* 1995; 13:323-332.
114. Sledge GW, Antman KH. Progress in chemotherapy for metastatic breast cancer. *Semin Oncol* 1992; 19:317-332.

115. Wingo PA, Tong T, Bolden S. Cancer statistics, 1995. *CA* 1995; 45:8-30.
116. Henwood JM, Brogden RN. Etoposide: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in combination chemotherapy of cancer. *Drugs* 1990; 30:438-490.
117. Ettinger DS. The place of ifosfamide in chemotherapy of small cell lung cancer: The eastern oncology group experience and a selected literature update. *Semin Oncol* 1995; 22(suppl 2): 23-27.
118. Warenius HM, Hurman DC, Cottier B. High-dose ifosfamide in small cell lung cancer [letter]. *Br J Cancer* 1986; 54:216.
119. Hanson HH. Management of small cell cancer of the lung. *Lancet* 1992; 339:846-849.
120. Nichols CR. The role of ifosfamide in germ cell tumors and non-small cell lung cancer. *Semin Oncol* 1995; 22(suppl 7):13-17.
121. Miller VA, Rigas JR, Grant SC, Pisters KMW, Kris MG. New chemotherapeutic agents for non-small cell lung cancer. *Chest* 1995; 107:306S-311S.
122. Gomm SA, Thatcher N, Cuthbert A, et al. High dose combination chemotherapy with ifosfamide, cyclophosphamide or cisplatin, mitomycin C and mustine with autologous bone marrow support in advanced non-small cell lung cancer. A phase I/II study. *Br J Cancer* 1991; 63: 293-297.
123. Pujol J-L, Rossi J-F, Le Chevalier T, et al. Pilot study of neoadjuvant ifosfamide, cisplatin, and etoposide in locally advanced non-small cell lung cancer. *Eur. J Cancer* 1990; 26:798-801.
124. Miller BA, Feuer EJ, Hankey BF. The significance of the rising incidence of breast cancer in the United States. In: DeVita VT, Hellman S, and Rosenberg SA, eds. *Important Advances in Oncology 1994.* Philadelphia: J.B. Lippencott. 1994:193-207.
125. Ayash LJ, Wheeler C, Fairclough D, et al. Prognostic factors for prolonged progression-free survival with high-dose chemotherapy with autologous stem cell support for advanced breast cancer. *J Clin Oncol* 1995; 13:2043-2049.
126. Sanchiz F, Milla A. High-dose ifosfamide and mesna in advanced breast cancer. A phase II study. *Cancer Chemother Pharm* 1990; 26(suppl):S91,S92.
127. Ghavamzadeh A. Treatment of metastatic breast cancer with the combination of ifosfamide, epirubicin and fluorouracil. *Cancer Chemother Pharm* 1990; 26(suppl):S66-S68.
128. McGuire, Rowinsky EK. Old drugs revisited, new drugs, and experimental approaches in ovarian cancer therapy. *Semin Oncol* 1991; 18:255-269.
129. Sutton GP, Blessing JA, Photopulos G, Berman ML, Homesley HD. Gynecologic oncology group experience with ifosfamide. *Semin Oncol* 1990; 17(suppl 4):6-10.
130. Motzer RJ, Cooper K, Geller NL, et al. The role of ifosfamide plus cisplatin-based chemotherapy as salvage therapy for patients with refractory germ cell tumors. *Cancer* 1990; 66:2476-2481.
131. Einhorn LH. Testicular cancer as a model for curable neoplasm: the Richard and Hilda Rosenthal Foundation award lecture. *Cancer Res* 1981; 41:3275-3280.
132. Clemm C, Hartenstein R, Willich N, Ledderose G, Wilmanns W. Combination chemotherapy with vinblastine, ifosfamide and cisplatin in bulky seminoma. *Acta Oncol* 1989; 28:231-235.
133. von Kalle A-K, Schaadt M, Diehl V. European experience with ifosfamide in lymphomas. *Semin Oncol* 1989; 16(suppl 3): 73-77.
134. Cabanillas F. Experience with ifosfamide combinations in malignant lymphomas. *Semin Oncol* 1989; 16(suppl 3):78-81.
135. Enblad G, Glimelius B, Hagberg H, Hindemalm C. Methyl-GAG, ifosfamide, methotrexate and etoposide (MIME) as salvage therapy for Hodgkin's disease and non-Hodgkin's lymphoma. *Acta Oncol* 1990; 29:297-301.
136. Elias A, Ryan L, Aisner J, Antman KH. Mesna, doxorubicin, ifosfamide, dacarbazine (MAID) regimen for adults with advanced sarcoma. *Semin Oncol* 1990; 17(suppl 4):41-49.
137. Cairo MS. The use of ifosfamide, carboplatin and etoposide in children with solid tumors. *Semin Oncol* 1995; 22(suppl 7):23-27.
138. Takamizawa A, Matsumoto S, Iwata T, et al. Synthesis and metabolic behavior of the suggested active species of isophosphamide having cytostatic activity. *J Med Chem* 1974; 17: 1237-1239.
139. Takamizawa A, Iwata T, Yamaguchi K, et al. Stereochemistry, metabolism, and antitumor

activity of 4-hydroperoxyisophosphamide (NSC-227114) and its stereoisomer. *Cancer Treatment Rep* 1976; 60:361–368.
140. Boal JH, Williamson M, Boyd V, Ludeman SM, Egan W. 31P nmr studies of the kinetics of bisalkylation by isophosphoramide mustard: comparisons with phosphoramide mustard. *J Med Chem* 1989; 32:1768–1773.
141. Chang TKH, Weber GF, Crespi CL, Waxman DJ. Differential activation of cyclophosphamide and ifosfamide by cytochromes P-450 2B and 3A in human liver microsomes. *Cancer Res* 1993:5629–5637.
142. Walker D, Flinois J-P, Monkman S, et al. Identification of the major human hepatic cytochrome P-450 involved in inactivation and *N*-dechloroethylation of ifosfamide. *Biochem Pharmacol* 1994; 47:1157–1163.
143. Boddy AV, Idle JR. Combined thin-layer chromatography-photography-densitometry for the quantification of ifosfamide and its principal metabolites in urine, cerebrospinal fluid and plasma. *J Chromatogr* 1992; 575:137–142.
144. Norpoth K. Studies on the metabolism of ifosfamide (NSC-109724) in man. *Cancer Treatment Rep* 1976; 4:437–443.
145. Kamen BA, Frenkel E, Colvin OM. Ifosfamide: should the honeymoon be over? [letter] *J Clin Oncol* 1995; 13:307–309.
146. Goren MP, Wright RK, Pratt CB, Pell FE. Dechloroethylation of ifosfamide and neurotoxicity. *Lancet* 1986; 2:1219,1220.
147. Skinner R, Sharkey IM, Pearson ADJ, Craft AW. Ifosfamide, mesna and nephrotoxicity in children. *J. Clin Oncol* 1993; 11:173–190.
148. Zamlauski-Tucker MJ, Morris ME, Springate JE. Ifosfamide metabolite chloroacetaldehyde causes fanconi syndrome in the perfused rat kidney. *Toxicol Appl Pharmacol* 1994; 129: 170–175.
149. Low JE, Borch RF, Sladek NE. Further studies on the conversion of 4-hydroxyoxazaphosphorines to reactive mustards and acrolein in inorganic buffers. *Cancer Res* 1983; 43: 5815–5820.
150. Hohorst HJ, Bielicki L, Voelcker G. The enzymatic basis of cyclophosphamide specificity. *Adv Enzyme Reg* 1986; 25:99–122.
151. Alarcon RA, Meienhofer J, Atherton E. Isophosphamide as a new acrolein-producing antineoplastic isomer of cyclophosphamide. *Cancer Res* 1972; 32:2519–2523.
152. Gibaldi M, Perrier D. *Pharmacokinetics.* 2nd ed. New York: Marcel Dekker, 1982.
153. Pantarotto C, Martini A, Belvedere G, Donelli MG, Frigerio A. Studies on the disposition of cyclophosphamide (NSC-26271) in tumor bearing mice by means of gas chromatography-chemical ionization-mass fragmentography. *Cancer Treatment Rep* 1976; 60:493–500.
154. Juma FD, Rogers HJ, Trounce JR. The pharmacokinetics of cyclophosphamide, phosphoramide mustard and nor-nitrogen mustard studied by gas chromatography in patients receiving cyclophosphamide therapy. *Br J. Clin Pharmacol* 1980; 10:327–335.
155. Kurowski V, Wagner T. Comparative pharmacokinetics of ifosfamide, 4-hydroxyifosfamide, chloroacetaldehyde and 2- and 3-dechloroethylifosfamide in patients on fractionated intravenous ifosfamide therapy. *Cancer Chemother Pharm* 1993; 33:36–42.
156. Masurel D, Wainer IW. Analytical and preparative high-performance liquid chromatographic separation of the enantiomers of ifosfamide, cyclophosphamide and trofosfamide and their determination in plasma. *J Chromatogr* 1989; 490:133–143.
157. Granvil CP, Gehrcke B, Konig WA, Wainer IW. The determination of the enantiomers of ifosfamide and its 2- and 3-*N*-dechloroethylated metabolites in plasma and urine using enantioselective gas chromatography with mass spectrometric detection. *J. Chromatogr* 1993; 622: 21–31.
158. Wright JE, Tetyakov O, Ayash L, Elias A, Rosowsky A, Frei E III. Analysis of 4-hydroxycyclophosphamide in human blood. *Anal Biochem* 1995; 224:154–158.
159. Anderson LW, Ludeman SM, Colvin OM, et al. Quantitation of 4-hydroxycyclophosphamide/aldophosphamide in whole blood. *J Chromatrogr* 1995; 667:247–257.
160. Chan KK, Hong PS, Tutsch K, Trump DL. Clinical pharmacokinetics of cyclophosphamide and metabolites with and without SR-2508. *Cancer Res* 1994; 54:6421–6429.
161. Bryant BM, Jarman M, Baker MH, Smith IE, Smyth JF. Quantification by gas chromatog-

raphy on *N,N'*-di-(2-chloroethyl)-phosphoramidic acid in the plasma of patients receiving isophosphamide. *Cancer Res* 1980; 40:4734–4738.
162. Kurowski V, Cerny T, Küpfer A, Wagner T. Metabolism and pharmacokinetics of oral and intravenous ifosfamide. *J Cancer Res Clin Oncol* 1991; 117(suppl IV):S148–S153.
163. Kaijser GP, Beijnen JH, Jeunink EL, et al. Determination of chloroacetaldehyde, a metabolite of oxazaphosphorine cytostatic drugs, in plasma. *J Chromatogr* 1993; 614:253–259.
164. Moore MJ. Clinical pharmacokinetics of cyclophosphamide. *Drug Dispos* 1991; 20:194–208.
165. Kaijser GP, Beijnen JH, Bult A, Underberg WJM. Ifosfamide metabolism and pharmacokinetics. *Anticancer Res* 1994; 14:517–532.
166. Lind MJ, Margison JM, Cerny T, Thatcher N, Wilkinson PM. Comparative pharmacokinetics and alkylating activity of fractionated intravenous and oral ifosfamide in patients with bronchogenic-carcinoma. *Cancer Res* 1989; 49:753–757.
167. Lewis LD. Ifosfamide pharmacokinetics. *Invest New Drugs* 1991; 9:305–311.
168. Lind MJ, Ardiet C. Pharmacokinetics of alkylating agents. *Cancer Surveys* 1993; 17:157–188.
169. Allen LM, Creaven PJ, Nelson RL. Studies on the human pharmacokinetics of ifosfamide (NSC-109724). *Cancer Treatment Rep* 1976; 60:451–458.
170. Thomas Wagner, Ifosfamide clinical pharmacokinetics. *Clin Pharmacokinet* 1994; 26: 439–456.
171. Boos J, Welslau W, Ritter J, Blaschke G, Schellong G. Ifosfamid und die metabolite der seitenkettenoxidation-ausscheidung im urine bei verschiedenen pädiatrischen therapieprotokollen. *Klin Pädiatr* 1992; 204:299–305.
172. Grever MR, Schepartz SA, Chabner BA. The National Cancer Institute: cancer drug discovery and development program. *Semin Oncol* 1992; 19:622–638.
173. Brock N. Oxazaphosphorine cytostatics: structure-activity relationships. Selectivity and metabolism, regional detoxification. In: Reinhoudt DN, Connors TA, Pinedo HM, van den Poll KW, eds. *Structure–Activity Relationships of Anti-Tumor Agents.* The Hague: Martinius Nijhoff Publishers. 1983:239–267.
174. Chen L, Waxman DJ. Intratumoral activation and enhanced chemotherapeutic effect of oxazaphosphorines following cytochrome P-450 gene transfer: development of a combined chemotherapy/cancer gene therapy strategy. *Cancer Res* 1995; 55:581–589.
175. Abel G, Cox PJ, Farmer PB, et al. Isolation and identification of a metabolic intermediate in the selective dechloroethylation of one of the four stereoisomers of 4-methylcyclophosphamide. *Cancer Res* 1978; 38:2592–2599.
176. Kutscher B, Niemeyer U, Engel J, et al. Synthesis and antitumor activity of two ifosfamide analogs with a five-membered ring. *Arzneim-Forsch* 1995; 45:323–326.
177. Foster AB, Jarman M, Kinas RW, et al. 5-Fluoro- and 5-chlorocyclophosphamide: synthesis, metabolism, and antitumor activity of the cis and trans isomers. *J Med Chem* 1981; 24: 1399–1403.
178. Ayash LJ, Wright JE, Tretyakov O, et al. Cyclophosphamide pharmacokinetics: Correlation with cardiac toxicity and tumor response. *J. Clin Oncol* 1992; 10:995–1000.
179. Farmer PB, Cox PJ. Synthesis and antitumor activity of 6-trifluoromethylcyclo-phosphamide and related compounds. *J Med Chem* 1975; 18:1106–1110.
180. Ludeman SM, Boyd VL, Regan JB, Gallo KA, Zon G, Ishii K. Synthesis and antitumor activity of cyclophosphamide analogues. 4. Preparation, kinetic studies, and anticancer screening of "phenylketophosphamide" and similar compounds related to the cyclophosphamide metabolite aldophosphamide. *J Med Chem* 1986; 29:716–727.
181. Stec WJ. Cyclophosphamide and its congeners. *J Organophosphorus Chem* 1982; 13:145–174.
182. Montgomery JA, Struck RF. Synthesis and structure-activity relationships of pre-activated analogs of cyclophosphamide (NSC-26271). *Cancer Treatment Rep* 1976; 60:381–393.
183. Takamizawa A, Matsumoto S, Iwata T, et al. Studies on cyclophosphamide metabolites and their related compounds. 2. Preparation of an active species of cyclophosphamide and related compounds. *J Med Chem* 1975; 18:376–383.
184. Takamizawa A, Matsumoto S, Iwata T, et al. Synthesis and metabolic behavior of the suggested active species of isophosphamide having cytostatic activity. *J Am Chem Soc* 1973; 95:985,986.
185. Kubota T, Hanatani YH, Tsuyuki K, et al. Antitumor effect and metabolic activation of cyclophosphamide and 4-hydroperoxycyclophosphamide in the human breast carcinoma (MX-1)-nude mouse system. *Gann* 1983; 74:437–444.

186. Jones RJ. Purging with 4-hydroperoxycyclophosphamide [review]. *J Hematother* 1992; 1: 343-348.
187. Peter G, Wagner T, Hohorst H-J. Studies on 4-hydroxycyclophosphamide (NSC-18185): a simple preparation method and its application for the synthesis of a new class of "activated" sulfur-containing cyclophosphamide (NSC-26271) derivatives. *Cancer Treatment Rep* 1976; 60:429-435.
188. Niemeyer U, Engel J, Scheffler G, et al. Chemical characterization of ASTA Z 7557 (INN mafosfamide, CIS-4-sulfidoethylthio cyclophosphamide), a stable derivative of 4-hydroxycyclophosphamide. *Invest New Drugs* 1984; 2:133-139.
189. Kwon C-H, Borch RF, Engel J, Niemeyer U. Activation mechanisms of mafosfamide and the role of thiols in cyclophosphamide metabolism. *J Med Chem* 1987; 30:395-399.
190. Klein HO, Wickramanye PD, Christian E, Coerper C. Preclinical evaluation of toxicity and therapeutic effacy of a stabilized cytostatic metabolite of cyclophosphamide (ASTA Z 7557, INN mafosfamide). *Invest New Drugs* 1984; 2:191-199.
191. Abele R, Aapro MS, Haeflinger JM, Alberto P. Phase I study of cyclohexylamine and lysine salt of mafosfamide. *Cancer Chemother Pharm* 1986; 16:182,183.
192. Bruntsch U, Groos G, Hiller TA, Wandt, Tigges FJ. Phase-I study of mafosfamide-cyclohexylamine (ASTA-Z-7557, NSC 345 842) and limited phase-I data on mafosfamide-lysine. *Invest New Drugs* 1985; 3:293-296.
193. Laporte JP, Douay L, Lopez M, et al. One hundred twenty-five adult patients with primary acute leukemia autografted with marrow purged by mafosfamide: a 10-year single institutional experience. *Blood* 1994; 84:3810-3818.
194. Kwon C-H, Borch RF. Effects of *N*-substitution on the activation mechanisms of 4-hydroxycyclophosphamide analogues. *J Med Chem* 1989; 32:1491-1496.
195. Wang Y, Farquhar D. Aldophosphamide acetal diacetate and structural analogues: synthesis and cytotoxicity studies. *J Med Chem* 1991; 34:197-203.
196. Beran M, Borje SA, Wang Y, McCredie KB, Farquhar D. The effects of acetaldophosphamide, a novel stable aldophosphamide analogue, on normal human and leukemic progenitor cells in vitro: implications for use in bone marrow purging. *Cancer Res* 1988; 48:339-345.
197. Andersson BS, Wang Y-Q, McCredie KB, Farquhar D. Suitability of a new stable acetal analogue of aldoifosfamide for purging leukemic cells from human bone marrow. *Leukemia* 1990; 4:435-440.
198. Borch RF, Valente RR. Synthesis, activation and cytotoxicity of aldophosphamide analogues. *J Med Chem* 1991; 34:3052-3058.
199. Sladek NE, Dockham PA, Lee M-O. Human and mouse hepatic aldehyde dehydrogenases important in the biotransformation of cyclophosphamide and the retinoids. *Adv Exp Med Biol* 1990; 284:97-104.
200. Sahovic E, Colvin M, Hilton L, Ogawa M. Role for aldehyde dehydrogenase in survival of progenitors for murine blast cell colonies after treatment with 4-hydroperoxycyclophosphamide in vitro. *Cancer Res* 1988; 48:1223-1226.
201. Friedman OM, Wodinsky I, Myles A. Cyclophosphamide (NSC-26271)-related phosphoramide mustards-recent advances and historical perspective. *Cancer Treatment Rep* 1976; 60:337-346.
202. Boyd VL, Robbins JD, Egan W, Ludeman SM. ^{31}P Nuclear magnetic resonance spectroscopic observation of the intracellular transformations of oncostatic cyclophosphamide metabolites. *J Med Chem* 1986; 29:1206-1210.
203. Lenssen U, Hohorst HJ. Zur frage der permiabilität von *N,N*-bis(2-chloräthyl)-phosphorsäurediamid in tumorzellen. *J Cancer Res Clin Oncol* 1979; 93:161-164.
204. Sonawat H, Liebfritz D, Engel J. Hilgard P. Biotransformation of mafosfamide in P388 mice leukemia cells: intracellular ^{31}P-nmr studies. *Biochim Biophys Acta* 1990; 1052:36-41.
205. Struck RF, Schmid SM, Waud WR. Antitumor activity of halogen analogs of phosphoramide, isophosphoramide and triphosphoramide mustards, and cytotoxic metabolites of cyclophosphamide, ifosfamide, and trofosfamide. *Cancer Chemother Pharm* 1994; 34:191-196.
206. Yuan Z-M, Smith PB, Brundrett RB, Colvin M, Fenselau C. Glutathione conjugation with phosphoramide mustard with cyclophosphamide. A mechanistic study using tandem mass spectrometry. *Drug Metab Dispos* 1991; 19:625-629.
207. Dirven HAAM, van Ommen B, van Bladeren PJ. Involvement of human glutathione S-trans-

ferase isozymes in the conjugation of cyclophosphamide metabolites with glutathione. *Cancer Res* 1994; 54:6215–6220.
208. Vaury C, Lane é R, Noguiez P, et al. Human glutathione S-transferase M1 null genotype is associated with a high inducibility of cytochrome P450 1A1 gene transcription. *Cancer Res* 1955; 55:5520–5523.
209. Meier T, Allenbacher A, Mueller E, et al. Ifosfamide induced depletion of glutathione in human peripheral blood lymphocytes and protection by mesna. *Anti-cancer Drugs* 1994; 5:403–409.
210. Lind MJ, McGown AT, Hadfield JA, Thatcher N, Crowther D, Fox BW. The effect of ifosfamide and its metabolites on intracellular glutathione levels *in vitro* and *in vivo*. *Biochem Pharmacol* 1989; 38:1835–1840.
211. Insittoris E, Tretter L, Gaal D. Severe depletion of cellular thiols and glutathione-related enzymes of a carmustine-resistant L-1210 strain associates with collateral sensitivity to cyclophosphamide. *Cancer Chemother Pharm* 1993; 33:85–88.
212. Pohl J, Bertram B, Hilgard P, Nowrousian MR, Stüben J, Weißler M. D-19575-A sugar-linked isophosphoramide mustard derivative exploiting transmembrane glucose transport. *Cancer Chemother Pharm* 1995; 35:364–370.
213. Coleman CN. Hypoxia in tumors: a paradigm for the approach to biochemical and physiological heterogeneity. *JNCI* 1988; 80:310–317.
214. Kennedy KA, Teicher BA, Rockwell S, Sartorelli AC. The hypoxic tumor cell: a target for selective cancer chemotherapy. *Biochem Pharmacol* 1980; 29:1–8.
215. Bickel MH. The pharmacology and biochemistry of N-oxides. *Pharmacol Rev* 1969; 21: 325–355.
216. Adams GE, Stratford IJ, Wallace RG, Wardman P, Watts. Toxicity of nitro compounds towards hypoxic mammalian cells in vitro: dependence on reduction potential. *JNCI* 1980; 64: 555–560.
217. Moore HW, Czerniak R. Naturally occurring quinones as potential bioreductive alkylating agents. *Med Res Rev* 1981; 1:249–280.
218. Begg AC, Shrieve DC, Smith KA, Terry NHA. Effects of hypoxia, pH and growth stage on cell killing in Chinese hamster V79 cells *in vitro* by activated cyclophosphamide. *Cancer Res* 1985; 45:3454–3459.
219. Grau C, Bentzen SM, Overgaard J. Cytotoxic effects of misonidazole and cyclophosphamide on aerobic and hypoxic cells in a C_3H mammary carcinoma. *Br J Cancer* 1990; 61:61–64.
220. Tannock I. Response of aerobic and hypoxic cells in a solid tumor to adriamycin and cyclophosphamide and interaction of the drugs with radiation. *Cancer Res* 1982; 42:4921–4926.
221. Hill RP, Stanley JA. The response of hypoxic B16 melanoma cells to in vivo treatment with chemotherapeutic agents. *Cancer Res* 1975; 35:1147–1153.
222. Firestone A, Mulcahy RT, Borch RF. Nitroheterocycle reduction as a paradigm for intramolecular catalysis of drug delivery to hypoxic cells. *J Med Chem* 1991; 34:2933–2935.
223. Mulcahy RT, Gipp JJ, Schmidt JP, Joswig C, Borch RP. Nitrobenzyl phosphorodiamidates as potential hypoxia-selective alkylating agents. *J Med Chem* 1994; 37:1610–1615.
224. Tietze LF, Neumann M. Möllers T, et al. Proton-mediated liberation of aldophosphamide from a nontoxic prodrug: a strategy for tumor selective activation of cytocydal drugs. *Cancer Res* 1989; 49:4179–4184.
225. Waxman DJ, Sundseth SS, Srivastava PK, Lapenson DP. Gene-specific oligonucleotide probes for alpha, mu, pi, and microsomal rat glutathine S-transferases: analysis of liver transferase expression and its modulation by hepatic enzyme inducers and platinum anticancer drugs. *Cancer Res* 1992; 52:5797–5802.
226. Antman KS, Griffin JD, Elias A, et al. Effect of recombinant human granulocyte-macrophage colony-stimulating factor on chemotherapy-induced myelosuppression. *New Engl J Med* 1988; 319:593–598.
227. Antman K, Eder JP, Frei E III. High-dose chemotherapy with bone marrow support for solid tumors [review]. *Imp Adv Oncol* 1987:221–235.
228. Wilcox WS. The last surviving cancer cell: the chances of killing it. *Cancer Chemother Rep* 1966; 50:541–542.
229. Frei E III. Curative cancer chemotherapy [review]. *Cancer Res* 1985; 45:6523–6537.

3 Development of the Nitrosoureas

David B. Ludlum, PhD, MD

CONTENTS

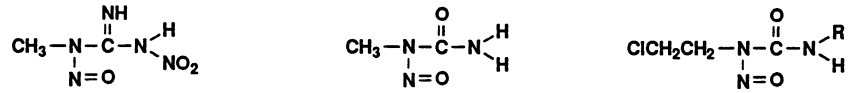
1. INTRODUCTION

Several of the most important principles in cancer drug development are illustrated by the discovery of the nitrosoureas as antitumor agents: the success of random screening in finding new agents; the importance of the assay system in evaluating new agents; the use of structure-activity relationships in developing more active agents when a lead compound is discovered; and the understanding and further developments that arise from mechanism of action studies. These contributions are all discussed in the following chapter. Additionally, since the nitrosoureas are still under active investigation, it is reasonable to hope that further advances in basic research may lead to additional improvements in the clinical use of these agents.

2. INITIAL DISCOVERY AND ANALOG SYNTHESIS

In the 1950s, after the alkylating agents had become established as useful chemotherapeutic agents, the National Cancer Institute organized a comprehensive screening program to discover new compounds with antitumor activity (*1*). Several animal tumors were investigated for use in the screening assay, and the L1210 murine leukemia line was chosen for much of the testing; literally thousands of synthetic and naturally occurring compounds were tested for activity against this tumor. In 1960, Greene and Greenberg reported that the synthetic compound, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), resulted in a moderate increase in the life-span of mice carrying intraperitoneal L1210 cells (*2*). As discussed in Section 3, the choice of this animal species for screening purposes probably favored the discovery of the nitrosoureas—the therapeutic index is high in rodents because these animals have high

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ



MNNG MNU CENU

Fig. 1. Structures of MNNG, MNU, and CENU. CENUs in clinical use in this country include: BCNU (R = chloroethyl), CCNU (R = cyclohexyl), and MeCCNU (R = methylcyclohexyl).

levels of a DNA repair enzyme, O^6-alkylguanine-DNA alkyltransferase, which protects normal cells from some of the DNA damage caused by the nitrosoureas.

Since MNNG represented a new class of compounds, the National Cancer Institute decided to investigate its activity further and to synthesize analogs of MNNG in an attempt to find more active derivatives. A synthetic program was started at the Southern Research Institute in Birmingham, AL, and new compounds were tested for activity against L1210 cells (*3,4*).

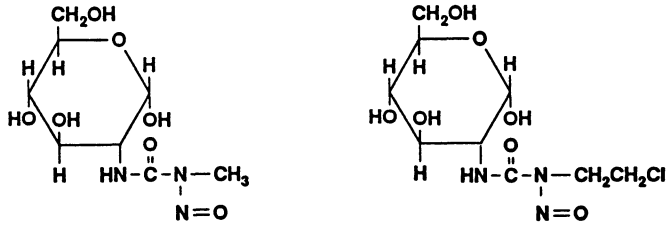
One of the first congeners tested, *N*-methyl-*N*-nitrosourea (MNU), whose structure is shown in Fig. 1, proved to be significantly more active against ip injected L1210 cells than MNNG (*5*). Even more importantly, given the scarcity of agents effective against central nervous system malignancies, this compound was active against intracerebrally implanted L1210 cells as well (*5*).

These findings led to a systematic study of the effects of varying the structure of MNU on the antitumor activity of this compound; *see* Fig. 1. Replacing the methyl group in the N-position with alkyl groups that did not contain a halogen atom had little effect on activity (*6*). However, replacing this group with a 2-chloroethyl or a 2-fluoroethyl group resulted in a major increase in activity (*3,4,6,7*). Compounds with a 2-bromoethyl group in this position were less active than the 2-chloroethyl derivatives, and compounds with a 2-iodoethyl group were inactive. 2-Chloroethyl derivatives were chosen for development over 2-fluoroethyl derivatives, because the latter can give rise to the toxic metabolite, 2-fluoroacetic acid (*4*).

The compound containing two 2-chloroethyl groups, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU), proved to be especially active; it cured mice that had been injected with a lethal dose of L1210 cells either intraperitoneally or intracerebrally (*7*). Ultimately, this compound was the first nitrosourea to be chosen for clinical trial.

With the advantage of 2-chloroethyl substitution at the N-position established, attention was paid to the effects of varying substituents at the N'-position. By varying the hydrophobicity of this group, it was possible to change the lipid solubility of the entire molecule. This was important because the activity of the nitrosoureas against intracerebrally implanted L1210 was attributed to their fat solubility allowing them to penetrate cell membranes by passive diffusion. A compound bearing the hydrophobic cyclohexyl group at the N'-position, *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (CCNU), was especially effective against intracerebrally implanted L1210 cells and was chosen for further development on this basis (*8*). Hansch and his colleagues established the relationship between lipid solubility and antitumor activity for a wide range of nitrosoureas (*9,10*).

An additional objective of the synthetic program was to find nitrosoureas that would be effective against solid tumors. To this end, nitrosoureas were tested for



STZ CLZ

Fig. 2. Structures of STZ and CLZ.

activity against a panel of solid animal tumors and human tumor xenografts (*11*). *N*-(2-chloroethyl)-*N'*-(trans-4-methylcyclohexyl)-*N*-nitrosourea (MeCCNU) proved to be more active than BCNU or CCNU against one of these, the Lewis lung carcinoma, and was chosen for further development for this reason (*11*).

At about the same time that the synthetic nitrosoureas were being developed, a naturally occurring methylnitrosourea derivative, streptozotocin (STZ), was also found to have antitumor activity (*12*). This interesting compound, whose structure is shown in Fig. 2, had less bone marrow toxicity than MNU. This observation led Montgomery and his colleagues to synthesize the chloroethyl analog of STZ, chlorozotocin (CLZ), whose structure is also shown in Fig. 2 (*13*).

3. INITIAL CLINICAL TRIALS

Because of their activity against animal tumors, clinical trials of the *N*-(2-chloroethyl)-*N'*-alkyl-*N*-nitrosoureas (CENUs) were begun with great enthusiasm. Especially encouraging were the results obtained in the treatment of brain tumors. In keeping with animal data showing that CENUs could cure mice that had been injected intracerebrally with L1210 cells, these agents had moderate activity against CNS neoplasms in humans (*14–16*). Both BCNU and CCNU were effective, but the more lipid-soluble CCNU had no clear superiority over BCNU. The responses were more modest than hoped for, but they were nevertheless definite, and the CENUs remain one of the few classes of drugs effective against gliomas and other CNS tumors.

The CENUs also had activity against Hodgkin's and non-Hodgkin's lymphoma, malignant melanoma, tumors of the gastrointestinal tract, and some other solid tumors (*17*), and are currently used in combination with other agents in the treatment of these malignancies (*18*). Nevertheless, it was disappointing that the CENUs were not more active in the clinic in view of their high activity in animal models (*19*). As described below, the probable explanation for this is that levels of the DNA repair enzyme, O^6-alkylguanine-DNA alkyltransferase, are higher in the normal tissues of rodents than in humans, thus enhancing the therapeutic ratio in the animal models that were used to evaluate the CENUs.

The naturally occurring nitrosourea, STZ, was also introduced into clinical trials in the 1970s. This compound had previously been shown to be diabetogenic in animals (*20*) and, consistent with that finding, had activity against islet cell carcinomas in humans (*21*). Furthermore, STZ was found to be less toxic to the bone marrow than

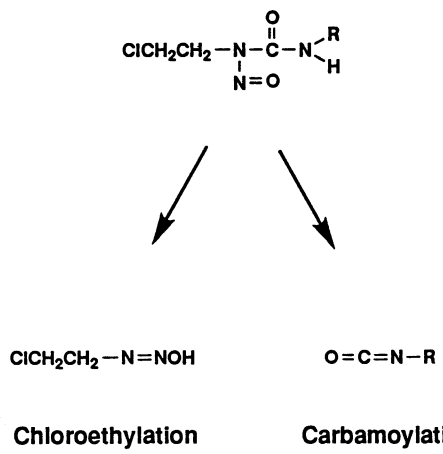


Fig. 3. Decomposition of a CENU. Chloroethylation results from the production of a chloroethylating species like chloroethyl diazonium hydroxide, and carbamoylation results from the production of an isocyanate.

the CENUs. As discussed below, the presence of the sugar moiety on STZ prevents protein carbamoylation by this compound, and it was anticipated that other nitrosoureas containing the same sugar would not be myelosuppressive. Unfortunately, however, the chloroethyl analog of STZ, chlorozotocin, was found to have bone marrow toxicity (*22*).

4. MECHANISM OF ACTION STUDIES

Early investigations of the nitrosoureas suggested that they were in a different category from the "alkylating agents," a group of compounds that are known to react with DNA and that are thought to produce their therapeutic effects through DNA modification. Not only did the nitrosoureas have a different chemical structure from the classical alkylating agents, many of which are related to nitrogen mustard, but their spectrum of activity in animal models was different and even their bone marrow toxicity in humans differed from that of the nitrogen mustards, being slower in onset.

It became apparent early in the investigation of the nitrosoureas that these compounds decomposed rapidly in aqueous solution, and that they had the capacity both to alkylate DNA and to carbamoylate proteins (*23*). The decomposition scheme shown in Fig. 3 accounted for this; the chloroethyl diazonium hydroxide moiety shown at the left could alkylate DNA and the isocyanate moiety shown at the right could carbamoylate proteins (*23*). The ability to carbamoylate proteins clearly distinguished the nitrosoureas from the mustards at a molecular level.

An alternate or additional point of view was that the DNA modification produced by the nitrosoureas was qualitatively different from that produced by the mustards and that differences in biological activity reflected this difference. In studying the reactions of MNU with DNA, Lijinsky et al. had shown that a fully deuterated methyl group was transferred intact from MNU to DNA (*24*). We reasoned that an analogous transfer of a chloroethyl group from a CENU would confer alkylating ability on the DNA itself and would lead to secondary reactions, including DNA crosslinking (*25*). The first indication that such reactions occurred came from the isolation of a

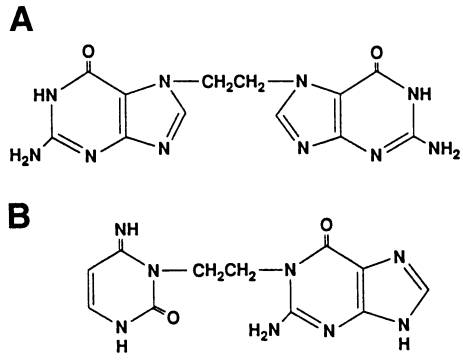


Fig. 4. DNA crosslinks formed by the CENUs. **A**. 1,2-bis(7-guanyl)-ethane; **B**. 1-(3-cytosinyl),2-(1-guanyl)ethane.

derivative of cytosine, 3,N^4-ethanocytosine, that had evidently been formed by an initial transfer of a chloroethyl group to a base N, followed by an intramolecular condensation reaction (*25,26*).

Kohn then showed that DNA modified by CENUs underwent reversible denaturation, indicating that interstrand crosslinks had indeed been formed (*27*). This, of course, led to a search for the nature of the crosslink. Ultimately, the two crosslinked entities shown in Fig. 4, 1,2-bis(7-guanyl)ethane and 1-(3-cytosinyl),2-(1-guanyl)ethane, were isolated from DNA treated with BCNU (*28,29*). For steric reasons, it seemed probable that 1,2-bis(7-guanyl)ethane (the "GG crosslink") was intrastrand in nature and that 1-(3-cytosinyl),2-(1-guanyl)ethane (the "GC crosslink") was responsible for interstrand crosslinking, but this has never been firmly established.

It seemed probable that 1,2-bis(7-guanyl)ethane was formed by the sequential attachment of a chloroethyl group to the N-7 position of one guanine followed by its reaction with the N-7 position of a second guanine. Neither of the base positions that are connected by the $-CH_2CH_2-$ bridge in 1-(3-cytosinyl), 2-(1-guanyl)ethane is very reactive, however, and it was not immediately clear how this crosslink was formed.

Meanwhile, in an important discovery, Erickson et al. had shown that the sensitivity of human tumor cells to the nitrosoureas was inversely related to their ability to remove alkyl groups from the O^6- position of guanine; cells that had higher levels of O^6-alkylguanine-DNA alkyltransferase ("alkyltransferase") were resistant to the action of the nitrosoureas (*30*). Consequently, we proposed the scheme shown in Fig. 5 to explain both the formation of the GC crosslink and the role of alkyltransferase in conferring resistance to the action of the nitrosoureas (*29*).

The first step in the crosslinking reaction shown in Fig. 5 is the transfer of a chloroethyl group from the CENU to the O^6-position of guanine in DNA. This is followed by an intramolecular condensation to form the intermediate, 1,O^6-ethanoguanine, which ultimately reacts with the N-3 position of cytosine to form the GC crosslink. However, alkyltransferase can remove the chloroethyl group from the O^6-position of guanine before crosslinking can occur. If this happens, the DNA is truly repaired; the modified O^6-chloroethyl guanine is restored to its original unmodified form, and the cell is protected from toxicity. Alkyltransferase also recognizes the second intermediate in this pathway, 1,O^6-ethanoguanine, but in this case, the protein becomes

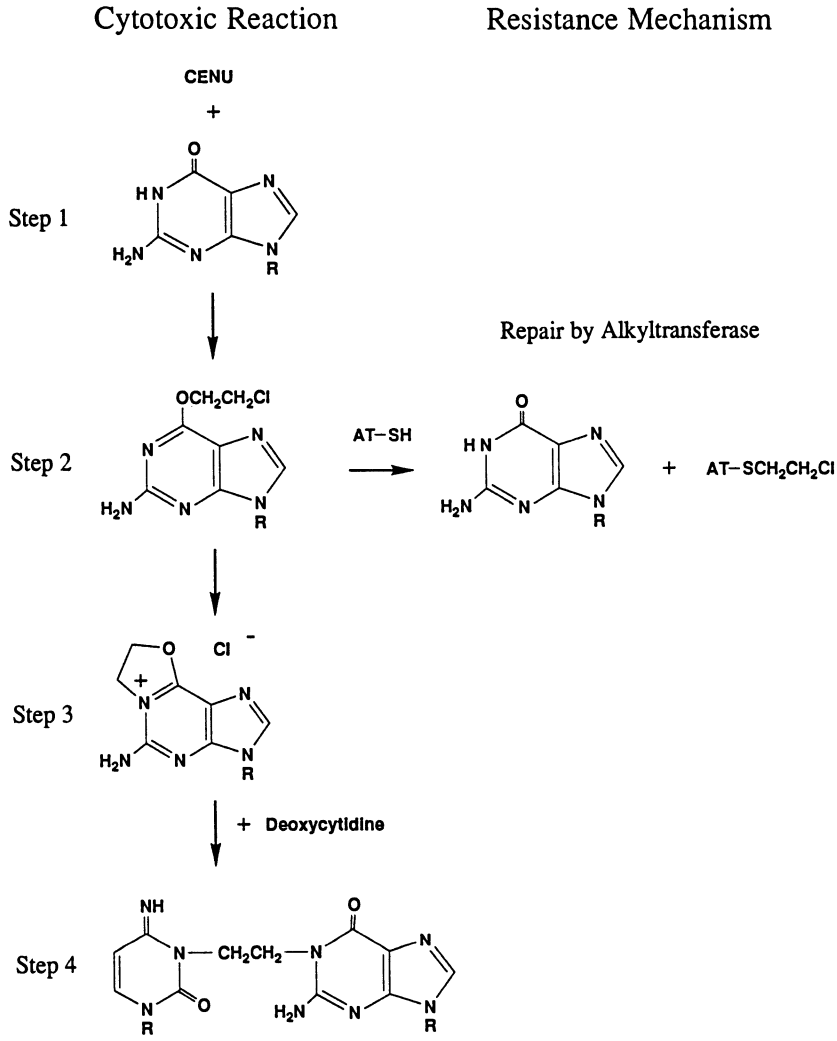


Fig. 5. Reaction of a CENU with guanine in DNA. The attachment of a chloroethyl group to the O^6-position of guanine leads to a crosslink in a four-step reaction. However, this process can be interrupted by alkyltransferase, which causes resistance by removing the chloroethyl group before crosslinking can occur. R = deoxyribose contained in the DNA structure.

bound to DNA, and additional steps are necessary to restore the DNA to its original condition (*31*).

The formation of the O^6-chloroethyl guanine in DNA was finally demonstrated by Parker et al. (*32*), and the other steps shown in Fig. 5 have been fully established (*33*). GC crosslinks are depleted in cells that are resistant to the action of CENUs (*34*), and the evidence that GC crosslink formation is cytotoxic is altogether convincing. In fact, the GC crosslink is probably the first crosslink whose formation has been directly linked to cytotoxicity. This information is now being put to use, since inhibitors of alkyltransferase are being tested for their ability to restore the sensitivity of tumor cells to CENUs (*see* Section 5).

However, formation of the GC crosslink is not essential for antitumor activity, because the methylating agents, MNU and STZ, are also active. These agents methylate the O^6-position of guanine, which suggests that O^6-alkylation itself is cytotoxic (*35*). Nevertheless, the CENUs produce a variety of other DNA modifications, and it seems probable that some of these modifications that do not involve the O^6-position of guanine also contribute to their cytotoxicity.

Some evidence for this was obtained several years ago in studies of adapted *Escherichia coli* (*36*). When either *uvrA*-deficient or *recA*-deficient mutants were adapted by growth in MNNG, they became more resistant to the cytotoxic action of MNNG and to *N*-ethyl-*N*-nitrosourea, but they remained sensitive to BCNU. More recently, Wu et al. have investigated the effects of transfecting alkyltransferase into wild-type and excision repair-deficient Chinese hamster ovary (CHO) cells (*37*). Both of these cell lines are deficient in alkyltransferase and are sensitive to MNNG, but the excision repair-deficient strain is more sensitive to *N*-(2-chloroethyl)-*N*1-nitrosourea (CNU) than is the wild type. When human alkyltransferase is transfected into either cell line, sensitivity to MNNG is greatly diminished. However, the transfected excision repair-deficient cells are still more sensitive to CNU than are the transfected wild-type cells. The authors suggest that this difference represents the ability of the excision repair mechanism to recognize a CNU-induced GG intrastrand crosslink that is cytotoxic to these cells (*37*).

Other studies have suggested that purine glycosylases may also play a role in the resistance phenomenon. HPLC profiles of the DNA modifications present in resistant glial cells that have been treated with CNU show that several adducts besides the GC crosslink are absent or diminished in amounts in comparison with the amounts present in sensitive cells (*34*). Since both alkyltransferase and 3-methyladenine DNA glycosylase II are upregulated in the *E. coli* adaptive response to methylating agents (*38*), it seems likely that glycosylases may also participate in the resistance of mammalian cells to DNA-modifying agents. Bacterial 3-methyladenine DNA glycosylase II, has in fact, been shown to act on all of the CNU-modified bases in Fig. 6 (*39,40*) At least two of these, 1,2-bis(7-guanyl)ethane and N^2,3-ethanoguanine, probably disrupt the DNA structure in a way that may be cytotoxic. Furthermore, biochemical assays have shown that glycosylase levels are elevated in a resistant glial cell line that is apparently depleted in some of the modified bases shown in Fig. 6 (*41*).

In summary, the haloethylnitrosoureas exert their cytotoxic action by modifying DNA. The formation of one lesion, 1-(3-cytosinyl),2-(1-guanyl)ethane, is clearly a lethal event, and it is likely that other DNA modifications are cytotoxic as well. At the same time, DNA repair is an important cause of resistance: one repair enzyme, alkyltransferase, prevents the formation of the lethal GC crosslink, and other repair enzymes probably act on other DNA modifications that are cytotoxic.

Our understanding of the mechanism of action of these agents is not complete, however. For example, the first step in DNA modification, transfer of the chloroethyl group to a DNA base, is more complicated than indicated in Fig. 3. By a mechanism that is not yet fully established, the carbon-halogen bond in the CENU is broken during alkylation, and the carbon that was originally attached to the halogen becomes the one that attaches to the DNA base (*42,43*). Also, one would expect that the distribution of DNA adducts would be the same for different CENUs if the agents acted

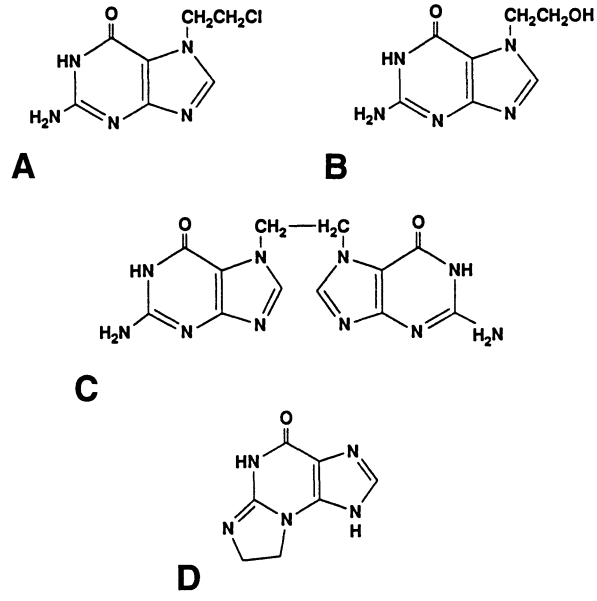


Fig. 6. Modified bases released from CENU-treated DNA by bacterial 3-methyladenine DNA glycosylase II. **A**. 7-(2-chloroethyl)guanine; **B**. 7-(2-hydroxyethyl)guanine; **C**. 1,2-bis(7-guanyl)ethane; **D**. N^2,3-ethanoguanine.

by generating a common alkylating intermediate as shown in Fig. 3, but the distribution is actually different for different agents (*44*).

Also not explained by a simple alkylation mechanism is the observation that these agents have a preference for guanine-rich sequences in DNA (*45*). Clearly, neighboring bases affect the alkylation sites, and this raises the possibility that CENUs could be targeted to specific regions, perhaps increasing their toxicity to tumor cells more than to normal cells.

Finally, the role of carbamoylation is not completely elucidated. CENUs with carbamoylating activity inhibit several enzymes involved in DNA repair, presumably by reacting with proteins involved in the repair process. Thus, it is possible that the ability of some CENUs to damage DNA and, at the same time, to inhibit repair may contribute to their antitumor activity (*46*).

5. FURTHER DEVELOPMENTS

Since alkyltransferase activity is closely linked to CENU resistance, attention has turned to methods of inhibiting this enzyme as a means of restoring sensitivity (*47*). Inhibition is favored by the fact that the reaction is stoichiometric. A single molecule of alkyltransferase has the ability to remove just one alkyl group from an O^6-alkylguanine in DNA; the alkyl group that is removed is transferred to the alkyltransferase, which is thereby inactivated.

Two approaches to inactivation have been used. In one approach, STZ is given somewhat before the CENU; in the interval before CENU administration, alkyltransferase is consumed in repairing the DNA methylation caused by STZ (*48,49*). In the second approach, a low-mol-wt inhibitor is given to inactivate the alkyltransferase

directly (*50,51*). Both approaches have been shown to work, but the latter seems to be particularly promising and is being pursued actively at the present time.

The use of low-mol-wt inhibitors began with the observation that exposure of mammalian cells to O^6-methylguanine and certain other O^6-alkylguanines caused a major decrease in cellular alkyltransferase activity (*50,51*). O^6-Methylguanine was shown to be a substrate for alkyltransferase, although it is a very poor substrate in comparison with O^6-methylguanine in DNA (*50*). Relatively high concentrations of O^6-methylguanine were required to produce inhibition, and a search for more effective inhibitors was initiated.

O^6-Benzylguanine was found to be much more active than O^6-methylguanine, producing as much inhibition at a concentration of 2.5 μM as O^6-methylguanine did at 0.2 mM (*52*). Other O^6-alkylguanine inhibitors have been synthesized, and their ability to inactivate alkyltransferase has been tested; compounds having easily displaced allyl or benzyl groups are most active (*53,54*). Research in this area is being pursued actively, and the critical question will be whether the alkyltransferase inhibitors sensitize tumor cells to the cytotoxic action of the CENUs more than they sensitize normal cells.

Meanwhile, the search for more active CENUs continues. Derivatives that include carrier steroid or amino acid groups that may target the CENU to particular tumor cell populations have been synthesized as reviewed recently (*55*). At a molecular level, attempts have been made to increase the sequence specificity of these agents by linking them to peptides that bind in the minor groove of DNA (*56,57*). Although these agents do show a different specificity of reaction, they have not yet shown any increase in antitumor activity.

6. SUMMARY

As mentioned in Section 1., the development of the nitrosoureas has illustrated several important aspects of cancer drug discovery. First, the lead compound was discovered by a random screening process that focused attention on a chemical structure different from any that had been used previously in the treatment of cancer. Careful synthetic work combined with an ongoing evaluation of antitumor activity led to the development of significantly more active compounds.

Both the initial screening and the drug development program emphasize the importance of the choice of assay system for evaluating antitumor activity. In this case, the major role of alkyltransferase in both causing tumor resistance and protecting normal cells from toxicity illustrates the problems that may arise from testing compounds in a model that has different DNA repair capabilities from those found in human cells. As an aside, this particular problem might now be avoided by engineering animal tumor models with human DNA repair genes.

Finally, mechanism of action studies have helped greatly in understanding the limitations of the CENUs and, perhaps, in overcoming them. Study of the CENUs has provided us with a specific example of a cytotoxic DNA modification whose formation can be prevented by a particular cellular enzyme. At the same time, indications that other CENU-induced DNA modifications may be cytotoxic suggest that effective antitumor agents may produce a variety of DNA modifications that depend on different repair modalities to produce complete resistance.

ACKNOWLEDGMENTS

The author would like to express his appreciation for the contributions of his many colleagues to the work from our laboratory that is reviewed here. Support by grant CA-44499 from the National Cancer Institute, Department of Health, Education and Welfare is also gratefully acknowledged.

REFERENCES

1. Schepartz SA. Early history and development of the nitrosoureas. *Cancer Treatment Rep* 1976; 60:647-649.
2. Greene MO, Greenberg J. The activity of nitrosoguanidines against ascites tumors in mice. *Cancer Res* 1960; 44:1166-1171.
3. Montgomery JA. Chemistry and structure-activity studies of the nitrosoureas. *Cancer Treatment Rep* 1976; 60:651-664.
4. Montgomery JA. The development of the nitrosoureas: A study in congener synthesis. In: Prestayko AW, Crooke ST, Baker LH, Carter SK, Schein PS, eds. *Nitrosoureas: Current Status and New Developments*. New York: Academic. 1981:3-8.
5. Skipper HE, Schabel FM Jr, Trader MW, Thomson JR. Experimental evaluation of potential anticancer agents. VI. Anatomical distribution of leukemic cells and failure of chemotherapy. *Cancer Res* 1961; 21:1154-1164.
6. Johnston TP, McCaleb GS, Montgomery JA. The synthesis of antineoplastic agents. XXXII. N-nitrosoureas. I. *J Med Chem* 1963; 6:669-681.
7. Johnston TP, McCaleb GS, Oplinger PA, Montgomery JA. The synthesis of potential anticancer agents. XXXVI. N-nitrosoureas. II. Haloalkyl derivatives. *J Med Chem* 1966; 9: 892-910.
8. Schabel FM Jr. Nitrosoureas: A review of experimental antitumor activity. *Cancer Treatment Rep* 1976; 60:665-698.
9. Hansch C, Smith N, Engle R, and Wood, H. Quantitative structure-activity relationships of antineoplastic drugs: Nitrosoureas and triazenoimodazoles. *Cancer Chemother Rep* 1972; 56: 443-456.
10. Hansch C, Leo A, Schmidt C, Jow PY, Montgomery JA. Antitumor structure-activity relations. Nitrosoureas vs. L-1210 leukemia. *J Med Chem* 1980; 23:1095-1101.
11. Goldin A. Historical overview of nitrosourea development. In: Serrou B, Schein PS, Imbach J-L, eds. *Nitrosoureas in Cancer Treatment*. Amsterdam: Elsevier. 1981:3-11.
12. Evans JS, Gerritsen GC, Mann KM, Owen SP. Antitumor and hyperglycemic activity of streptozotocin (NSC-37914) and its cofactor, U-15,774. *Cancer Chemother Rep* 1965; 48:1-6.
13. Johnston TP, McCaleb GS, Montgomery JA. Synthesis of chlorozotocin, the 2-chloroethyl analog of the anticancer antibiotic streptozotocin. *J Med Chem* 1975; 18:104-106.
14. Levin VA. Chemotherapy of recurrent brain tumors. In: Prestayko AW, Crooke ST, Baker LH, Carter SK, Schein PS, eds. *Nitrosoureas: Current Status and New Developments*. New York: Academic. 1981:259-268.
15. Rosenstock JG. Lipid-soluble nitrosoureas in the management of children with primary and recurrent brain tumors. In: Prestayko AW, Crooke ST, Baker LH, Carter SK, Schein PS, eds. *Nitrosoureas: Current Status and New Developments*. New York: Academic. 1981:269-276.
16. Walker ME. Adjuvant therapy of brain tumors with nitrosoureas. In: Prestayko AW, Crooke ST, Baker LH, Carter SK, Schein PS, eds. *Nitrosoureas: Current Status and New Developments*. New York: Academic. 1981:277-284.
17. Carter SK, Schabel FM Jr, Broder LE, Johnston TP. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) and other nitrosoureas in cancer treatment: A Review. *Adv Cancer Res* 1972; 16: 273-332.
18. Colvin M. Alkylating agents and platinum antitumor compounds. In: Holland JF, Frei III E, Bast RC Jr, Kufe DW, Morton DL, Weichselbaum RR, eds. *Cancer Medicine,* 3rd ed. Philadelphia: Lea & Febiger, 1993:733-754.

19. Wasserman TH, Slavik M, Carter SK. Clinical comparison of the nitrosoureas. *Cancer* 1975; 36: 1258–1268.
20. Rakieten N, Rakieten ML, Moreshwar V. The diabetogenic action of streptozotocin (NSC-37917). *Cancer Chemother Rep* 1963; 29:91–98.
21. Byrne PJ, Schein PS. Streptozotocin. In: Prestayko AW, Crooke ST, Baker LH, Carter SK, Schein PS, eds. *Nitrosoureas: Current Status and New Developments.* New York: Academic. 1981:367–377.
22. Hoth DF, Duque-Hammershaimb L. Chlorozotocin: clinical trials. In: Prestayko AW, Crooke ST, Baker LH, Carter SK, Schein PS, eds. *Nitrosoureas: Current Status and New Developments.* New York: Academic. 1981:387–398.
23. Wheeler GP. Mechanism of action of nitrosoureas. In: Sartorelli AC, Johns DG, eds. *Handbook of Experimental Pharmacology*, vol. XXXVII/2. New York: Springer-Verlag, 1975:65–84.
24. Lijinsky W, Garcia H, Keefer L, Loo J, Ross AE. Carcinogenesis and alkylation of rat liver nucleic acids by nitrosomethylurea and nitrosoethylurea administered by intraportal injection. *Cancer Res* 1972; 32:893–897.
25. Ludlum DB, Kramer BS, Wang J, Fenselau C. Reaction of 1,3-bis-(2-chloroethyl)-1-nitrosourea with synthetic polynucleotides. *Biochemistry* 1975; 14:5480–5485.
26. Kramer BS, Fenselau CC, Ludlum DB. Reaction of BCNU (1,3-bis[2-chloroethyl]-1-nitrosourea) with polycytidylic acid. Substitution of the cytosine ring. *Biochem Biophys Res Commun* 1974; 56:783–788.
27. Kohn KW. Interstrand cross-linking of DNA by 1,3-bis-(2-chloroethyl)-1-nitrosourea and other 1-(2-haloethyl)-1-nitrosoureas. *Cancer Res* 1977; 37:1450–1454.
28. Tong WP, Ludlum DB. Formation of the cross-linked base, diguanylethane, in DNA treated with *N,N'*-bis(2-chloroethyl)-N-nitrosourea. *Cancer Res* 1981; 41:380–382.
29. Tong WP, Kirk MC, Ludlum DB. Formation of the cross-link, 1-[N^3-deoxycytidyl],2-[*N*-deoxyguanosinyl]-ethane, in DNA treated with *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU). *Cancer Res* 1982; 42:3102–3105.
30. Erickson LC, Laurent G, Sharkey NA, Kohn KW. DNA cross-linking and monoadduct repair in nitrosourea-treated human tumour cells. *Nature* 1980; 288:727–729.
31. Gonzaga PE, Brent TP. Affinity purification and characterization of human O^6-alkylguanine-DNA alkyltransferase complexed with BCNU-treated, synthetic oligonucleotide. *Nucleic Acids Res* 1989; 17:6581–6590.
32. Parker S, Kirk MC, Ludlum DB. Synthesis and characterization of O^6-(2-chloroethyl)guanine: a putative intermediate in the cytotoxic reaction of chloroethylnitrosoureas with DNA. *Biochem Biophys Res Commun* 1987; 148:1124–1128.
33. Ludlum DB. DNA alkylation by the haloethylnitrosoureas: nature of modifications produced and their enzymatic repair or removal. *Mutat Res* 1990; 233:117–126.
34. Bodell WJ, Tokuda K, Ludlum DB. Differences in DNA alkylation products formed in sensitive and resistant human glioma cells treated with *N*-(2-chloroethyl)-*N*-nitrosourea. *Cancer Res* 1988; 48:4489–4492.
35. Bennett RA Pegg AE. Alkylation of DNA in rat tissues following administration of streptozotocin. *Cancer Res* 1981; 41:2786–2790.
36. Kacinski BM, Rupp WD, Ludlum DB. Repair of haloethylnitrosourea-induced DNA damage in mutant and adapted bacteria. *Cancer Res* 1985; 45:6471–6474.
37. Wu Z, Chan C-L, Eastman A, Bresnick E. Expression of human O^6-methylguanine-DNA methyltransferase in a DNA excision repair-deficient Chinese hamster ovary cell line and its response to certain alkylating agents. *Cancer Res* 1992; 52:32–35.
38. Lindahl T, Sedgwick B, Sekiguchi M, Nakabeppu Y. Regulation and expression of the adaptive response to alkylating agents. *Annu Rev Biochem* 1988; 57:133–157.
39. Habraken Y, Carter CA, Sekiguchi M, Ludlum DB. Release of N^2,3-ethanoguanine from haloethylnitrosourea-treated DNA by *Escherichia coli* 3-methyladenine DNA glycosylase II. *Carcinogenesis* 1991; 12:1971–1973.
40. Habraken Y, Carter CA, Kirk MC, Ludlum DB. Release of 7-alkylguanines from *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea-modified DNA by 3-methyladenine DNA glycosylase. *Cancer Res* 1991; 51:499–503.

41. Matijesevic Z, Bodell WJ, Ludlum DB. 3-Methyladenine DNA glycosylase activity in a glial cell line sensitive to the haloethylnitrosoureas in comparison with a resistant cell line. *Cancer Res* 1991; 51:1568–1570.
42. Parker S, Kirk MC, Ludlum DB, Koganty RR, Lown JW. Reaction of 1,3-bis-(2-chloroethyl) -1-nitrosourea (BCNU) with guanosine: Evidence for a new mechanism of DNA modification. *Biochem Biophys Res Commun* 1986; 139:31–36.
43. Naghipur A, Ikonomou MG, Kebarle P, Lown JW. Mechanism of action of (2-haloethyl)-nitrosoureas on DNA: Discrimination between alternative pathways of DNA base modification by 1,3-bis-(2-fluoroethyl)-1-nitrosourea by using specific deuterium labeling and identification of reaction products by HPLC/tandem mass spectrometry. *J Am Chem Soc* 1990; 112: 3178–3187.
44. Tong WP, Kohn KW, Ludlum DB. Modifications of DNA by different haloethylnitrosoureas. *Cancer Res* 1982; 42:4460–4464.
45. Hartley JA, Gibson NW, Kohn KW, Mattes WB. DNA sequence specificity of guanine-N7 alkylation by three antitumor chloroethylating agents. *Cancer Res* 1986; 46:1943–1947.
46. Kann HE Jr. Carbamoylating activity of the nitrosoureas. In: Prestayko AW, Crooke ST, Baker LH, Carter SK, Schein PS, eds. *Nitrosoureas: Current Status and New Developments*. New York: Academic, 1981:95–105.
47. Pegg AE. Mammalian O^6-alkylguanine-DNA alkyltransferase: Regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res* 1990; 50:6119–6129.
48. Futscher BW, Micetich KC, Barnes DM, Fisher RI, Erickson LC. Inhibition of a specific DNA repair system and nitrosourea cytotoxicity in resistant human cancer cells. *Cancer Commun* 1989; 1:65–73.
49. Gerson SL. Modulation of human lymphocyte O^6-alkylguanine-DNA alkyltransferase by streptozotocin *in vivo*. *Cancer Res* 1989; 49:3134–3138.
50. Dolan ME, Morimoto K, Pegg AE. Reduction of O^6-alkylguanine-DNA alkyltransferase activity in HeLa cells treated with O^6-alkylguanines. *Cancer Res* 1985; 45:6413–6417.
51. Karran P, Williams SA. The cytotoxic and mutagenic effects of alkylating agents on human lymphoid cells are caused by different DNA lesions. *Carcinogenesis* 1985; 6:789–792.
52. Dolan ME, Moschel RC, Pegg AE. Depletion of mammalian O^6-alkylguanine-DNA alkyltransferase activity by O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc Natl Acad Sci USA* 1990; 87:5368–5372.
53. Moschel RC, McDougall MG, Dolan ME, Stine L, Pegg AE. Structural features of substituted purine derivatives compatible with depletion of human O^6-alkylguanine-DNA alkyltransferase. *J Med Chem* 1992; 35:4486–4491.
54. Chae MY, McDougall MG, Dolan ME, Swenn K, Pegg AE, Moschel RC. Substituted O^6-benzylguanine derivatives and their inactivation of human O^6-alkylguanine-DNA alkyltransferase. *J Med Chem* 1994; 37:342–347.
55. McCormick JE, McElhinney RS. Nitrosoureas from chemist to physician: Classification and recent approaches to drug design. *Eur J Cancer* 1990; 26:207–221.
56. Chen FX, Zhang Y, Church KM, Bodell WJ, Gold B. DNA crosslinking, sister chromatid exchange and cytotoxicity of *N*-2-chloroethylnitrosoureas tethered to minor groove binding peptides. *Carcinogenesis* 1993; 14:935–940.
57. Gold B, Church KM, Wurdeman RL, Zhang Y, Chen FX. Control over the sequence specificity of DNA alkylation: syntheses and reactions with ^{32}P-end-labelled DNA of *N*-alkyl-*N*-nitrosoureas linked to minor groove binding lexitropsins. IARC Sci Publications 1991; 105:439–442.

4 Platinum Complexes

Lloyd R. Kelland, PhD

CONTENTS

1. HISTORICAL ASPECTS

It is now over 20 years since the platinum-based drug cisplatin (cis-diamminedichloro platinum [II]) was added to the armamentarium available to the oncology physician. Interestingly, the chemical identity of cisplatin was first established in the mid-19th century (and known as Peyrone's chloride) and, were it not for the well-documented serendipitous observations of Barnett Rosenberg while performing experiments investigating the effects of electric fields on bacteria, its antitumor properties may have remained undiscovered (*1*). Cisplatin has long been accepted to confer significant antitumor activity against a variety of neoplasms; in particular, as a result of the introduction of cisplatin-containing chemotherapy regimes, over 80% of men presenting with testicular cancer are now cured of their disease (*2*). Initial studies at the Royal Marsden Hospital (London) by Wiltshaw and Carr also established that cisplatin conferred promising activity against ovarian cancer (*3*). Combination regimens including cisplatin (typically with cyclophosphamide) generally produce clinical complete remissions in approx 50% of patients with advanced disease. Moreover, long-term followup studies in patients with advanced ovarian cancer have shown that combination chemotherapy containing cisplatin enhanced survival by at least 10% at 5 and 10 yr postdiagnosis compared to the accepted best available drugs of the pre-cisplatin era (*4*). In addition to these dramatic effects on the long-term survival of cancer sufferers, cisplatin also offers substantial palliative benefit to patients with small-cell lung, bladder, cervical, and head and neck carcinomas (*2*).

In spite of demonstrable dramatic efficacy against these neoplasms, from early days, cisplatin was observed to suffer from two major drawbacks: (1) its particularly severe normal tissue toxicities, and (2) the propensity of many tumors to be resistant *ab initio* or to acquire resistance to its tumor inhibitory properties. Therefore, there has been substantial effort to discover and develop analogs of cisplatin that address

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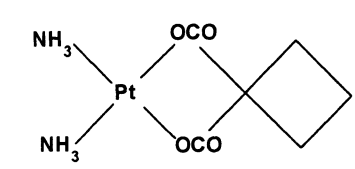
Edited by: B. Teicher Humana Press Inc., Totowa, NJ



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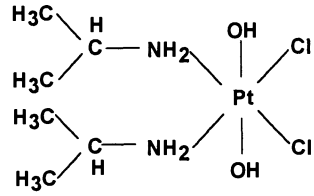

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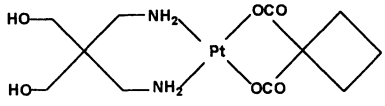

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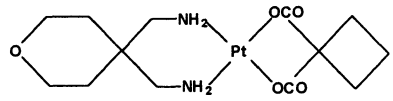

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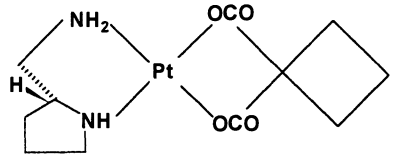

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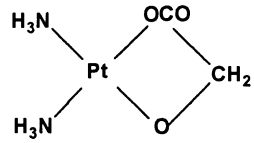
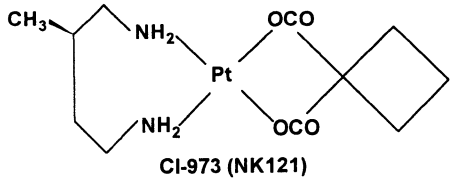


254-S



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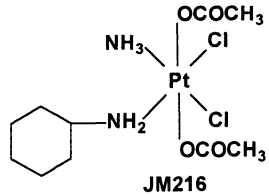


JM216

Fig. 1. Structures of analogs designed principally to reduce the toxicity of platinum-based chemotherapy.

these two limitations. This chapter summarizes early efforts to improve on cisplatin, the current clinical status of analog development (a total of 13 platinum complexes are currently undergoing or have recently undergone clinical trial), and future prospects for the discovery of new platinum-based anticancer drugs, particularly with respect to the circumvention of resistance mechanisms known to limit the efficacy of cisplatin.

2. THE STATUS OF DEVELOPMENT OF CISPLATIN ANALOGS

2.1. Analogs Developed to Reduce the Toxicities of Cisplatin

In contrast to the circumvention of tumor resistance to cisplatin (*see* Section 2.2.), efforts to ameliorate the drug's severe adverse effects (predominantly nephro-, gastrointestinal-, and neurotoxicities) have met with considerably more success (Fig. 1).

Table 1
Dose-Limiting Toxicities of Cisplatin Analogs

Analog	*Dose-limiting toxicity*	*Other notable toxicity*
Carboplatin	Thrombocytopenia	
Iproplatin	Thrombocytopenia	Diarrhea
Zeniplatin	Neutropenia	Nephrotoxicity
Enloplatin	Neutropenia	Nephrotoxicity
DWA2114R	Leukopenia	
254-S	Leukopenia, thrombocytopenia	Nephrotoxicity
CI-973	Neutropenia	
JM216	Leukopenia, thrombocytopenia	
Tetraplatin	Neuropathy	
Oxaliplatin	Neuropathy	
Lobaplatin	Thrombocytopenia	
Cycloplatam	Myelosuppression	
L-NDDP	Neutropenia, thrombocytopenia	

A summary of the dose-limiting toxicities of these analogs is shown in Table 1. To date, the most successful advance has been provided by carboplatin (cis-diammine-1, 1-cyclobutane dicarboxylato platinum [II]; Paraplatin), which remains the only cisplatin analog to have been licensed worldwide and to have entered routine clinical practice (*5*).

2.1.1. Carboplatin

From the initial clinical trials with carboplatin in the early 1980s, it was clear that the drug was substantially less toxic than cisplatin, not only in terms of nephrotoxicity, but also in terms of nausea, vomiting, and neurotoxicity. Myelosuppression, primarily thrombocytopenia (*see* Table 1) is dose-limiting (*6*). Today, the main area of contention with carboplatin revolves around whether this less toxic analog should replace cisplatin as the first-line treatment for patients where cisplatin-based therapy has proven efficacy (i.e., those presenting with ovarian or testicular cancer). Numerous randomized trials (especially in patients with advanced ovarian cancer) have been performed; overviews of these trials have also been published (*7*). Conclusions drawn from such studies should bear in mind that:

1. Accrual to some of the larger studies did not include early stage (IC,II) good prognosis groups;
2. The median followup of some of the studies is still at too early a stage to comment on comparative effects on long-term survival; and
3. The doses of cisplatin and carboplatin have varied from trial to trial (it is generally accepted that carboplatin is approximately fourfold less dose-potent than cisplatin; thus doses of 400 vs 100 mg/m^2, respectively, should be compared).

Nonetheless three major conclusions may be drawn (*7*):

1. Carboplatin is less toxic to the kidneys, nerves, and gastrointestinal system than cisplatin;
2. Response rates in ovarian cancer are broadly comparable; and

3. Survival rates among similar prognostic groups at comparable doses (*see above*) are similar.

Recently published long-term followup results of the first randomized trial of cisplatin (100 mg/m² q 4 wk for 5 courses and 30 mg/m² for another 5 courses) vs carboplatin (400 mg/m² q 4 wk for 10 courses), performed at the Royal Marsden Hospital, London, between October 1981 and June 1984 in 131 patients with advanced epithelial ovarian cancer, have shown that the two drugs produce similar long-term survival (*8*). With a median followup of 9 yr, 5-yr survival rates were 15% (95% confidence interval [CI], 8–26%) for cisplatin (median survival duration of 19.5 mo) and 19% CI, 11–30%) for carboplatin (median survival duration of 13 mo); none of these differences were statistically significant (*8*).

Although these analyses have led some to propose that the less toxic carboplatin rather than cisplatin should currently be considered the standard choice of platinum compound in the first-line treatment of advanced ovarian cancer (typically in combination with cyclophosphamide, e.g., *9*), this view has not yet gained universal acceptance. Similarly, carboplatin has been shown to possess equivalent activity to cisplatin in germ-cell tumors of the testis and is less toxic (*10*).

A further advantage of carboplatin might be in studies of dose intensity (*see* Section 3.2.2.), since the drug largely lacks nonhematological toxicity (thus allowing high doses to be given with hematologic support by growth factors). Second, as developed by Calvert and colleagues, dosage calculations in terms of the area under the plasma concentration vs time curve (AUC) based on pretreatment renal function are applicable (*11*); dose (mg) = AUC × (glomerular filtration rate + 25). Carboplatin AUC values of 5 mg/mL × min for previously untreated patients were recommended.

Carboplatin undoubtedly offers patients a more acceptable level of morbidity compared to cisplatin, but clinical data suggest that the two agents share crossresistance. For example, in the above-described Royal Marsden Hospital randomized trial (*8*), where the study design allowed crossover between the two arms, patients who crossed over from one platinum to the other owing to progressive or nonresponding disease exhibited a low (14.3%; 95% CI, 4.8–30.25%) response rate to the other platinum compound.

2.1.2. Iproplatin (CHIP)

Similarly to carboplatin, iproplatin (JM9, CHIP, [cis-dichloro-trans-dihydroxo-bis {isopropylamine} platinum {IV}) (Fig. 1) was developed on the basis of a favorable preclinical toxicity profile to that of cisplatin with accompanying retention of antitumor efficacy (*12*). To date, iproplatin is the only platinum analog in addition to carboplatin to have undergone extensive phase II/III evaluation. The results of these trials, including large randomized trials with carboplatin, support the following conclusions (*13–15*):

1. As with carboplatin, the dose-limiting toxicity of iproplatin is thrombocytopenia.
2. Iproplatin is active in cisplatin-responsive disease (e.g., ovarian cancer), but shares crossresistance with cisplatin and carboplatin, and thus does not offer significant activity in cisplatin refractory disease. In patients with ovarian cancer, previously treated with either cisplatin or carboplatin, response rates to iproplatin were 12% in 60 patients resistant to cisplatin and 11% in 18 patients resistant to carboplatin (*14*).

3. In randomized trials of iproplatin vs carboplatin in ovarian (*14*) (120 patients) and cervical (*15*) (394 patients) cancers, iproplatin was more toxic (especially in terms of gastrointestinal effects) and less active than carboplatin. In the ovarian trial, median survival was 114 wk for patients in the carboplatin arm (400 mg/m² q 28 d) and 68 wk for patients receiving iproplatin (300 mg/m² q 28 d) ($p = 0.008$).

Based on such clinical data, iproplatin appears to offer no advantage over carboplatin, and therefore, its future use would appear to be limited.

2.1.3. Other Analogs

Since the introduction of carboplatin and iproplatin in the early to mid-1980s, several other analogs designed mainly to reduce the toxicity of cisplatin-based chemotherapy have entered clinical trial (Fig. 1). Many of the complexes (e.g., zeniplatin, enloplatin, and the Japanese-based DWA2114R and CI-973) possess identical leaving group chemistry (oxygenated leaving groups conferring good aqueous solubility and greater stability) to carboplatin. However, and perhaps surprisingly, both zeniplatin and enloplatin caused some nephrotoxicity in patients while in phase I clinical trial. Their future clinical role would appear to be limited. In addition, another analog, 254-S (cis-diammine [glycolato] platinum, synthesized in Japan) has also produced one severe episode of renal toxicity. 254-S is currently undergoing phase II/III trials in Japan, generally using a schedule of 100 mg/m² q 4 wk (*16*). In a phase II trial in patients ($n = 15$) with advanced germ-cell testicular cancer, prostatic cancer ($n = 16$), or transitional-cell carcinoma of the urinary tract ($n = 35$), objective response rates were 80, 18.8 and 28.6%, respectively. DWA2114R in phase II trials has also shown response rates broadly comparable to that observed for cisplatin, e.g., a 44% response rate was observed in patients ($n = 34$) with advanced ovarian cancer (*17*).

In addition to studies based in Japan, CI-973 was evaluated in a five-daily dose schedule phase I at the Fox Chase Cancer Center, Philadelphia (*18*). In common with the Japanese phase I, neutropenia was dose-limiting, and in contrast to carboplatin, thrombocytopenia was infrequent. No responses were recorded; the recommended phase II dose of CI-973 was 30 mg/m²/d for 5 d. In another US-based phase I study using a bolus q 28-d schedule, similar results were reported; granulocytopenia was dose-limiting, no responses were observed, and recommended phase II doses were 230 and 190 mg/m² for previously untreated and treated patients, respectively (*19*).

2.1.4. Orally Active Platinum Drugs: JM216

The success of carboplatin is testimony to the importance of considering patient quality of life issues in cancer chemotherapy. Since cisplatin and carboplatin require iv administration, scientists at the Institute of Cancer Research, London, in conjunction with the Johnson Matthey Technology Centre and Bristol Myers Squibb, have attempted to facilitate patient comfort and convenience further during platinum-based chemotherapy through the discovery and development of platinum complexes capable of oral administration. Moreover, through potential treatment on an outpatient basis, hospitalization costs may be substantially reduced. Such studies have led to the introduction of the first clinically used orally administrable platinum complex: bis-acetato-ammine-dichloro-cyclohexylamine platinum (IV) (JM216) (Fig. 1). Preclinically, JM216 possesses several encouraging properties (*20,21*):

1. Rodent toxicology resembled carboplatin rather than cisplatin; myelosuppression was dose-limiting;
2. Ferret emesis studies showed that JM216 was substantially less emetic than cisplatin;
3. Orally administered JM216 showed antitumor activity broadly comparable to intravenously administered cisplatin/carboplatin in murine tumor and human ovarian carcinoma xenograft models;
4. JM216 exhibited greater antitumor activity using a daily × 5 schedule q 21 d compared to administering the same total dose as a bolus q 21 d; and
5. In vitro, JM216 showed evidence of circumvention of acquired cisplatin resistance, especially in tumor cells where resistance was attributable to reduced drug transport.

JM216 entered phase I clinical trial at the Royal Marsden Hospital, London, in August 1992. Two phase I trials have now been completed; a single dose q 21 days and a schedule of daily administration on days 1–5, q 21 d. In agreement with preclinical rodent studies, myelosuppression (including both leukopenia and thrombocytopenia) was the dose-limiting toxicity. Emesis has been controllable, and no nephro- or neurotoxicity has been observed. A partial response and a reduction in the ovarian tumor marker CA125 were observed in a patient with ovarian cancer, which recurred after treatment with cisplatin (*22*). In late 1994, JM216 entered phase II trials in Europe and North America in patients with ovarian and lung carcinomas.

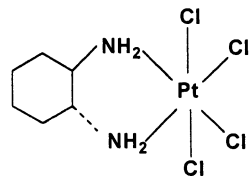
2.2. Analogs Designed Principally to Circumvent Tumor Resistance

As stated above, compared to efforts aimed at reducing the side effects of platinum-based chemotherapy, progress in overcoming tumor resistance to cisplatin has, thus far, met with little success in the clinic. Thus, circumvention of resistance remains the major goal in current platinum drug discovery programs.

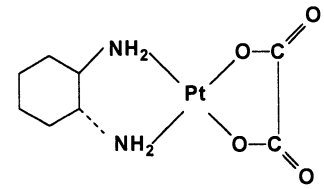
2.2.1. 1,2-Diaminocyclohexane (DACH) Platinum Complexes

The earliest lead to compounds that might offer clinical utility against cisplatin resistance arose over 20 years ago in studies by Burchenal and colleagues through their discovery of so-called DACH containing platinum complexes (*23*). Notably, these complexes exhibited activity in murine L1210 leukemia tumor models possessing acquired resistance to cisplatin. However, since this observation has not been translated to all other murine or human acquired cisplatin-resistant tumor models (e.g., in our own studies using the murine ADJ-PC6 plasmacytoma [*24*] and various human ovarian carcinoma xenografts [*25*]), doubt has been cast on the predictive utility of this mouse leukemia for human malignancies. Following formulation difficulties with early DACH complexes selected for clinical trial (e.g., JM82, PHIC), two more water-soluble derivatives have recently undergone phase I clinical trial: tetraplatin and oxaliplatin (Fig. 2; Table 1) (*26–28*). Disappointingly, in the phase I trials of both tetraplatin (*26,27*) and oxaliplatin (*28*), severe neurotoxicity has proven a limitation to their therapeutic efficacy. At this time, it is uncertain whether tetraplatin (where severe neurotoxicity was observed in patients receiving a cumulative dose above 200 mg/m^2 using a day 1, day 8 q 28 d schedule [*26*] and above 165 mg/m^2 using a daily × 5 schedule [*27*] will be progressed to phase II evaluation.

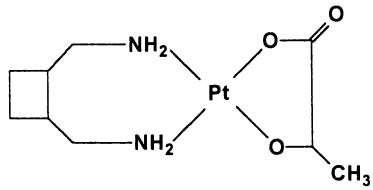
Oxaliplatin has also exhibited neurotoxicity during a phase I trial (*28*). Comprehensive phase II data for single-agent oxaliplatin have not yet been reported. However, four partial responses were observed in the phase I study (in patients with eso-



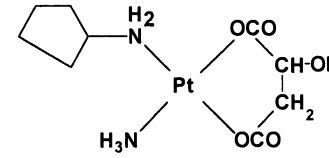
Tetraplatin (Ormaplatin)



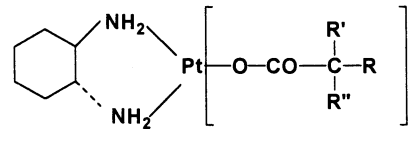
Oxaliplatin (1-OHP)



Lobaplatin (D-19466)



Cycloplatam



L-NDDP

Fig. 2. Structures of analogs designed principally to circumvent tumor resistance.

phageal [2 cases], lung [1], and urothelial cancer [1]) (*28*). In addition, oxaliplatin (20 mg/m²/d for 5 d q 21 d) has been used in a randomized chronomodulated vs fixed infusion rate chemotherapy regime along with 5-fluorouracil (600 mg/m²/d for 5 d q 21 d) and folinic acid (leucovorin) (300 mg/m²/d for 5 d q 21 d) in patients with colorectal cancer metastases (*29*). In 47 patients, drug delivery was kept constant, whereas in 45 patients it was chronomodulated (5 fluorouracil and leucovorin at 0400 h and oxaliplatin at 1600 h). Whereas peripheral sensitive neuropathy was dose-limiting in the chronomodulated arm (attributed to the oxaliplatin), a significantly higher (and encouraging) objective response rate (53%, 95% CI = 38–68% compared to 32%; 95% CI = 18–46%) was observed in the chronomodulated arm. However, the contribution of oxaliplatin to the activity of this complex treatment protocol is difficult to ascertain.

Another approach to the clinical evaluation of DACH complexes has been provided through liposomal preparations of DACH complexes. A phase I trial with L-NDDP has reported dose-limiting toxicities of neutropenia and thrombocytopenia (*30*). However, drug stability formulation, and delivery problems remain to be overcome before this approach can be fully evaluated.

2.2.2. Other Analogs (Fig. 2)

Two other platinum analogs that, at least from preclinical data, may offer some utility against acquired cisplatin resistance, are in early clinical trials. Lobaplatin (D-19466) has completed phase I evaluation in Germany and the Netherlands (*31,32*). Thrombocytopenia was dose-limiting; responses (one partial, one complete) occurred in two patients with ovarian cancer (both pretreated with cisplatin and carboplatin) in the 5-d trial (*31*), and one partial response was observed in another ovarian cancer patient pretreated with platinum in a trial using a schedule of 72-h infusion every 4 wk (*32*). The results of phase II trials using this interesting complex are awaited. As with carboplatin, resulting plasma AUCs following administration of lobaplatin appeared to be dependent on renal function.

To date, clinical trials with cycloplatam have been conducted only in Russia and await full reporting.

3. FUTURE PROSPECTS

Tumor resistance remains the major factor that limits the clinical effectiveness of cisplatin/carboplatin. Various strategies to circumvent clinical resistance, based largely on increasing knowledge of how cisplatin exerts its cell killing effects and of mechanisms of resistance, may be envisaged. These include:

1. Development of analogs of cisplatin;
2. Dose intensification of cisplatin/carboplatin:
3. Pharmacological modulation of resistance mechanisms; and
4. Combination with other active anticancer drugs with nonoverlapping toxicities and mechanisms of resistance.

3.1. Mechanisms of Resistance to Cisplatin

Cisplatin reacts primarily by stepwise exchange of its two labile chlorides (leaving ligands) for water or hydroxyl ions (Fig. 3). The final positively charged, highly reactive, diaquo species (which is the same for cisplatin and carboplatin) is then capable of reacting with nucleophilic sites on DNA, RNA, or proteins. The presence of high chloride ion concentrations in extracellular fluid (approx 100 m*M*) suppresses the aquation reactions and allows the uncharged complex to penetrate cell membranes. However, on entering a cell, where the cytoplasmic chloride concentration is much lower (as low as 4 m*M*), the chloride ligands begin to exchange. Replacement of the two chloride ligands of cisplatin by the bidentate cyclobutane dicarboxylic acid (CBDCA) ligand in carboplatin results in a complex more than 100-fold resistant to the above aquation reactions; in chloride-free phosphate buffer, pH 7.0, cisplatin had a half-life of 2.4 h compared to a half-life of 268 h for carboplatin (*33*).

The cell killing effects of cisplatin appear to be the result of the formation of an assortment of stable bifunctional adducts on DNA, which then block replication and/or inhibit transcription (*34,35*). The most common adduct (60–65%) involves binding of platinum to the nitrogen on position 7 of the imidazole ring of adjacent deoxyguanosines along the same strand of DNA (the GpG 1,2-intrastrand adduct). In addition, ApG (20–25%) and G*pX*pG and A*pX*pG (5–6%; where *X* is any base) 1,3-intrastrand adducts, monofunctional adducts (2–3%), DNA–protein crosslinks, and G-G interstrand crosslinks (ISCs, 2%) are also found. Moreover, recent evidence

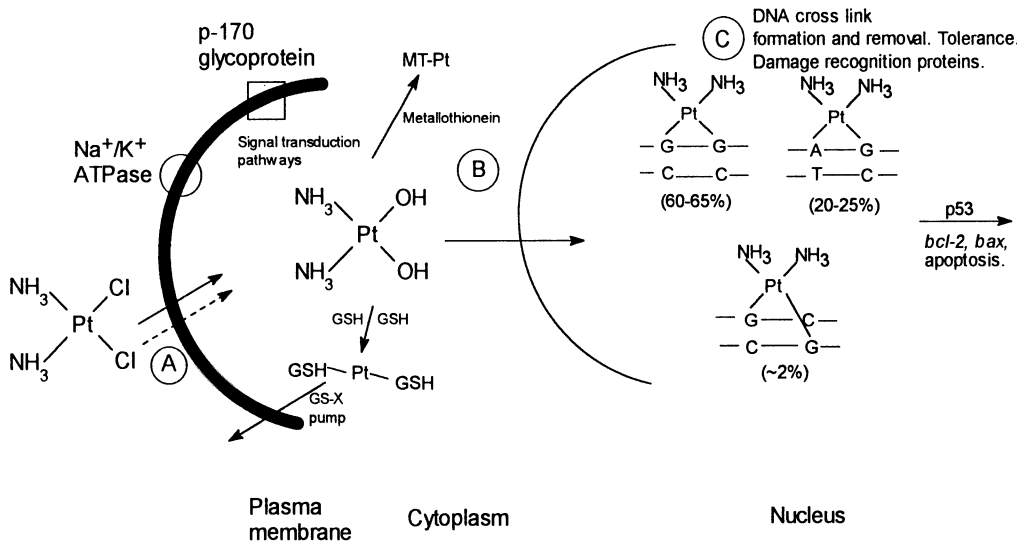


Fig. 3. Platinum drugs: mechanism of action and mechanisms of resistance.

measuring the sequence specificity of cisplatin DNA binding in the N-*ras* gene from cells exposed to cisplatin suggests that a novel adduct in the sequence 5'-TACT-3' may also be formed (*36*). However, controversy still remains regarding the relative role of each of the various DNA adducts induced by cisplatin (especially intra- vs interstrand adducts) in mediating cell killing. On the one hand, there is supportive evidence emphasizing the importance of 1,2 intrastrand adducts. The inactive transplatin (*37*) is sterically unable to form the major GpG and ApG 1,2-intrastrand adducts formed by cisplatin. Instead, a high proportion of DNA monoadducts are formed (up to 85% of adducts following a 1–2 h drug incubation with DNA (*38*). These are mainly detoxified through rapid reaction with glutathione. A minority slowly rearrange to form bifunctional 1,3 or 1,4 G-G ISCs or DNA ISCs. Furthermore, the GpG intrastrand adduct has been shown to be poorly repaired compared to Gp*X*pG and monofunctional adducts (*39,40*). Interestingly, both the G-G and A-G intrastrand adducts unwind DNA by 13°, whereas the G*X*G (*1,3*) intrastrand adduct unwinds DNA by 23°; bending of the DNA double helix is similar (32–35°) for all three adducts (*41*).

Although they represent only approx 2% of total adducts formed by cisplatin, other findings suggest that ISCs may be important determinants of cytotoxicity. Early studies in L1210 leukemia cells, using cis- and trans-platinum, showed a relationship between cell killing and levels of ISCs (*42*); although both isomers produce ISCs in naked DNA, only cisplatin produces substantial ISCs in whole cells. Moreover, two independent studies determining repair at the level of individual genes in paired cisplatin-sensitive and cisplatin-resistant human ovarian carcinoma cell lines have shown a marked increase in the gene-specific removal of ISCs in resistant lines, but no difference between sensitive and resistant lines in the removal of intrastrand adducts (*43,44*). Recent data have also indicated that the nature of the ISCs formed by transplatin and cisplatin in purified DNA differs with cisplatin favoring crosslink formation between guanines and transplatin between guanine and complementary cytosines (*45*).

Table 2
Genes/Proteins That May Play a Role in Determining Tumor Sensitivity/Resistance to Cisplatin

Gene/protein	*Comment*	*Reference*
CPR^{200}	Increased drug efflux	*52*
SQM1	Decreased drug accumulation	*53*
ERCC1	Increased DNA repair	*79,80*
HMG domain proteins (including HMG1 and 2)	Increased repair(?)/shielding from repair(?)	*73–76; 79,80*
c-Ha-*ras*	Increased drug resistance Decreased uptake/increased MT	*81*
c-*myc*	Increased drug resistance	*82*
c-*fos*	anti-*fos* ribozyme reverses resistance	*83*
HSP60	Heat-shock protein, chaperonin	*87*
p53	Mutations associated with drug resistance Cell-cycle checkpoint control/DNA repair	*60,86,88*

The type of crosslinks that eventually lead to death of the cell following exposure to cisplatin and how they bring about cell death are unknown. However, it appears likely that a programmed cell death, or apoptosis, is triggered in a variety of cell types (*46*), including in our own panel of human ovarian carcinoma cell lines (*47*).

Largely through the use of in vitro murine and human tumor cell lines, three major mechanisms of resistance to cisplatin have been identified:

1. Decreased intracellular transport of drug;
2. Increased cytoplasmic detoxification through increased levels of thiol-rich species, such as glutathione and/or metallothioneins; and
3. Enhanced removal of platinum-induced adducts from DNA and/or increased tolerance to platinum-DNA adducts (Fig. 3A, B, and C) (*see 48* for a review).

In addition, a number of genes and proteins (some directly associated with the three main cellular mechanisms of resistance described above) have been shown to be involved in determining the cellular sensitivity of tumor cells to cisplatin. These are summarized in Table 2.

3.1.1. Decreased Drug Transport

Many (but not all) acquired cisplatin-resistant cell lines (including our own 41McisR human ovarian carcinoma) (*49*) exhibit a decrease in platinum accumulation (typically two- to fourfold) compared to their respective parent line (*see 50* for a review). However, the mechanism by which platinum drugs enter cells remains unclear, with supportive laboratory evidence for roles for both passive diffusion (e.g., the uptake of cisplatin is not saturable) and active transport (e.g., uptake can be modulated by a variety of pharmacological agents including the Na^+/K^+-ATPase inhibitor, ouabain). This has led some to postulate that cisplatin may enter cells by both passive diffusion and partly through facilitated transport via a gated channel (*50*). In contrast to the increased efflux of drug observed in tumor cells with multiple resistance to other commonly used anticancer drugs (such as doxorubicin, paclitaxel, etoposide, and the vinca alkaloids, mediated through 170- and/or 190-kDa mem-

brane glycoproteins), cisplatin resistance mediated at the level of the plasma membrane appears to occur mainly through reduced drug influx (*50*). None of our own acquired cisplatin-resistant human ovarian carcinoma cell lines exhibit increased levels of P-glycoprotein (*51*).

Although at least two studies have reported changes in membrane proteins that appear to be associated with decreased drug uptake (*52, 53*; Table 2), these proteins (a 200-kDa glycoprotein and a 46-kDa protein, SQM1) have not, as yet, been identified in additional cell lines. Further, it is not yet clear whether reduced drug uptake also plays a role in determining clinical resistance to platinum drugs.

3.1.2. Increased Cytoplasmic Detoxification

As with reduced drug accumulation, many (but again, not all) in vitro studies have provided evidence for a role for the cytoplasmic thiol-containing tripeptide glutathione (GSH) in mediating platinum drug resistance (e.g., *54*). In our own studies using eight human ovarian carcinoma cell lines exhibiting a 100-fold range in intrinsic sensitivity to cisplatin, GSH (but not glutathione *S*-transferase [GST]) levels showed a significant correlation with cisplatin sensitivity; the most resistant line possessing fourfold higher GSH levels than the most sensitive (*55*). Evidence for a direct interaction between cisplatin and GSH inside tumor cells involving 2 mol of GSH complexed with 1 mol of platinum to form bis(glutathionato)-platinum has also been reported (*56*). Elimination of this complex from tumor cells has been proposed to occur via an ATP-dependent glutathione *S*-conjugate export pump (the GS-X pump, which has recently been shown to be functionally overexpressed in acquired cisplatin-resistant HL-60 human leukemia cells [*57*] and has been linked to another efflux protein, multidrug resistance related protein (MRP), associated with a form of multidrug resistance [*58*]). Cytoplasmic detoxification of platinum-based drugs may also occur through binding to metallothioneins (MT), a class of cysteine-rich, low-mol-wt isoproteins. Increased levels of MT have been reported in at least some acquired cisplatin-resistant cell lines (*59*).

A number of studies have attempted to correlate clinical platinum resistance of ovarian cancer to increased levels of GST (*60–63*) or MT (*64*). However, apart from one study (*62*), which used a semiquantitative intensity of staining method and reported a higher intensity of staining for GST π in resistant tumors, these results do not support a role for either GST or MT in clinical platinum drug resistance.

3.1.3. Increased DNA Repair/Tolerance

Studies using both bacterial enzymes (*Escherichia coli* UVRABC nuclease [*65*]) and mammalian cell extracts [*66*] indicate that platinum-DNA adducts are removed from DNA by nucleotide excision repair. The *E. coli* UVRABC nuclease has been shown to incise the eighth phosphodiester bond 5′ and the fourth bond 3′ to G-G intrastrand crosslinks, thereby excising an oligomer containing the adduct [*65*]. Resistance to cisplatin in many acquired resistant cell lines, including our own studies, appears to be owing to either an enhanced repair/removal of platinum adducts from DNA or an increased tolerance to such adducts (*67,68*). Moreover, the possible relevance of DNA repair in determining responses to platinum-based therapy in ovarian cancer patients has been highlighted in studies measuring RNA levels encoding for the ERCC1 human DNA repair gene (*69,70*). Patients who were clinically resistant to cisplatin- (or carboplatin)-based therapy had a 2.6-fold significantly higher expression

level of ERCC1 in their tumor biopsy than did patients who responded to that therapy. There is some evidence to suggest that the hypersensitivity of testicular cancer cell lines, which correlates with the clinical sensitivity of this tumor, might also be related to defective removal of platinum-DNA adducts (*71,72*).

3.1.4. Other Proteins/Genes

Over recent years, proteins, termed structure-specific recognition proteins (generally of approx 28 and 80–100 kDa), have been identified in mammalian cell extracts that bind specifically to the two major types of cisplatin-induced DNA 1,2,-intrastrand crosslinks and do not bind to adducts induced by transplatin (*73,74*). These proteins have been shown to possess high mobility group (HMG) domain motifs (e.g., SSRP1) (*74*); in addition, HMG1 and HMG2 themselves (proteins of 28.5 and 26.5 kDa, respectively) can recognize and bind to DNA platinated with cisplatin (*75,76*). The exact function of these damage recognition proteins, however, is presently unknown. Models have been proposed for their involvement in promoting the repair of cisplatin-damaged DNA or, in contrast, in blocking access of repair enzymes to damaged DNA. Support for the model involving shielding of ISCs from DNA repair enzymes has arisen from studies in yeast mutants where deletion of the HMG domain protein, lxr1, led to a twofold decrease in sensitivity to cisplatin (*77*). In addition, HMG domain proteins have recently been shown to inhibit specifically the repair of cisplatin-induced 1,2-intrastrand crosslinks by human excision nuclease (*78*). Alternatively, some support for the repair supposition is provided by observations that some (but not all) cisplatin-resistant tumor cell lines (some of which have an increase in DNA-repair capacity) also exhibit an increase in levels of damage recognition proteins (*73,79*). In addition, a damage recognition protein complex, B1, has been shown to contain a protein involved in an early stage of mammalian excision repair, human single-stranded binding protein (HSSB) (*80*).

Other genes/proteins that may have an involvement in determining cellular sensitivity to platinum drugs include various oncoproteins (e.g., p21*RAS*, *MYC*, *FOS/JUN*, and *BCL2/BAX/BCLx*), the p53 tumor suppressor gene, and a 60-kDa mitochondrial chaperonin heat shock protein (HSP60) (*see* Table 2) (*81–88*). Transformation of mouse fibroblasts with *ras* oncogenes produced a four- to eightfold increase in cisplatin resistance associated with a 40% decrease in accumulation and a 3.3-fold elevation in MT levels (*81*). Cisplatin has been shown to induce cell death through a programmed death pathway (apoptosis); at least some pathways of cell death appear to be activated through the wild-type p53 gene (*84*). Interestingly, testicular tumors (which are hypersensitive to cisplatin) have been reported rarely to exhibit p53 mutations, whereas tumors less responsive to chemotherapy commonly acquire p53 mutations (*85*). Moreover, p53 mutations have recently been shown to be associated with decreased sensitivity of human lymphoma cells to DNA-damaging agents, including cisplatin (*86*), and nuclear immunoreactivity of p53 in ovarian carcinomas has been associated with shorter overall survival in at least some studies (e.g., *60*). Elevated constitutive levels of HSP60 have been observed to correlate with clinical resistance of ovarian cancer (*87*). Modulation of various intracellular signal transduction pathways (e.g., those mediated by protein kinase C or via the epidermal growth factor) may also influence sensitivity and resistance to cisplatin (*89*). Intriguingly, there is also evidence suggesting that some forms of resistance to cisplatin may only be operative in vivo and may not be apparent in two-dimensional tissue-culture systems (*90*).

3.2. Circumvention of Resistance

3.2.1. Platinum Analogs

In addition to the many platinum analogs currently in clinical trial (*see* Section 2.), including the DACH-containing complexes tetraplatin and oxaliplatin, and lobaplatin and JM216, which all circumvent cisplatin resistance in at least some (typically L1210 leukemia) preclinical models, further analog development is ongoing. Since cisplatin and carboplatin both possess symmetrical diammine carrier ligands, our efforts in collaboration with chemists at the Johnson Matthey Technology Centre, have centered on asymmetric ammine/amine (or mixed amine) platinum complexes (e.g., JM216) (*see* Section 2.1.4.), which may bind to DNA in a different manner to that of cisplatin (e.g., to differing sequences of DNA or to produce a differing spectrum of adducts). Consequently, recognition by the HMG domain damage recognition proteins and/or repair proteins might differ. Moreover, in additional efforts to design novel platinum complexes which should bind to DNA in a manner distinct from that of cisplatin (and carboplatin) we, and others, have pursued the idea of activating the trans geometry of platinum complexes (*91–93*), whereas Farrell and colleagues have synthesized a series of novel neutral bis(platinum) complexes where two cis-diammine platinum groups are linked by an alkyldiamine of variable length (*94*).

The realization that some trans platinum complexes are as active as their cis counterparts in vitro and, moreover, for the trans ammine(amine) dichlorodihydroxo platinum(IV) complexes, in vivo (*92*), contravenes the original structure–activity rules for platinum complexes based on cis and transplatin (*37*). Our studies have centered on JM335 (trans ammine [cyclohexylamine]dichlorodihydroxo platinum[IV]), which is the first transplatinum complex to demonstrate marked in vivo activity against both murine (including two acquired cisplatin-resistant models) and human (ovarian carcinoma) subcutaneous tumors. Other recently reported novel synthetic approaches include the demonstration of in vivo antitumor activity (P388 leukemia) for platinum(II) organoamides, which do not possess a hydrogen substituent on any nitrogen donor atom (general formula; $Pt(NRCH_2)_2L_2$ where R = polyfluorophenyl and L = pyridine or substituted pyridine) (*95*) and for a series of 2-substituted-4,5-bis(aminomethyl)-1,3-dioxolane platinum(II) complexes (*96*).

3.2.2. Dose Intensification of Cisplatin/Carboplatin

In recent years, attempts have been made to increase the doses of cisplatin administered to patients in the hope of achieving improved response rates. Although the renal and gastrointestinal toxicities of cisplatin may be ameliorated through iv hydration and forced diuresis and 5HT3 inhibitor antiemetics, respectively, disabling neurotoxicity has proven to be dose-limiting (*97*). Carboplatin may be better suited to such studies (especially in combination with hematological support with growth factors) (*98*), since the main toxicities of carboplatin are hematological and dosage calculations based on kidney function are applicable (*11*). However, although a randomized trial of high- (100 mg/m^2) vs low- (50 mg/m^2) dose cisplatin in advanced ovarian cancer (both administered with cyclophosphamide every 21 d) has shown a clear survival advantage for the higher dose schedule (*99*), results with carboplatin (particularly for doses producing AUC values higher than 5–7 mg/mL × min) are presently less clear (*100*). Further clinical trials, including comparison of carboplatin AUC values of 6 vs 12, are ongoing. Thus, it remains an area of controversy concerning whether

increased doses above a certain level will be reflected in improved patient survival. Our own data, using a panel of human ovarian carcinoma cell lines, have shown a difference in intrinsic cellular sensitivity to carboplatin and cisplatin of 30- and 100-fold, respectively (*101*), and, typically, acquired cisplatin-resistant cell lines are 5- to 10-fold resistant (*48,102*). Thus, dose escalations of a similar magnitude may be necessary in the clinic. In addition, attempts have been made to increase the dose intensity of platinum in patients by combining platinum drugs with generally nonoverlapping toxicities (e.g., as recently reported for carboplatin and oxaliplatin [*103*]).

3.2.3. Modulation of Platinum Resistance Mechanisms

To date, attempts at the modulation of platinum resistance mechanisms have generally been conducted in preclinical models and have not reached the stage of clinical trials. However, a diverse range of agents has shown promise (*see 104* for a review), and at least some may reach clinical trial in the near future. Examples include modulation at the level of the plasma membrane using the antifungal agent, amphotericin B, reduction of GSH levels using buthionine sulfoximine (which is already undergoing clinical trial with the nonnephrotoxic bifunctional alkylating agent, melphalan), and inhibition of DNA repair using aphidicolin.

3.2.4. Combinations with Other Anticancer Agents: Paclitaxel

Although the vast majority of currently available anticancer drugs do not exhibit significant activity against ovarian tumors that are, or have become, resistant to cisplatin, in recent years, paclitaxel (taxol), a natural tubulin binding taxane extracted from the bark of the Pacific Yew, has shown a promising level of activity (*105*). Response rates in the region of 20–30% have been reported in platinum refractory ovarian cancer; clinical studies combining cisplatin and paclitaxel and, more recently, carboplatin and paclitaxel, are also ongoing.

4. SUMMARY

While much has been achieved in making platinum-based chemotherapy more acceptable to the patient (principally through the introduction of carboplatin) the key issue of circumvention of tumor resistance to cisplatin has, disappointingly, met with limited clinical success. In particular, the thorough clinical evaluation of platinum complexes possessing the DACH ligand appears to be precluded at present owing to dose-limiting neurotoxicity. Other attempts to develop analogs to reduce the toxicity of cisplatin (e.g., iproplatin, zeniplatin, 254-S) have shown no clear advantages over carboplatin. However, laboratory-based studies have shed significant light in recent years on the underlying mechanisms by which tumors acquire resistance to cisplatin; this should guide the way toward either improved platinum drugs targeted against particular mechanisms of resistance or pharmacological modulation of resistance mechanisms by nonplatinum drugs.

ACKNOWLEDGMENTS

Studies described from the CRC Centre for Cancer Therapeutics at the Institute of Cancer Research were supported by grants from the UK Cancer Research Campaign, the Medical Research Council, the Johnson Matthey Technology Center (JMTC), and Bristol Myers Squibb Oncology. Thanks are owed to Barry Murrer (JMTC) and

my colleagues Ken Harrap, Mark McKeage, Ian Judson, Florence Raynaud, Prakash Mistry, and Sarah Morgan for numerous constructive discussions, and Swee Sharp and Jeff Holford for preparation of figures and proofreading.

REFERENCES

1. Rosenberg B. Fundamental studies with cisplatin. *Cancer (Phila)* 1985; 55:2303-2316.
2. Loehrer PJ, Einhorn LH. Cisplatin. *Anna Intern Med* 1984; 100:704-713.
3. Wiltshaw E, Carr B. *Cis*-platinumdiamminedichloride. In: Connors TA, Roberts JJ, eds. *Platinum Coordination Complexes in Cancer Chemotherapy*. Heidelberg: Springer-Verlag. 1974:178-182.
4. Neijt JP, ten Bokkel Huinink WW, van der Burg MEL, van Oosterom AT, Willemse PHB, Vermorken JB, van Lindert ACM, Heintz APM, Aartsen E, van Lent M, Trimbos JB, de Meijer AJ. Long-term survival in ovarian cancer. *Eur J Cancer* 1991; 27:1367-1372.
5. Harrap KR. Preclinical studies identifying carboplatin as a viable cisplatin alternative. *Cancer Treat Rev* 1985; 12:21-33.
6. Calvert AH, Harland SJ, Newell DR, Siddik ZH, Harrap KR. Phase I studies with carboplatin at the Royal Marsden Hospital. *Cancer Treat Rev* 1985; 12:51-57.
7. Williams CJ, Stewart L, Parmar M, Guthrie D. Meta-analysis of the role of platinum compounds in advanced ovarian carcinoma. *Semin Oncol* 1992; 19:120-128.
8. Taylor AE, Wiltshaw E, Gore ME, Fryatt I, Fisher C. Long term follow up of the first randomised study of cisplatin versus carboplatin for advanced epithelial ovarian cancer. *J Clin Oncol* 1994; 12:2066-2070.
9. Alberts DS, Canetta R, Mason-Liddil N. Carboplatin in the first-line treatment of ovarian cancer. *Semin Oncol* 1990; 17:54-60.
10. Horwich A, Mason M, Dearnaley DP. Use of carboplatin in germ cell tumors of the testis. *Semin Oncol* 1992; 19:72-77.
11. Calvert AH, Newell DR, Gumbrell LA, O'Reilly S, Burnell M, Boxall FE, Siddik ZH, Judson IR, Gore ME, Wiltshaw E. Carboplatin dosage: prospective evaluation of a simple formula based on renal function. *J Clin Oncol* 1989; 7:1748-1756.
12. Foster BJ, Harding BJ, Wolpert-DeFilippes MK, Rubinstein LY, Clagett-Carr K, Leyland-Jones B. A strategy for the development of two clinically active cisplatin analogs:CBDCA and CHIP. *Cancer Chemother Pharmacol* 1990; 25:395-404.
13. Sessa C, Vermorken J, Renard J, Kaye S, Smith D, Huinink WTB, Cavalli F, Pinedo H. Phase II study of iproplatin in advanced ovarian carcinoma. *J Clin Oncol* 1988; 6:98-105.
14. Trask C, Silverstone A, Ash CM, Earl H, Irwin C, Bakker A, Tobias JS, Souhami RL. A randomized trial of carboplatin versus iproplatin in untreated advanced ovarian cancer. *J Clin Oncol* 1991; 9:1131-1137.
15. McGuire WP, Arseneau J, Blessing JA, DiSaia PJ, Hatch KD, Given FT Jr, Teng NNH, Creasman WT. A randomized comparative trial of carboplatin and iproplatin in advanced squamous carcinoma of the uterine cervix: A Gynecologic Oncology Group Study. *J Clin Oncol* 1989; 7:1462-1468.
16. Akaza H, Togashi M, Nishio Y, Miki T, Kotake T, Matsumura Y, Yoshida O, Aso Y. Phase II study of *cis*-diammine(glycolato)platinum, 254-S, in patients with advanced germ cell testicular cancer, prostatic cancer and transitional-cell carcinoma of the urinary tract. *Cancer Chemother. Pharmacol.* 1992; 31:187-192.
17. Majima H. Clinical studies with cisplatin analogues, 254-S, DWA2114R and NK121, In: Howell S, ed. *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy*. New York: Plenum. 1991:345-355.
18. O'Dwyer PJ, Hudes GR, Walczak J, Schilder R, LaCreta F, Rogers B, Cohen I, Kowal C, Whitfield L, Boyd RA. Phase I and pharmacokinetic study of the novel platinum analogue CI-973 on a 5-daily dose schedule. *Cancer Res* 1992; 52:6746-6753.
19. Theriault RL, Cohen IA, Esparza L, Kowal C, Raber MN. Phase I clinical evaluation of [SP-4-3(R)]-[1,1-cyclobutanedicarboxylate(2-)](2-methyl-1,4-butanediamine-N,N^1) platinum in patients with metastatic tumors. *Cancer Chemother Pharmacol* 1993; 31:333-337.

20. Kelland LR, Abel G, McKeage MJ, Jones M, Goddard PM, Valenti M, Murrer BA, Harrap KR. Preclinical antitumor evaluation of bis-acetato-ammine-dichloro-cyclohexylamine platinum(IV): an orally active platinum drug. *Cancer Res* 1993; 53:2581–2586.
21. McKeage MJ, Kelland LR, Boxall FE, Valenti MR, Jones M, Goddard PM, Gwynne J, Harrap KR. Schedule dependency of orally administered bis-acetato-ammine-dichloro cyclohexylamine-platinum (IV) (JM216) *in vivo*. *Cancer Res* 1994; 54:4118–4122.
22. McKeage MJ, Mistry P, Ward J, Boxall FE, Loh S, O'Neill C, Ellis P, Kelland LR, Morgan SE, Murrer BA, Santabarbara P, Harrap KR, Judson IR. A phase I and pharmacokinetic study of oral bis-acetato-ammine-dichloro cyclohexylamine platinum(IV) (JM216) on a single dose (q21day) administration schedule. *Cancer Chemother Pharmacol* 1995; 36:451–458.
23. Burchenal JH, Kalaher K, Dew K, Lokys L. Rationale for development of platinum analogs. *Cancer Treat Rep* 1979; 63:1493–1498.
24. Goddard PM, Valenti MR, Harrap KR. The role of murine tumour models and their acquired platinum-resistant counterparts in the evaluation of novel platinum antitumour agents: a cautionary note. *Ann Oncol* 1991; 2:535–540.
25. Harrap KR, Jones M, Siracky J, Pollard L, Kelland LR. The establishment, characterization and calibration of human ovarian carcinoma xenografts for the evaluation of novel platinum anticancer drugs. *Ann Oncol* 1990; 1:65–76.
26. Schilder RJ, LaCreta FP, Perez RP, Johnson SW, Brennan JM, Rogatko A, Nash S, McAleer C, Hamilton TC, Roby D, Young RC, Ozols RF, O'Dwyer PJ. Phase I and pharmacokinetic study of Ormaplatin (Tetraplatin, NSC 363812) administered on a day 1 and day 8 schedule. *Cancer Res* 1994; 54:709–717.
27. O'Rourke TJ, Weiss GR, New P, Burris HA III, Rodriguez G, Eckhardt J, Hardy J, Kuhn JG, Fields S, Clark GM, von Hoff DD. Phase I clinical trial of ormaplatin (tetraplatin, NSC 363812). *Anticancer Drugs* 1994; 5:520–526.
28. Extra JM, Espie M, Calvo F, Ferme C, Mignot, L, Marty M. Phase I study of oxaliplatin in patients with advanced cancer. *Cancer Chemother Pharmacol* 1990; 25:299–303.
29. Levi FA, Zidani R, Vannetzel J-M, Perpoint B, Focan C, Faggiulo R, Chollet P, Garfi C, Itzhaki M, Dogliotti L, Iacobelli S, Adam R, Kunstlinger F, Gastiaburu J, Bismuth H, Jasmin C, Misset J-L. Chronomodulated versus fixed infusion-rate delivery of ambulatory chemotherapy with oxaliplatin, fluorouracil, and folinic acid (leucovorin) in patients with colorectal cancer metastases: a randomized multi-institutional trial. *J Natl Cancer Inst* 1994; 86:1608–1617.
30. Perez-Soler R, Lopez-Berestein G, Lautersztain J, Al-Baker S, Francis K, Macias-Kiger D, Raber MN, Khokhar AR. Phase I clinical and pharmacological study of liposome-entrapped *cis*-Bis-neodecanoato-*trans*-R,R-1,2-diaminocyclohexane platinum(II). *Cancer Res* 1990; 50: 4254–4259.
31. Gietema JA, de Vries EGE, Sleijfer DTH, Willemse PHB, Guchelaar H-J, Uges DRA, Aulenbacher P, Voegeli R, Mulder NH. A phase I study of 1,2-diamminomethyl-cyclobutane-platinum (II) lactate (D-19466; lobaplatin) administered daily for 5 days. *Br J Cancer* 1993; 67: 396–401.
32. Gietema JA, Guchelaar H-J, deVries EGE, Aulenbacher P, Sleijfer Dth, Mulder NH. A phase I study of lobaplatin (D-19466) administered by 72h continuous infusion. *Anticancer Drugs* 1993; 4:51–55.
33. Knox RJ, Friedlos F, Lydall DA, Roberts JJ. Mechanism of cytotoxicity of anticancer platinum drugs: Evidence that cisdiamminedichloro platinum(II) and cisdiammine(1,1-cyclobutanedicarboxylato) platinum (II) differ only in the kinetics of their interaction with DNA. *Cancer Res* 1986; 46:1972–1979.
34. Fichtinger-Schepman AMJ, van der Veer JL, den Hartog JHJ, Lohman PHM, Reedijk J. Adducts of the antitumor drug cis-Diamminedichloroplatinum(II) with DNA: formation, identification and quantitation. *Biochemistry* 1985; 24:707–713.
35. Eastman A. Reevaluation of interaction of cis-dichloro(ethylenediamine) platinum(II) with DNA. *Biochemistry* 1986; 25:3912–3915.
36. Grimaldi KA, McAdam SR, Souhami RL, Hartley JA. DNA damage by anti-cancer agents resolved at the nucleotide level of a single copy gene: evidence for a novel binding site for cisplatin in cells. *Nucleic Acids Res* 1994; 22:2311–2317.
37. Connors TA, Cleare MJ, Harrap KR. Structure-activity relationships of the antitumor platinum coordination complexes. *Cancer Treat Rep* 1979; 63:1499–1502.

38. Eastman A, Barry MA. Interaction of trans-diamminedichloroplatinum(II) with DNA: formation of monofunctional adducts and their reaction with glutathione. *Biochemistry* 1987; 26: 3303–3307.
39. 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.
40. Szymkowski DE, Yarema K, Essigmann JM, Lippard SJ, Wood RD. An intrastrand d(GpG) platinum crosslink in duplex M13 DNA is refractory to repair by human cell extracts. *Proc Natl Acad Sci USA* 1992; 89:10,772–10,776.
41. Bellon SF, Coleman JH, Lippard SJ. DNA unwinding produced by site-specific intrastrand cross-links of the antitumor drug *cis*-Diamminedichloroplatinum(II). *Biochemistry* 1991; 30: 8026–8035.
42. Zwelling LA, Anderson T, Kohn KW. DNA-protein and DNA interstrand cross-linking by cis- and trans- platinum(II) Diamminedichloride in L1210 mouse leukemia cells and relation to cytotoxicity. *Cancer Res* 1979; 39:365–369.
43. Johnson SW, Perez RP, Godwin AK, Yeung AT, Handel LM, Ozols RF, et al. Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. *Biochem Pharmacol* 1994; 47:689–697.
44. Zhen W, Link CJ Jr, O'Connor PM, Reed E, Parker R, Howell SB, Bohr VA. Increased gene-specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines. *Mol Cell Biol* 1992; 12:3689–3698.
45. Brabec V, Leng M. DNA interstrand cross-links of trans-diamminedichloroplatinum(II) are preferentially formed between guanine and complementary cytosine residues. *Proc Natl Acad Sci USA* 1993; 90:5345–5349.
46. Eastman A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells* 1990; 2:275–280.
47. Ormerod MG, O'Neill CF, Robertson D, Harrap KR. Cisplatin induces apoptosis in a human ovarian carcinoma cell line without concomitant internucleosomal degradation of DNA. *Exp Cell Res* 1994; 211:231–237.
48. Andrews PA, Howell SB. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. *Cancer Cell* 1990; 2:35–43.
49. Loh SY, Mistry P, Kelland LR, Abel G, Murrer BA, Harrap KR. Reduced drug accumulation as a major mechanism of acquired resistance to cisplatin in a human ovarian carcinoma cell line: circumvention studies using novel platinum(II) and (IV) ammine/amine complexes. *Br J Cancer* 1992; 66:1109–1115.
50. Gately DP, Howell SB. Cellular accumulation of the antitumour agent cisplatin: a review. *Br J Cancer* 1993; 67:1171–1176.
51. Sharp SY, Rowlands MG, Jarman M, Kelland LR. Effects of a new antioestrogen, idoxifene, on cisplatin- and doxorubicin-sensitive and -resistant human ovarian carcinoma cell lines. *Br J Cancer* 1994; 70:409–414.
52. Kawai K, Kamatani N, Georges E, Ling V. Identification of a membrane glycoprotein overexpressed in murine lymphoma sublines resistant to cis-diamminedichloroplatinum(II). *J Biol Chem* 1990; 265:13,137–13,142.
53. Bernal SD, Speak JA, Boeheim K, Dreyfuss AI, Wright JE, Teicher BA, Rosowsky A, Tsao SW, Wong YC. Reduced membrane protein associated with resistance of human squamous carcinoma cells to methotrexate and cis-platinum. *Mol Cell Biochem* 1990; 95:61–70.
54. Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC, Anderson ME. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. *Proc Natl Acad Sci USA* 1992; 89:3070–3074.
55. Mistry P, Kelland LR, Abel G, Sidhar S, Harrap KR. The relationships between glutathione, glutathione-S-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. *Br J Cancer* 1991; 64:215–220.
56. Ishikawa T, Ali-Osman F. Glutathione-associated *cis*-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. *J Biol Chem* 1993; 268:20,116–20,125.
57. Ishikawa T, Wright CD, Ishizuka H. GS-X pump is functionally overexpressed in *cis*-Diamminedichloroplatinum(II)-resistant human leukemia HL-60 cells and down-regulated by cell differentiation. *J Biol Chem* 1994; 269:29085–29093.
58. Muller M, Meijer C. Zaman GJR, Borst P, Scheper RJ, Mulder NH, et al. Overexpression of

the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc Natl Acad Sci USA* 1994; 91:13,033-13,037.
59. Kelley SL, Basu A, Teicher BA, Hacker MP, Hamer DH, Lazo JS. Overexpression of metallothionein confers resistance to anticancer drugs. *Science* 1988; 241:1813-1815.
60. van der Zee AGJ, Hollema H, Suumeijer AJH, Krans M, Sluiter WJ, Willemse PHB, Aalders JG, de Vries EGE. Value of P-glycoprotein, glutathione S-transferase pi, c-*erb*B-2, and p53 as prognostic factors in ovarian carcinomas. *J Clin Oncol* 1995; 13:80-78.
61. Murphy D, McGown AT, Hall A, Cattan A, Crowther D, Fox BW. Glutathione S-transferase activity and isoenzyme distribution in ovarian tumour biopsies taken before or after cytotoxic chemotherapy. *Br J Cancer* 1992; 66:937-942.
62. Green JA, Robertson LJ, Clark AH. Glutathione S-transferase expression in benign and malignant ovarian tumours. *Br J Cancer* 1993; 68:235-239.
63. van der Zee AG, van Ommen B, Meijer C, Hollema H,, van Bladeren PJ, de Vries EG. Glutathione S-transferase activity and isoenzyme composition in benign ovarian tumours, untreated malignant ovarian tumours, and malignant ovarian tumours after platinum/cyclophosphamide chemotherapy. *Br J Cancer* 1992; 66:930-936.
64. Murphy D, McGowan AT, Crowther D, Mander A, Fox BW. Metallothionein levels in ovarian tumours before and after chemotherapy. *Br J Cancer* 1991; 63:711-714.
65. Beck DJ, Popoff S, Sancar A, Rupp WD. Reactions of the UVRABC excision nuclease with DNA damaged by diamminedichloroplatinum(II). *Nucleic Acids Res* 1985; 13:7395-7412.
66. Hansson J, Wood RD. Repair synthesis by human cell extracts in DNA damaged by cis- and trans- diammine dichloroplatinum(II). *Nucleic Acids Res* 1989; 17:8073-8091.
67. Shellard SA, Hosking LK, Hill BT. Anomalous relationship between cisplatin sensitivity and the formation and removal of platinum-DNA adducts in two human ovarian carcinoma cell lines in vitro. *Cancer Res* 1991; 51:4557-4564.
68. O'Neill CF, Orr RM, Kelland LR, Harrap KR. Comparison of platinum binding to DNA and removal of total platinum adducts and interstrand crosslinks in three human ovarian carcinoma cell lines sensitive and resistant to cisplatin. *Cell Pharmacol* 1995; 2:1-7.
69. Dabholkar M, Bostick-Bruton F, Weber C, Bohr VA, Egwuagu C, Reed E. ERCC1 and ERCC2 expression in malignant tissues from ovarian cancer patients. *J Natl Cancer Inst* 1992; 84:1512-1517.
70. Dabholkar M, Vionnet J, Bostick Bruton F, Yu JJ, Reed E. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. *J Clin Invest* 1994; 94:703-708.
71. Kelland LR, Mistry P, Abel G, Freidlos F, Loh SY, Roberts JJ, et al. Establishment and characterization of an in vitro model of acquired resistance to cisplatin in a human testicular nonseminomatous germ cell line. *Cancer Res* 1992; 52:1710-1716.
72. Hill BT, Scanlon KJ, Hansson J, Harstrick A, Pera M, Fichtinger-Schepman AMJ, et al. Deficient repair of cisplatin-DNA adducts identified in human testicular teratoma cell lines established from tumours from untreated patients. *Eur. J Cancer* 1994; 30:832-837.
73. Chu G, Chang E. Cisplatin-resistant cells express increased levels of a factor that recognizes damaged DNA. *Proc Natl Acad Sci USA* 1990; 87:3324-3328.
74. Bruhn SL, Pil PM, Essigmann JM, Housman DE, Lippard SJ. Isolation and characterization of human cDNA clones encoding a high mobility group box protein that recognizes structural distortions to DNA caused by binding of the anticancer agent cisplatin. *Proc Natl Acad Sci USA* 1992; 89:2307-2311.
75. Hughes EN, Engelsberg BN, Billings PC. Purification of nuclear proteins that bind to cisplatin-damaged DNA: identity with high mobility group proteins 1 and 2. *J Biol Chem* 1992; 267:13,520-13,527.
76. Pil PM, Lippard SJ. Specific binding of chromosomal protein HMG1 to DNA damaged by the anticancer drug cisplatin. *Science* 1992; 256:234-237.
77. Brown SJ, Kellett PJ, Lippard SJ. lxr1, a yeast protein that binds to platinated DNA and confers sensitivity to cisplatin. *Science* 1993; 261:603-605.
78. Huang J-C, Zamble DB, Reardon JT, Lippard SJ, Sancar A. HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. *Proc Natl Acad Sci USA* 1994; 91:10,394-10,398.

79. Chao CC-K, Huang S-L, Lee L-Y, Lin-Chao S. Identification of inducible damage-recognition proteins that are overexpressed in HeLa cells resistant to cis-diamminedichloroplatinum(II). *Biochem J* 1991; 277:875–878.
80. Clugston CK, McLaughlin K, Kenny MK, Brown R. Binding of human single-stranded DNA binding protein to DNA damaged by the anticancer drug cis-diamminedichloroplatinum (II). *Cancer Res* 1992; 52:6375–6379.
81. Isonishi S, Hom DK, Thiebaut FB, Mann SC, Andrews PA, Basu A, Lazo JS, Eastman A, Howell SB. Expression of c-Ha-ras oncogene in mouse NIH 3T3 cells induces resistance to cis-platin. *Cancer Res* 1991; 51:5903–5909.
82. Sklar MD, Prochownik EV. Modulation of cis-platinum resistance in Friend erythroleukemia cells by *c-myc*. *Cancer Res* 1991; 51:2118–2123.
83. Scanlon KJ, Jiao L, Funato T, Wang W, Tone T, Rossi JJ, Kashani-Sabet M. Ribozyme-mediated cleavage of *c-fos* mRNA reduces gene expression of DNA synthesis enzymes and metallothionein. *Proc Natl Acad Sci USA* 1991; 88:10,591–10,595.
84. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993; 74:957–967.
85. Peng H, Hogg D, Malkin D, Bailey D, Gallie BL, Bulbul M, et al. Mutations of the p53 gene do not occur in testis cancer. *Cancer Res* 1993; 53:3574–3578.
86. Fan S, El-Diery WS, Bae I, Freeman J, Jondle D, Bhatia K, Fornace AJ Jr, Magrath I, Kohn KW, O'Connor PM. p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res* 1994; 54:5824–5830.
87. Kimura E, Enns RE, Alcaraz JE, Arboleda J, Salmon DJ, Howell SB. Correlation of the survival of ovarian cancer patients with mRNA expression of the 60-kD heat shock protein HSP-60. *J Clin Oncol* 1993; 11:891–898.
88. Smith ML, Chen I, Zhan Q, Bae I, Chen C, Gilmer TM, et al. Interaction of the p53-regulated protein GAdd45 with proliferating cell nuclear antigen. *Science* 1994; 266:1376–1380.
89. Howell SB, Isonishi S, Christen RC, Andrews PA, Mann SC, Hom D. Signal transduction pathway regulation of DDP sensitivity. In: Howell SB, ed. *Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy*. New York: Plenum. 1991:173–179.
90. Teicher BA, Herman TS, Holden SA, Wang YY, Pfeffer MR, Crawford JW, et al. Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. *Science* 1990; 247:1457–1461.
91. Farrell N, Kelland LR, Roberts JD, Van Beusichem M. Activation of the *trans* geometry in platinum antitumor complexes: a survey of the cytotoxicity of *trans* compounds containing planar ligands in murine L1210 and human tumor panels and studies on their mechanism of action. *Cancer Res* 1992; 52:5065–5072.
92. Kelland LR, Barnard CFJ, Mellish KJ, Jones M, Goddard PM, Valenti M, Bryant A, Murrer BA, Harrap KR. A novel *trans*-platinum coordination complex possessing in vitro and in vivo antitumour activity. *Cancer Res* 1994; 54:5618–5622.
93. Coluccia M, Nassi A, Loseto F, Boccarelli A, Mariggio MA, Giordano D, et al. A trans-platinum complex showing higher antitumor activity than the Cis congeners. *J Med Chem* 1993; 36:510–512.
94. Farrell N, Qu Y, Hacker MP. Cytotoxicity and antitumor activity of bis(platinum) complexes. A novel class of platinum complexes active in cell lines resistant to both cisplatin and 1,2-diaminocyclohexane complexes. *J Med Chem* 1990; 33:2179–2184.
95. Webster LK, Deacon GB, Buxton DP, Hillcoat BL, James AM, Roos IAG, et al. cis-Bis(pyridine) platinum(II) organoamides with unexpected growth inhibition properties and antitumor activity. *J Med Chem* 1992; 35:3349–3353.
96. Kim D, Kim G, Gam J, Cho Y, Kim H, Tai J, et al. Synthesis and antitumor activity of a series of [2-substituted-4,5-bis(aminomethyl)-1,3-dioxolane] platinum(II) complexes. *J Med Chem* 1994; 37:1471–1485.
97. Ozols RF, Ostchega Y, Myers CE, Young RC. High-dose cisplatin in hypertonic saline in refractory ovarian cancer. *J Clin Oncol* 1985; 3:1246–1250.
98. Calvert AH, Newell DR, Gore ME. Future directions with carboplatin: Can therapeutic monitoring, high-dose administration, and hematologic support with growth factors expand the spectru compared with cisplatin? *Semin Oncol* 1992; 19:155–163.

99. Kaye SB, Lewis CR, Paul J, Duncan ID, Gordon HK, Kitchener HC, Cruickshank DJ, Atkinson RJ, Soukop M, Rankin EM, Cassidy J, Davis JA, Reed NS, Crawford SM, MacLean A, Swapp GA, Sarkar TK, Kennedy JH, Symonds RP. Randomised study of two doses of cisplatin with cyclophosphamide in epithelial ovarian cancer. *Lancet* 1992; 340:329-333.
100. Jodrell DI, Egorin MJ, Canetta RM, Langenberg P, Goldbloom EP, Burroughs JN, Goodlow JL, Tan S, Wiltshaw E. Relationships between carboplain exposure and tumor response and toxicity in patients with ovarian cancer.*J Clin Oncol* 1992; 10:520-528.
101. Hills CA, Kelland LR, Abel G, Siracky J, Wilson AP, Harrap KR. Biological properties of ten human ovarian carcinoma cell lines: calibration *in vitro* against platinum complexes. *Br J Cancer* 1989; 59:527-534.
102. Kelland LR, Mistry P, Abel G, Loh SY, O'Neill CF, Murrer BA, Harrap KR. Mechanism-related circumvention of acquired cis-diamminedichloroplatinum(II) resistance using two pairs of human ovarian carcinoma cell lines by ammine/amine platinum(IV) dicarboxylates. *Cancer Res* 1992; 52:3857-3864.
103. Llory JF, Soulie P, Cvitkovic E, Misset JL. Feasibility of high-dose platinum delivery with combined carboplatin and oxaliplatin. *J Natl Cancer Inst* 1994; 86:1098-1099.
104. Timmer Bosscha, H., Mulder, N.H., and de Vries, E.G. Modulation of cis-diamminedichloroplatinum(II) resistance: a review. *Br J Cancer* 1992; 66:227-238.
105. Trimble EL, Adams JD, Vena D, Hawkins MJ, Friedman MA, Fisherman JS, et al. Paclitaxel for platinum-refractory ovarian cancer: Results from the first 1,000 patients registered to national Cancer Institute Treatment Referral Center 9103. *J Clin Oncol* 1993; 11:2405-2410.

5 Anthracyclines

Trevor W. Sweatman, PhD and Mervyn Israel, PhD

CONTENTS

1. INTRODUCTION: DAUNORUBICIN AND DOXORUBICIN: PROTOTYPICAL ANTHRACYCLINES

The clinical activity of actinomycin D against Wilm's pediatric kidney tumor and choriocarcinoma in the early to mid-1950s resulted in a widespread effort to discover other antibiotic substances for potential use in the treatment of malignancies. As a result, cancer chemotherapy was altered immeasurably more than 30 years ago by the independent discoveries by Grein (*1*), Dubost (*2*), and their colleagues of an anthracycline antibiotic, derived from a *Streptomyces* soil mold, with significant experimental activity against leukemias. Structurally this intensely red product whose common name, daunorubicin, reflects both its Italian (daunomicina) and French (rubidomycine) origins, consists of a planar anthraquinone attached to a daunosamine sugar; the latter has been shown to stabilize intercalation of the molecule with DNA, which for many years was considered to be the principal mechanism of anthracycline action (*3*). Subsequently, although not necessarily through DNA interaction, this structural requirement has proven essential for the multiple potential mechanisms of action now identified for anthracyclines, including inhibition of DNA topoisomerase I (*4*) and II (*5*), inhibition of helicases (*6*), generation of toxic free radicals (*7*), alteration of membrane structure and function (*8–10*), and endonucleolytic cleavage (*11*).

Encouraging early activity data for daunorubicin (*12–14*) prompted a search for other anthracyclines with improved therapeutic activity, a quest that continues to the present day. In 1969, DiMarco and colleagues (*15*) reported that doxorubicin (Adriamycin®), a closely related (14-hydroxy) analog of daunorubicin (Fig. 1), possessed superior preclinical activity to daunorubicin, with a broad spectrum of activity against leukemias, lymphomas, and solid tumors (*16*). Notwithstanding more recent clinical data supporting daunorubicin activity against a variety of solid tumors (*17–19*),

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

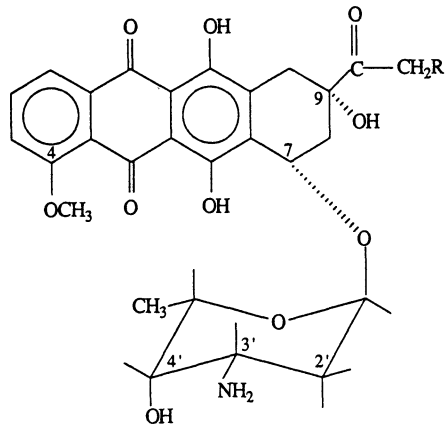


Fig. 1. Structure of prototypical anthracycline agents, daunorubicin and doxorubicin.

daunorubicin has become associated more closely with combination chemotherapy of leukemia, where it is considered to be less toxic than doxorubicin (*20*). By contrast, doxorubicin is employed not only as a single agent, but also as an important component of many combination chemotherapy regimens for the treatment of lymphoma, breast, and small-cell lung carcinoma, and sarcomas (*21*). Despite these successes, doxorubicin has proven ineffective against many tumors, including colon cancer, melanoma, chronic lymphocytic leukemias, CNS tumors, and renal cancer.

2. LIMITATIONS TO ANTHRACYCLINE THERAPY

2.1. Toxicities

In common with most anticancer agents that possess a narrow therapeutic index, early reports of anthracycline clinical activity (*12–14*) documented acute toxicities against rapidly dividing cells, e.g., myelosuppression, gastrointestinal toxicity (nausea and vomiting), alopecia, and stomatitis. Indeed, leukopenia is the dose-limiting toxicity with all clinically tested anthracyclines. Of equal importance was the recognition of the acute and chronic effects of doxorubicin and daunorubicin on the heart. Electrocardiographic changes have been documented during anthracycline administration (*22*), although the significance of these phenomena are equivocal (*23,24*). However, it is the insidious dose-dependent cardiomyopathy produced by these agents that has the greatest impact on their clinical utility. Cardiac tissue that, as a result of mitochondrial multiplicity and low antioxidant levels (*25*), appears particularly susceptible to lipid peroxidation (*26*), undergoes progressive dose-related irreversible vacuolization and myocyte necrosis in response to doxorubicin and daunorubicin exposure. Correlation of drug exposure with overt signs of congestive heart failure have led to the now familiar recommendations of 450–550 mg/m^2 being placed on total doxorubicin and daunorubicin dose exposure (*22,27*). However, this "safe" dose may be illusory; combination chemotherapy and thoracic irradiation are both known to exacerbate myocardial damage at lower cumulative anthracycline levels (*28*); Lipschultz and coworkers (*29,30*) have identified cardiac abnormalities in patients who have received total doxorubicin doses as low as 45 mg/m^2. In addition, the aging of large numbers of long-term cancer survivors has produced increasing evidence of heart

failure many years following doxorubicin or daunorubicin exposure (*31–35*). Thus, the dilemma for the oncologist is how best to administer these highly active antitumor agents while at the same time minimizing the potential for the cancer-free patient to subsequently succumb to late heart failure. To provide for greater safety in the continued use of daunorubicin and doxorubicin, there has been active research interest in the areas of dose schedule optimization, drug formulation, and antioxidant coadministration.

Despite almost three decades of use, optimal dosage regimens have yet to be fully defined (*36,37*). In consideration that drug fractionation or drug infusion reduces peak plasma anthracycline levels without reducing overall drug exposure (as measured by the area under the concentration time curve; AUC), a marked reduction in cardiotoxicity has been reported following weekly (*27,38*) or 48–96 h doxorubicin infusions (*39,40*). However, in demonstrating a correlation between high peak serum anthracycline concentrations and therapeutic response (*41–43*), others have suggested better tumor drug penetration by higher drug levels (*38,44–46*). Given the wide interpatient variability in anthracycline pharmacokinetics and the relatively small number of subjects in the studies conducted to date, deValeriola (*36*) has advocated larger prospective studies to validate these data.

Although liposomes remain a controversial area, following initial problems with physicochemical stability and marked extravasation of liposomes into the reticuloendothelial system, their potential value in reducing anthracycline cardiotoxicity has not been overlooked (*47*). Initial experimental studies with liposomal drug encapsulation have documented superior activity to the corresponding free agent (*48–51*). Among the number of clinical trials of liposomal encapsulated doxorubicin presently under way, significant antitumor activity and reduced toxicity of liposomal daunorubicin (DaunoXone®) have been demonstrated against Kaposi's sarcoma (*52,53*). Conversely, liposomal doxorubicin (Lipodox®) produced no evidence of activity against advanced renal cell carcinoma (*54*). The ultimate potential utility of liposomal drug formulations must await the outcome of such clinical trials.

Implication of intracellular free iron in complexing with the planar anthraquinone portion of the anthracycline molecule to liberate free radicals and produce cardiomyopathy (*55*) had led to the evaluation of a number of iron-chelating agents, principally those related to edetic acid (EDTA), as cardioprotective agents. Dexrazoxane (ICRF-187; Zinecard®) has undergone the most extensive preclinical and clinical evaluation. It has proven effective in reducing experimental free radical generation (*56,57*) and in reducing clinical cardiotoxicity of breast cancer therapy (*58–60*), although at the expense of a slight increase in myelotoxicity. However, the effects of this chelator on antitumor efficacy remain equivocal (*61,62*); for this reason, Zinecard, which has recently received FDA approval for use in conjunction with doxorubicin against metastatic breast cancer, is presently limited to use in patients who have already received >300 mg/m^2 doxorubicin (*63*).

2.2. Drug Resistance

Inability to achieve cytotoxic drug concentrations in the tumor with consequent treatment failure may relate, in part, to pharmacokinetic considerations of drug sanctuary or metabolism to inactive products, but more than likely to problems associated with the development of cellular drug resistance in subpopulations of tumor cells.

Since the first reports of in vitro drug resistance in 1968 (*64*) and 1970 (*65*), experimental investigation of mammalian cells has highlighted the many ways in which drug cytotoxicity can be circumvented. Of the numerous potential resistance mechanisms that have been identified, only two, the multidrug resistance (MDR) phenotype and the multidrug resistance-associated protein (MRP), have demonstrated some correlation with clinical drug resistance. Thus, the well-characterized MDR phenotype, with a membrane-associated P-glycoprotein (P-gp) encoded in human cells by the MDR 1 gene (*66,67*), confers crossresistance to a broad spectrum of natural product drugs, including the anthracyclines. Although distribution of P-gp in secretory cells of the kidney, liver, pancreas, jejunum, and colon suggests some physiological role in normal tissue, it is now recognized that overexpression of MDR 1 mRNA or P-gp is associated with poor prognosis in human tumors, e.g., acute leukemia (*68*). A further membrane-associated efflux mechanism, the MRP pump, has now been characterized (*69,70*) with the ability to extrude natural product drugs from the cell against the concentration gradient. Unlike P-gp, whose function can be interrupted by MDR-reversing agents, such as verapamil or cyclosporin A, MRP action is not significantly affected by these reversing agents (*71,72*). In common with P-gp, overexpression of MRP has demonstrated some clinical correlation with disease prognosis, e.g., for leukemias (*73*) and neuroblastoma (*74*). Both of these resistance mechanisms have received extensive literature reviews.

Although these membrane protein pumps confer on the tumor cell the ability to reduce intracellular drug concentrations, for anthracyclines there is a further potential mechanism of resistance that does not involve drug trafficking, but rather altered intracellular topoisomerase I and II enzyme levels. These essential nuclear enzymes, which are intimately involved in cell division by controlling the topology of the DNA during strand separation and replication, catalyze the relaxation/supercoiling and catenation/decatenation of DNA. In common with a number of anticancer agents, such as the epipodophyllotoxins, aminoacridines, and mitoxantrone, daunorubicin and doxorubicin inhibit cell division through formation of stable drug–DNA–topoisomerase II complexes (*75*). There is also evidence that some of the morpholinylanthracycline analogs produce the same effect through an interaction with topoisomerase I rather than topoisomerase II. Such agents differ from actinomycin D (Dactinomycin), which acts against both enzymes, by demonstrating no apparant interaction with topoisomerase II. In consequence of these drug–enzyme interactions, the inhibited religation of DNA is manifest as protein-associated single- and double-strand breaks (*76*). Resistance to topoisomerase-directed drugs is imparted by a decrease in the topoisomerase protein and/or a decline in catalytic activity (*77*). Although topoisomerases remain a fertile research area, with extensive literature citation, at the present time there is no convincing correlation between altered topoisomerase activity and clinical drug resistance.

Two approaches have been used to circumvent drug resistance clinically, combination chemotherapy, and resistance modifying agents. As noted above, the P-gp pump confers crossresistance on the cell, thereby negating combination chemotherapy involving other natural product drugs. A number of agents have shown the ability to competitively inhibit P-gp activity in vitro; these include steroids, nonimmunosuppressive cyclosporin A derivatives, and calcium channel antagonists, such as verapamil. Unfortunately, verapamil has itself proven toxic at the clinical levels required for

Table 1
Comparison of Anthracycline Analog Clinical Activity with that of Doxorubicin

Analog	*Clinical findings*
Older agents	
Carminomycin	No advantage over doxorubicin
Esorubicin	Inferior to doxorubicin
Marcellomycin	Toxicity
Quelamycin	Toxicity
Rubidizone	Toxicity
Current agents	
Aclarubicin	No advantage over doxorubicin
Pirarubicin	Comparable activity (less myelosuppression)
Epirubicin	Comparable activity (less toxicity)
Idarubicin	Activity data equivocal

reversal of drug resistance. Additionally, interaction of calcium channel antagonists with cardiotoxicity has been demonstrated experimentally by an increase in doxorubicin-induced myocyte damage in neonatal rat myocytes exposed to verapamil (*78*). Clinical trials are presently under way with other resistance modifiers.

3. NEWER ANTHRACYCLINE DRUG DEVELOPMENT

Although anthracyclines are of significant clinical utility in chemotherapy, cardiotoxicity and tumor drug insensitivity (natural or acquired) are clearly major impediments to optimal clinical prognosis. Historically, the development of new anthracycline antitumor agents has focused primarily on improving the spectrum of clinical activity, reducing the cardiotoxicity, and overcoming known mechanisms of clinical drug resistance. Approaches to new anthracycline analogs include primarily isolation and evaluation of fermentation-derived antibiotic substances and semisynthetic modification of parental antibiotics. Relatively little of clinical utility has come from the former approach, the most significant contributions being represented by carminomycin (Table 1) and aclacinomycin (*vide infra*). Many more significant leads have come from structure–activity relationship studies of synthetically derived products. However, despite the considerable promise of this class of antitumor agents and the extensive research conducted to date, there have been no new anthracyclines yet introduced to the marketplace whose clinical activity is superior to that of doxorubicin or daunorubicin. Based on these facts, a recent review article by Weiss (*79*) posed the question, "Will we ever find a better doxorubicin?"

Of necessity, many of the earlier anthracycline analogs were developed for clinical trials at a time when our understanding of drug resistance was in its infancy. Selection of potential therapeutic agents was based initially on in vitro activity, usually against P388 leukemia cells. Use of such tools failed to provide detailed knowledge of the structure–activity relationships of the various analogs relative to drug resistance and, as a consequence, many interesting leads may have been overlooked (*80*). However, the field of drug resistance has now matured to the extent that many mammalian cell

lines that are drug resistant through a variety of well-defined mechanisms, e.g., cells expressing the MDR, MRP, and altered topoisomerase II (at-MDR) phenotypes, are now available as research tools. These are proving invaluable in the preclinical evaluation of new anthracycline analogs and in a more rigorous evaluation of the structure–activity relationships.

3.1. The First Wave of Second-Generation Analogs

Over the 30-yr span since the original discovery of doxorubicin and daunorubicin, several dozen anthracycline analogs have achieved the milestone of clinical evaluation as a result of promising preclinical studies (Table 1). Consistent with the then prevailing mechanistic view for anthracyclines, the earliest analogs were all based on the paradigm of avid DNA complexation. In general, these agents failed to show significant therapeutic advantage over the prototypical antibiotics, and trials with some were discontinued. However, as indicated below, several of the agents are currently marketed in various regions of the world. These appear to offer comparable antitumor activity, but somewhat lesser toxicity than the parental agent; none of them has demonstrated any clear therapeutic advantage over doxorubicin, especially against anthracycline-resistant tumors.

3.2. Clinically Approved Analogs

Aclacinomycin (Aclarubicin®), originally developed in Japan, is presently available in France, although studies in the US have failed to demonstrate any therapeutic advantage over doxorubicin and daunorubicin (*79*). Likewise, tetrahydropyranyldoxorubicin (Pirarubicin®) is marketed in France and Japan and has undergone clinical trials in the U.S. Data for this agent, recently reviewed (*81*), have demonstrated only comparable antitumor activity to doxorubicin. The major difference between them appears to be one of lesser acute toxicity (leukopenia, alopecia, nausea, vomiting) and, possibly, risk of cardiotoxicity (up to 700 mg/m^2) for Pirarubicin. However, these differences do not appear to provide a compelling case for further trials of this agent in the US.

4′-Epidoxorubicin (Epirubicin), presently marketed worldwide with the exception of the US, was originally selected on the basis of reduced experimental cardiotoxicity compared to doxorubicin (*82*). Clinical trials with this agent have confirmed that when doxorubicin and epirubicin are compared on a equimolar basis, the latter does indeed demonstrate less acute and cardiotoxicity (*83,84*), primarily as a result of pharmacokinetic differences between the two agents. Epirubicin has a much faster conversion to its 13-carbinol metabolite (4′-epidoxorubicinol), with extensive glucuronide conjugation facilitating the excretion process (*85*). However, when the two agents are compared on an equi-myelosuppressive basis, their acute toxicity is comparable (*86*) and epirubicin clearly produces fatal cardiotoxicity (*87,88*), the cumulative dose limit for this agent being 900–1000 mg/m^2 (about 9 mo of therapy). Thus, epirubicin shows some advantage in terms of toxicity, but in common with many of the clinically approved analogs, its spectrum of clinical activity is not sufficiently different from that of doxorubicin that this analog represents an therapeutic advantage against anthracycline-insensitive tumors.

4-Demethoxydaunorubicin (idarubicin; Idamycin®) is presently the only anthracycline analog that is marketed worldwide, being active against leukemias and breast

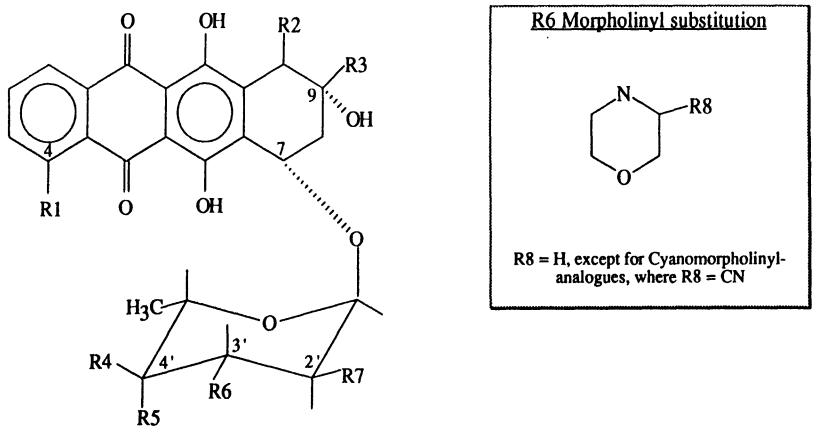
carcinoma both by iv and oral administration, although the latter formulation is not marketed in the US. In contrast to daunorubicin and doxorubicin, whose principal metabolic products, the respective 13-carbinol species, are less cytotoxic than the parental agent, 4-demethoxydaunorubicinol (idarubicinol) has demonstrated equipotency with idarubicin in cell-culture systems (*89*). However, this difference does not appear to have provided a significant advantage, since idarubicin demonstrates no clear-cut advantage over doxorubicin in terms of toxicity (*79*) or antitumor activity. Some iv drug studies demonstrate an advantage, whereas others fail to substantiate these findings (*90,91*). Comparison of oral idarubicin with iv doxorubicin has highlighted problems of variation in bioavailability (12–49%) of oral administered drug (*92*), resulting in lower response rates by this route (*93–95*). Data on in vitro activity against drug-resistant tumors are also equivocal, with some studies showing a lack of crossresistance between idarubicin and daunorubicin in MDR leukemic cell lines (*96*) and others finding crossresistance in relapsed tumor samples from children with acute lympoblastic leukemia (*97*). Certainly in clinical trials, idarubicin produced best antitumor activity in patients who had not previously received anthracycline therapy (*98*).

3.3. Newer Developmental Agents

If one were to summarize the record of accomplishments of anthracycline analog development up to this point, it is clearly one of unfulfilled promise, with the various "me too" agents showing only marginal clinical differences from their respective parental agents and none clearly demonstrating activity in anthracycline-insensitive or resistant tumors. Notwithstanding these disappointments, it has become increasingly apparent from structure–activity studies that only minor modifications of the anthracycline structure can result not only in inactive agents, but more importantly analogs with in vitro activity, not only against MDR tumor cell lines, but also those expressing MRP and at-MDR phenotypes. It should also be recognized that minor changes in structure can result in differences in the way in which these agents are metabolized and eliminated (*99*). The principal structural modifications have been made on: (1) the daunosamine sugar, whose structural features are implicated by many investigators (*see* Priebe et al. [*100*]) in DNA and cellular binding and intracellular drug transport, and/or (2) substitution at the C-14 position of the chromophore, which affects in vitro binding of drug with topoisomerase II, intracellular drug accumulation, and possibly intracellular drug targets.

Additionally, one cannot overlook the possible importance of the quinone structure in the anthracycline chromophore to the overall biological activity of such agents. Thus, the difference in the pattern of cytotoxicity seen for 5-iminodaunorubicin in the NCI's disease-oriented screen compared with doxorubicin is suggestive of a change in biological activity for this agent. Based on these data, Acton has proposed structure–activity studies using a series of phenazine-di-*N*-oxides as models (*101*); however, this worthwhile work is incomplete at present.

Several thousand analogs have been synthesized to data, and new ones appear with regularity in the scientific literature. As Acton (*101*) noted, "it has always been easier to find active analogues than to choose among them." For the sake of expediency then, we will consider only selected novel analogs that have either reached clinical trials or are in late-stage preclinical development and for which a considerable body of scientific data therefore exists. It is important to note that although these agents



Compound	R1	R2	R3	R4	R5	R6	R7
Morpholinyldoxorubicin	OCH_3	H	$COCH_2OH$	H	OH	see insert	H
MX2	OH	OH	CH_2CH_3	H	OH	see insert	H
AD 32	OCH_3	H	$COCH_2OCO(CH_2)_3CH_3$	H	OH	$NHCOCF_3$	H
AD 198	OCH_3	H	$COCH_2OCO(CH_2)_3CH_3$	H	OH	$NHCH_2C_6H_5$	H
ME2303	OCH_3	H	$COCH_2OCO(CH_2)_3$ COOH	H	OH	OH	F
Annamycin	H	H	$COCH_2OH$	OH	OH	OH	I

Fig. 2. Structures of anthracycline analogs in early clinical trials or advanced preclinical development.

are representative of the various types of modification that have been investigated, there may be other analogs from each group under active investigation around the world. Thus, the selected agents include: some of the morpholinyl-anthracycline derivatives that appear to possess different nuclear targets and to require metabolic activation for their full biological effect; the lipophilic *N*-alkyl- and *N*-acyl-analogs AD 32 and AD 198, which appear to possess novel mechanistic properties, in part unrelated to a topoisomerase II-directed target; two halogenated, deaminated topoisomerase II-directed analogs ME2303 and Annamycin; and the dual-mechanistic hybrid anthracycline–nitrosourea, AD 312. Chemical structures for all of these agents are to be found in Figs. 2 and 3.

3.3.1. Morpholinyl Anthracyclines: MX2

With the exception of studies with liposomally formulated daunorubicin and doxorubicin, there are currently no clinical trials of systemic anthracycline therapy in the US. However, a number of interesting morpholinyl analogs have been synthesized; some of these are currently undergoing clinical trials in Japan (*102*). This group of agents has arisen, in part, from the initial observations by Acton and colleagues (*103,104*) that cyclo-alkylation of the amino nitrogen on the daunosamine sugar, to reduce the basicity of the nitrogen and avoid P-gp-mediated efflux, dramatically increases antitumor activity (Fig. 2). Subsequently, a large number of analogs have been synthesized to investigate the effects of substitution on the morpholine ring. These efforts have yielded analogs that, in general, are 10–1000 times more potent than doxorubicin, in vitro. Of these, cyanomorpholinyl provided the most notable

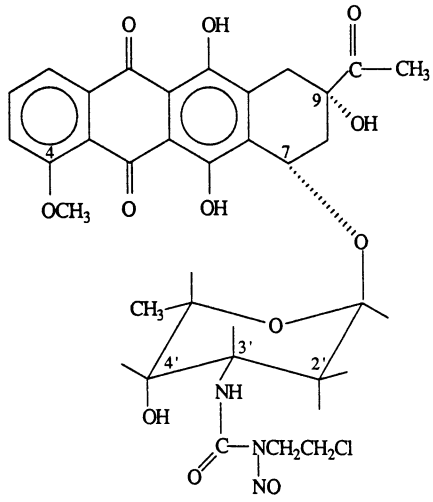


Fig. 3. Structure of the novel hydrid anthracycline-alkylator *N*-(2-chloroethyl)-*N*-nitrosoureidodaunorubicin (AD 312).

increase in biological activity with no evidence of either cardiotoxicity or crossresistance to doxorubicin (*103,105,106*). Unlike parental doxorubicin, methoxymorpholinyl-, morpholinyl- and cyanomorpholinyldoxorubicin all undergo covalent binding, producing DNA crosslinking in cells with an active metabolism, the last analog yielding cyanide in the process (*107*). This metabolically dependent DNA interaction, for which an intact morpholine ring structure appears critical, is considered the explanation for their greater antitumor activity vs doxorubicin in vivo, relative to in vitro studies (*108–110*). As noted earlier, mechanistic differences are evident between the actions of morpholinyl-doxorubicin and doxorubicin on topoisomerase (Table 2). Whereas doxorubicin produces protein-associated double-strand breaks in DNA, morpholinyl-doxorubicin yields only single-strand breaks (*111*). Subsequently, problems with the stability of the cyanomorpholinyl analog appear to have precluded its further development as a clinical agent. However, morpholine substitution remains an active area of interest; a number of analogs, e.g., MX2 and Ro31-3294, that incorporate this substituent have been investigated and have proven capable of circumventing P-gp resistance, at least in cell-culture systems. MX2 has reached the stage of clinical evaluation.

3′-Deamino-3′-morpholinyl-13-deoxo-10-hydroxycarminomycin (MX2) is presently in clinical trials in Japan against brain tumors, leukemias, lymphomas, and breast cancer. Preclinical data show that MX2, which is more lipophilic than doxorubicin or daunorubicin, has activity against implanted carcinomas and is effective against a doxorubicin-resistant P388 leukemia line in vitro and in vivo (*112*). This differs from the only partial effectiveness reported for the morpholinyldoxorubicin analog against such cells (*113*). Of potential significance, MX2 is orally active against P388 leukemia at serum levels comparable to those produced by iv drug administration; it also has some activity in an intracerebrally implanted L1210 leukemia model (*114*). The major dose-limiting toxicity of MX2 is myelosuppression, comparable to that of doxorubicin (*112*), although lower cardiotoxicity has been observed in a rabbit cardiotoxicity model (*112*). In escalating dose Phase I trials (1.5–54 mg/m^2, total dose), neutropenia

Table 2
Comparison of the Interactions of Morpholinyl-Anthracyclines and Doxorubicin with DNA and Mammalian Topoisomerase I and II

Anthracycline	*DNA intercalation*[a]	*DNA crosslinking*[b]	*DNA cleavage through*	
			Topoisomerase I[c]	*Topoisomerase II*[d]
Doxorubicin	+ +	−	−	+ +
Morpholinyldoxorubicin	+	−	+	−
Cyanomorpholinyldoxorubicin	−	+	(+)	−

[a]Drug intercalation was measured by use of a DNA topoisomerase I unwinding assay in the presence of excess enzyme.

[b]DNA crosslinking was measured by incubating the respective drugs with 5′-end-labeled DNA fragments and analyzing the product by agarose gel electrophoresis under denaturing conditions.

[c]Inhibition of DNA topoisomerase I catalytic activity was measured by relaxation of native supercoiled SV40 DNA in the presence of limited amounts of enzyme. The kinetics of the reactions in the presence and absence of test anthracyclines were compared.

[d]Inhibition of DNA topoisomerase II catalytic activity by the various test agents was measured in the presence of excess enzyme by cleavage of 3′-terminus end-labeled (^{32}P) *Ban*I-*Hpa*II SV40 DNA fragments and subsequent analysis by agarose gel electrophoresis.

For full experimental details, *see* ref(*111*).

and thrombocytopenia were evident as the dose-limiting toxicities (*102*); no objective responses were seen.

Despite these encouraging data, questions have been raised regarding the potential therapeutic variability of such drugs as the morpholinyl-anthracyclines, which rely, in part, on metabolic activation by cytochrome P450 enzymes for their full biological activity (*46*). If expression of this family of enzymes is as variable in tumors as it is in normal tissue, such as liver, this would predict potentially yet a further form of (partial) resistance to this type of agent. The full implications of this process for morpholinyl analogs must await clinical evaluation.

3.3.2. *N*-Acyl-Analogs: AD 32

A change in the basicity of the daunosamine nitrogen has also been effected by 3′-*N*-acyl substituents. However, simple *N*-acyl substituents on daunorubicin or doxorubicin, e.g., acetate, propionate, and so forth, are easily cleaved enzymatically and, as a consequence, such substituted antracyclines simply serve as prodrugs of the parent antibiotics. As discussed below, however, trifluoroacetylation yields a stable amide bond and results in an agent with remarkably different pharmacologic properties compared with doxorubicin. Various acyl substituents on the 14-carbinol moiety of trifluoracetylated anthracyclines have also been examined with a view to improving drug lipophilicity and more effective drug cell penetration. One such analog of current clinical interest is *N*-trifluoroacetyladriamycin-14-valerate (AD 32).

AD 32 differs from doxorubicin in having a five-carbon aliphatic ester at the 14-carbinol position and a trifluoroacetyl substitution on the glycosidic amino group. As a result of high lipid solubility, AD 32 rapidly traverses cell membranes, and drug-associated fluorescence accumulates in the cytoplasm, whereas doxorubicin is transported slowly into cells and becomes localized in nuclei and on chromosomes (*115,116*). AD 32 does not intercalate or bind to DNA by any other mechanism (*117*), long considered to be the principal mechanistic basis of cytotoxicity for anthracyclines. Thus, doxorubicin inhibits primarily DNA synthesis, whereas AD 32, despite a failure to complex with DNA, produces rapid inhibition of both DNA and RNA synthesis. Extensive studies have shown unequivocally that AD 32 does not serve as a doxorubicin prodrug. Furthermore, although extensively metabolized, neither can the biological activity of AD 32 be ascribed simply to a biotransformation product. Thus, whereas doxorubicin is a potent intercalator of DNA and an inhibitor of topoisomerase II (Table 3), AD 41 (*N*-trifluoroacetyladriamycin), the principal (in vitro and in vivo) biotransformation product of AD 32, undergoes only a minor degree of DNA intercalation and comparatively low inhibition of topoisomerase II activity (*118*). AD 32 is superior to doxorubicin in a broad range of rodent leukemia and solid tumor models with respect to both antitumor activity and degree of toxicity (*119–121*). In Lewis lung tumor-bearing animals, AD 32 persists in tumor tissue for a longer time than doxorubicin; this selectivity could result in greater exposure of tumor tissue to the drug or its active metabolites (*119*). Based on clinical studies of systemically administered drug conducted at the Dana-Farber Cancer Institute, this drug is well tolerated at doses up to 600 mg/m^2, with leukopenia and thrombocytopenia being the dose-limiting toxicities (*122–125*). Overall the toxicity profile of AD 32 in these earlier clinical studies showed several significant advantages over that of doxorubicin: no cardiotoxicity was observed, even at cumulative dose levels of 16.5 g/m^2; no contact

Table 3
The Effects of 3′-*N*-Acyl and 3′-*N*-Alkyl Substitution on the Biological Activity and DNA/Topoisomerase II Interactions of Selected Anthracycline Analogs and Their Principal Biotransformation Products

Anthracycline analog, abbreviation	*Activity*		*DNA intercalation*[c]	*Topoisomerase II-mediated cleavage*[d]
	In vivo[a]	*In vitro*[b]		
Doxorubicin	+ +	+ + +	+ + +	+ + +
Daunorubicin	+ +	+ + +	+ + +	+ + +
4′-Epi-doxorubicin (Epirubicin)	+ +	+ + +	+ + +	+ + +
3′-*N*-Acyl substituted analogs				
N-Trifluoroacetyladriamycin-14-valerate (AD 32)	+ + +	+ +	0	0
N-Trifluoroacetyladriamycin (AD 41)	+ + +	+ +	+	+
3′-*N*-Alkyl substituted analogs				
N-Benzyladriamycin-14-valerate (AD 198)	+ + +	+ +	+ +	0
N-Benzyladriamycin (AD 288)	+	+ +	+ + +	0

[a] In vivo activities (percentage increase in life-span) were compared against P388 cells at the respective optimal drug doses.
[b] In vitro activities (LD_{50} and LD_{90}) were compared against CEM and L1210 cells by growth inhibition and clonogenic assays, respectively.
[c] DNA intercalation was assessed by relaxation of Form 1pC15 DNA by topoisomerase I in the presence of various concentrations of the test analogs.
[d] Plasmid DNA in a cell-free system was used to compare drug-induced topoisomerase II-mediated DNA cleavage.

toxicity was evident during inadvertent paravenous extravasation; and less gastrointestinal irritation and alopecia were produced. These initial studies also documented clinical activity against several different tumor types (*124*).

Based on the penetrant qualities of AD 32 and, for an anthracycline-type drug, a remarkable absence of cardio- and contact-toxicity, the potential utility of this drug as an intracavitary agent has been pursued. Initial evaluation of AD 32 administration by topical application was undertaken using the intravesical (ive) route (*125–128*), with a view toward managing superficial bladder carcinoma. Based on these findings, AD 32 progressed to Phase I clinical trials of ive drug instillation in patients with superficial bladder cancer. Clinical pharmacologic monitoring shows comparable negligible systemic drug pharmacology to that observed in the animal model, consistent with a lack of systemic toxicity (*129*). Presently, complete responses have been sustained (mean followup = 2 yr) in 8/32 previously heavily treated patients who presented with recurrent tumor (*130*). Phase II/III studies of AD 32 ive for treatment of BCG-refractory carcinoma *in situ* are currently ongoing. In addition, Phase I studies of ive AD 32 employed in an adjunctive role to prevent reseeding of tumor, immediately following transurethral resection, are now under way with appropriate clinical pharmacology monitoring. Clinical studies with ive AD 32 are in progress as a neoadjuvant approach to the treatment of Stage T_a/T_1 papillary disease. The potential role of ive AD 32 in the treatment of muscle-invasive (Stage T_2) bladder cancer is currently under active consideration.

The potential use of AD 32 as an intraperitoneal (ip) agent against ovarian carcinoma has also been examined in preclinical studies, wherein ip instillation (q 14 d × 3) of AD 32 at concentrations up to 900 mg/m^2 in rats produced no evidence of inflammatory response, contact toxicity, or peritonitis (*131*), whereas parallel metabolic studies indicated a pharmacokinetic advantage for drug administration by this route (*132*). These data have been confirmed in a Phase I trial of ip AD 32 involving dose escalation up to the maximally tolerated dose of 600 mg/m^2 (*133*). Normalization of CA-125 antigen levels was evident in 26% of patients, and elimination or control of peritoneal ascites was accomplished in 44% of subjects, brief neutropenia being the dose-limiting toxicity. Additional clinical studies of ip AD 32 are now beginning. Experimental studies have now expanded into other topical applications for AD 32, including intrapleural (*134*), intraprostatic (*135,136*), and more recently, intralesional injection for tumors of the oral cavity and intraventricular administration for brain tumors.

3.3.3. *N*-Alkyl Analogs: AD 198

In addition to circumventing P-gp-mediated drug efflux, alkylation at the basic nitrogen site on the anthracycline daunosamine sugar can also modulate the ability of a drug to interact with topoisomerases and to intercalate DNA. A novel anthracycline analog representative of this structural change currently under development is *N*-benzyladriamycin-14-valerate (AD 198), which additionally contains the same lipophilic acyl substituent at the 14-carbinol position as seen in AD 32. Thus, this highly lipophilic adriamycin analog combines superior antitumor activity relative to doxorubicin in several syngeneic tumor models (Table 3) with significantly lower systemic toxicity (*137,138*). Furthermore, AD 198 demonstrates the ability to bypass multiple mechanisms of drug resistance, not only in vitro, but in vivo, as well. For example, against

a resistant subline of B16 melanoma totally unresponsive to doxorubicin, AD 198 remains as effective as against the parent sensitive line (*138*). *N*-Benzyladriamycin (AD 288) is the principal biotransformation product formed from AD 198, both in vitro and in vivo, with lower levels of *N*-benzyladriamycinol (AD 298) also being seen. Although AD 288 is more cytotoxic than doxorubicin in vitro, this agent, when given directly, is significantly less effective than AD 198 against P388 and L1210 leukemias in vivo (*137,139*). In vitro differences between AD 198 and doxorubicin include: irreversible G_2/M blockade of the cell cycle (*140*); a weaker binding with isolated purified DNA (*138,141*); production of DNA strand breaks on alkaline elution assays, despite an inability to inhibit isolated mammalian DNA topoisomerase II (*142*); potent membrane lytic activity (*138*); rapid and extensive drug accumulation and high retention in both sensitive and resistant cell lines (*139,143,144*); and lack of crossresistance in MDR phenotypic, as well as "atypical" (topo II-mutant) cell lines (*139,145,146*).

Although the mechanism(s) by which AD 198 exerts its cytotoxicity remains to be fully elucidated, it is clear that the overall effect contains both a nuclear and a cytoplasmic component, the latter occurring as a result of biotransformation. In this regard, a series of studies examining the cellular actions of AD 198 have highlighted the concept that anthracycline metabolites can be distinct from their parental agents both in the mechanism(s) of cytotoxicity and potential cellular resistance. Thus, AD 288, the major biotransformation product of AD 198, localizes in the nucleus an shows comparable DNA intercalation to that of doxorubicin, but is without activity against topoisomerase II, at least in isolated systems (Table 3). By contrast, AD 198, which is also without activity against isolated topoisomerase II, does not enter the nuclei of intact cells, but localizes in the perinuclear region of the cytoplasm in a punctate pattern suggestive of lysosomal/mitochondrial distribution (*147*). Cells expressing either the MDR or at-MDR phenotypes are crossresistant to AD 288. However they are sensitive to AD 198. Mouse macrophage-like J774.2 and P388 AD 198-resistant sublines, produced through selective (AD 198) pressure, independently express the mdr-1b isoform of P-gp as a result of the metabolic conversion of AD 198 to AD 288 under the selective conditions used; modified conditions of high concentration-short duration exposure do not result in P-gp overexpression. J774.2 AD 198-resistant variants show no change in topoisomerase II activity, based on the P4 DNA unwinding assay, an unsurprising result since AD 198 does not appear to interact with this enzyme. Additionally, no increased levels of glutathione-S-transferase activity and no difference in total cytoplasmic AD 198 accumulation are found when compared to parental cells (*148*). Reversal of AD 198 resistance by verapamil in the absence of changes in drug efflux suggests that resistance to this novel lipophilic anthracycline may be related to changes in intracellular drug compartmentalization, possibly into phagosomes that are abundant in macrophages. AD 198 has clearly demonstrated membrane perturbation effects, both in terms of structural integrity and, at minimally cytotoxic doses, in synergizing the cytotoxic effects of nuclear directed drugs, such as doxorubicin (*149*). Whether the effects of this highly lipophilic agent are nonspecific "detergent-like" or have targets within the membrane remains to be established, as does the apparent unique resistance mechanism seen in the mouse macrophage-like cell line.

Thus, AD 198 has proven capable of circumventing the clinically significant forms of drug resistance, and it appears to produce cytotoxicity, in part, through a novel mechanism. Based on its absence of cardiotoxicity, lower systemic toxicity when com-

pared with doxorubicin, superior antitumor activity seen in appropriate models, and its proven ability to circumvent mechanisms of drug resistance, AD 198 is expected soon to enter Phase I clinical trials in the U.S. Recent studies have demonstrated the ability of AD 198 to accumulate and persist at high concentration in rat lung tissue following iv administration (*150*). Such drug concentrations, in excess of the LD_{90} for AD 198 against a panel of human lung carcinoma cell lines (clonogenic assay; 3-h drug exposure), suggest the potential for lung tumor-directed therapy with this agent.

3.3.4. 3′-Deaminoanthracyclines: ME2303 and Annamycin

It is now evident from a number of different types of anthracycline analogs that substrate specificity for the P-gp-mediated efflux pump is, in part, dependent on the basicity of the C-3′ nitrogen on the daunosamine sugar, but that neither loss of charge at this position nor extreme lipophilicity of the drug molecule alone necessarily circumvents resistance in MDR cells (*151*). As an alternative to the N-substituted analogs like the morpholinyl-anthracyclines or AD 32 and AD 198, modulation of the basicity at this position has also been accomplished by a number of 3′-OH analogs. In addition, halogens, such as fluorine or iodine at the C-2′ position, show electron-withdrawing properties, which assist in stabilizing the glycosidic bond. Two such analogs, having a C-3′ hydroxyl substitution and a halogen at the C-2′ position, have undergone extensive preclinical/clinical evaluation.

2′-Fluoro-3′-hydroxyldoxorubicin-14-pimelate (ME2303) is one of a series of such substituted analogs (*152*), in this instance, a pimelate on the C-14 position functions to increase solubility (Fig. 1). ME2303 is superior to doxorubicin in vitro against a panel of resistant human and murine tumor cell lines (*153*). In vivo, ME2303 is effective by iv and ip administration against P388 leukemia and iv against colon carcinoma, Lewis lung carcinoma, and M5076 melanoma. Partial activity was also claimed in vivo against a doxorubicin-resistant P388 subline (*153*). Entry of this compound into Phase II clinical trials in Japan has been reported (*79*), although subsequent literature reports are lacking.

Substantial differences between 3′-deamino-4′-epi-3′-hydroxy-2′-iodo-4-demethoxyadriamycin (Annamycin) and ME2303 include an iodine rather than a fluorine on the C-2′ position, epimerization of the C-4′ hydroxyl function, and demethoxylation at C-4. These factors provide for increased drug lipophilicity and improved liposomal drug entrapment. Priebe and Perez-Soler have called these two factors, modification of the anthracycline structure to allow both circumvention of P-gp and easy packaging in liposomes for improved drug delivery, the "double advantage" (*154*). In vitro, liposomal Annamycin (L-Anna) has comparable potency to doxorubicin against sensitive P388, CEM, and KB-3-1 cells, but is more effective than doxorubicin against doxorubicin-resistant sublines (*80*). Uptake of L-Anna in these lines is less than for the sensitive cells, but is unaffected by inhibitors of P-gp, such as verapamil or cyclosporin A. Anthracycline concentrations in Annamycin-dosed mice bearing B16 melanomas are consistently higher than for doxorubicin-treated animals, especially in tumor and lung (*155*). Activity of Annamycin in vivo is related to the liposomal formulation used, with small unilamellar vesicles producing greater survival (74–95%) than L-Anna (63–73%) or Annamycin (39–45%) against Lewis lung carcinoma, M5076 reticulosarcoma, and KB-3-1 and KB-V1 human tumor xenografts

(*156*). Based on these data and a positive evaluation in preclinical toxicity studies, liposomal Annamycin has recently entered Phase I clinical trials in the US.

3.3.5. Novel Hybrid Anthracyclines: AD 312

N-(2-Chloroethyl)-*N*-nitrosoureidodaunorubicin (AD 312) combines within the molecule two structural features that are associated with antitumor efficacy, the anthracycline structure, which provides for DNA intercalation and protein-associated DNA strand breaks through an interaction with topoisomerase II, and a nitrosourea alkylating moiety, as in drugs like lomustine (CCNU) and carmustine (BCNU), to inhibit DNA synthesis and repair (*118,157*). Animal studies have confirmed the ability of the AD 312 molecule to distribute into tissues following iv administration (*158*). Glutathione conjugates characteristic of a nitrosourea alkylating agent are evident in rat urine following AD 312 administration (*159*); additionally, the functionality of DNA alkylation by AD 312 has been confirmed by in vitro assay (*157*). The relative lack of toxicity of AD 312 vs doxorubicin is evident in the Bertazzoli mouse model (*160*), wherein AD 312 at 5x the doxorubicin dose produces only minimal evidence of cardiotoxicity compared to the extensive cardiomyopathy seen with doxorubicin (*161*).

AD 312 is active in vitro against sensitive and doxorubicin-resistant P388 cells. In vivo, AD 312 is 100% curative of disease against parental or doxorubicin-resistant P388 leukemia cell lines, with single, multiple, and even following delayed treatment (*157*). AD 312 activity in the P388 doxorubicin-resistant subline, which has reduced levels of topoisomerase II enzyme (*146*), may be partly attributed to the nitrosourea portion of the molecule; the doxorubicin-resistant P388 subline is known to retain sensitivity to alkylating agents (*162*). Additionally, AD 312 shows significant activity against a P388 subline made resistant to BCNU. Significantly, the activity of AD 312 in this model is markedly superior to that of doxorubicin, daunorubicin, or admixtures of daunorubicin and BCNU or CCNU (*163,164*), and is effective at doses that are not associated with significant bone marrow suppression, the latter observation being remarkable for an agent than incorporates two normally potent hematotoxic moieties. Human xenograft studies have revealed AD 312 to possess markedly superior activity against doxorubicin-resistant ovarian (A2780/DOX5) and doxorubicin-insensitive advanced bladder (UCRU-BL13) carcinoma lines (*165*). Against the A549 human lung tumor model, AD 312 shows superior activity (56-> 80 d tumor growth delay) compared to doxorubicin or cisplatin (*165*). Activity against lung tumors is further confirmed in the aggressive FCCL-8 human-derived tumor model, wherein AD 312 produces dramatic tumor regression at all dose levels tested (27–40.8 mg/kg); doxorubicin is without significant effect in this assay (*166*). These finding and the apparent persistence of AD 312 in lung tissue following iv administration (*158*) suggest that AD 312 may have clinical utility against human lung carcinoma, in addition to other drug-refractory tumor types. Based on these data, the novel hydrid drug AD 312 is anticipated to enter clinical trials in the US soon.

4. SUMMARY: WHERE DO WE GO FROM HERE?

Despite the considerable toxicity problems associated with anthracyclines, continued use of such agents as front-line tumor therapy is testimony to their effectiveness relative to other chemotherapeutics. With the passage of time, there has neces-

sarily been a greater understanding of the processes involved in cardiotoxicity, and the use of cardioprotective agents, such as Dezrazoxane, offers the hope that this life-threatening side effect will ultimately be therapeutically controllable. Nevertheless, the emergence of drug-resistant cell populations, through mechanisms that we now recognize as potentially being infinitely more complex than simple overexpression of a P-gp membrane pump, remains as the greatest hurdle for improved anthracycline chemotherapy. All of the various anthracycline analogs outlined above appear, at least in experimental systems, to have potential for improved chemotherapy by their ability to circumvent some experimental resistance mechanisms; their ultimate test will come in the clinical trials where so many earlier aspirations have fallen short. However, the collective knowledge gained from these earlier disappointments, coupled with the many structure–activity studies that have now been conducted and the greater sophistication of current laboratory techniques, predicts a brighter future in anthracycline research. As outlined above, our capacity to control both cellular drug transport and lethal targets is at hand. Combination of one or more relatively nontoxic anthracyclines with different cellular target sites into a drug "cocktail" and/or formulation of drugs in liposomes may ultimately yield a better therapeutic result than our current concepts of anthracycline therapy. Conversely, the potential exists for "dual mechanistic" drugs that either combine two distinct antitumorals into one molecule or are structurally configured such that on biotransformation, they liberate a product with a different mechanism of action and cellular target site. An obvious need for improved anthracycline antitumor drugs remains. In answer to Weiss' question, "Will we ever find a better doxorubicin?" we believe the answer is yes, but probably not in terms of a major new systemic entity with a wide spectrum of activity, but rather for well-prescribed indications based on clear pharmacologic rationales and appropriate pharmacokinetic support.

REFERENCES

1. Grein A, Spella C, DiMarco A, Canevazzi G. Descrizione e classificazione di un attionamicette (Streptomyces Peucetius sp nova) produltore di un sostanza ad attivite antitumorale; la daunomicina. *Giorn Microbiol* 1963; 11:109–118.
2. Dubost M, Gauter P, Maral R, et al. Un novel antibiotique a proprietes cytostatiques; la rubidomycine. *CR Acad Sci Paris* 1963; 257:1813–1815.
3. Pigram WJ, Fuller W, Hamilton LD. Stereochemistry of intercalation: interaction of daunomycin with DNA. *Nature New Biol* 1972; 235:17–19.
4. Fogleson PD, Reckord C, Swink S. Doxorubicin inhibits human DNA topoisomerase I. *Cancer Chemother Pharmacol* 1992; 30:123–125.
5. Tewey KM, Rowe TC, Yang L, et al. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* 1984; 226:466–468.
6. Bachur NR, Yu F, Johnson R, et al. Helicase inhibition by anthracycline anticancer agents. *Mol Pharmacol* 1992; 41:993–998.
7. Bachur NR, Gordon SL, Gee MW. Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. *Mol Pharmacol* 1977; 13:901–910.
8. Murphree SA, Cunningham LS, Hwang KM, Sartorelli AC. Effects of adriamycin on surface properties of sarcoma 180 ascites cells. *Biochem Pharmacol* 1976; 25:1227–1231.
9. Tritton TR. Cell surface actions of Adriamycin. *Pharmacol Ther* 1991; 49:293–309.
10. Koseki Y, Israel M, Sweatman TW, et al. Inhibitory effects of immobilized adriamycin on cell growth and thymidine uptake in human CEM leukemic lymphocytes. *J Cell Pharmacol* 1991; 2:171–178.

11. Ling YH, Priebe W, Perez-Soler R. Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug resistant cells. *Cancer Res* 1993; 53:1583–1589.
12. Jacquillat C, Tanzer J, Boiron M, et al. Rubidomycin: a new agent active in the treatment of acute lymphoblastic leukemia. *Lancet* 1966; 2:27–28.
13. Boiron M, Jacquillat C, Weil M, et al. Daunorubicin in the treatment of acute myelocytic leukemia. *Lancet* 1969; 1:330–333.
14. Wiernik PH, Serpick AA. Randomized clinical trial of daunorubicin and a combination of prednisone, vincristine, 6-mercaptopurine and methotrexate in adult acute non-lymphocytic leukemia. *Cancer Res* 1972; 32:2023–2026.
15. DiMarco A, Gaetani M, Scarpinato B. Adriamycin (NSC-123,127): a new antibiotic with antitumor activity. *Cancer Chemother Rep (Part I)* 1969; 53:33–37.
16. DeVita VT, Hellman S, Rosenberg SA, eds. *Cancer. Principles and Practice of Oncology,* 4th ed. Philadelphia: JB Lippincott Company, 1993.
17. Woodcock TM, Allegra JC, Richman SP, et al. Pharmacology and phase I clinical studies of daunorubicin in patients with advanced malignancies. *Semin Oncol* 1984; 11:28–32.
18. Harvey J, Goodman A, McFadden M, et al. A phase I study of daunorubicin in advanced untreatable malignancies. *Semin Oncol* 1984; 11:33–35.
19. Von Hoff DD. Use of daunorubicin in patients with solid tumors. *Semin Oncol* 1984; 11: 23–27.
20. List AF. Multidrug resistance: clinical relevance in acute leukemia. *Oncology* 1993; 7:23–32.
21. Booser DJ, Hortobagyi GN. Anthracycline antibiotics in cancer therapy. *Drugs* 1994; 47: 223–258.
22. Von Hoff DD, Rozencweig M, Leyard M, et al. Daunomycin-induced cardiotoxicity in children and adults. A review of 110 cases. *Am J Med* 1977; 62:200–208.
23. Steinberg JS, Cohen AJ, Wasserman AG, et al. Acute arrhythmogenicity of doxorubicin administration. *Cancer* 1987; 60:1213–1218.
24. Wortman JE, Lucas VS Jr, Schuster E, et al. Sudden death during doxorubicin administration. *Cancer* 1979; 44:1588–1591.
25. Doroshow JH, Locker GY, Myers CE. Enzymatic defenses of the mouse heart against reactive oxygen metabolites; alterations produced by doxorubicin. *J. Clin Invest* 1980; 65:128–135.
26. Myers CE, McGuire WP, Liss RH, et al. Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumour response. *Science* 1977; 197:165–167.
27. Von Hoff DD, Layard MW, Basa P, et al. Risk factors for doxorubicin-induced congestive heart failure. *Ann Intern Med* 1977; 62:200–208.
28. Watts RG. Severe and fatal anthracycline cardiotoxicity at cumulative doses below 400 mg/m^2: evidence for enhanced toxicity with multi-agent chemotherapy. *Am J Hematol* 1991; 36:217,218.
29. Lipschultz SE, Colan SD, Gelber R, et al. Late cardiac effects of doxorubicin therapy for acute lymphoblastic leukemia in childhood. *N Engl J Med* 1991; 324:808–815.
30. Lipschultz SE, Lipsitz SR, Mone SM, et al. Female sex and drug dose as risk factors for late cardiotoxic effects of doxorubicin therapy for childhood cancer. *N Engl J Med* 1995; 332: 1738–1743.
31. Gottlieb SL, Edmiston WE Jr, Haywood LJ. Late, late doxorubicin cardiotoxicity. *Chest* 1980; 78:880–882.
32. Freter CE, Lee TC, Billingham ME, et al. Doxorubicin cardiac toxicity manifesting seven years after treatment. Case report and review. *Am J Med* 1986; 80:483–485.
33. Sunnenberg TD, Kramer B. Long-term effects of cancer chemotherapy. *Compr Ther* 1985; 11:58–67.
34. Goorin AM, Chauvenet AR, Perez-Atayde AR, et al. Initial congestive heart failure six to ten years after doxorubicin chemotherapy for childhood cancer. *J. Pediatr* 1990; 116:144–147.
35. Steinherz L, Steinherz P. Delayed cardiac toxicity from anthracycline therapy. *Paediatrician* 1991; 18:49–52.
36. DeValeriola DL. Dose optimization of anthracyclines. *Anticancer Res* 1994; 14:2307–2314.
37. Mitchell RB, Ratain MJ, Vogelzang NJ. Experimental rationale for continuous infusion chemotherapy. In: Lokich JJ, ed. *Cancer Chemotherapy by Infusion,* 2nd ed. Chicago: Precept Press. 1990:3.

38. Bielack SS, Erttmann R, Winkler K, Landbeck G. Doxorubicin: effect of different schedules on toxicity and antitumor efficacy. *Eur J Cancer Clin Oncol* 1989; 25:873–882.
39. Zalupski M, Metch B, Balcerzak S, et al. Phase III comparisons of doxorubicin and dacarbazine given by bolus versus infusion in patients with soft tissue sarcomas: a Southwest Oncology Group Study. *J Natl Cancer Inst* 1991; 83:926–932.
40. Hortobagyi GN, Frye D, Budzar AU, et al. Decreased cardiac toxicity of doxorubicin administered by continuous intravenous infusion in combination chemotherapy for metastatic breast carcinoma. *Cancer* 1989; 63:37–45.
41. Preisler HD, Gessner T, Azarnia N, et al. Relationship between plasma adriamycin levels and the outcome of remission induction therapy for acute non-lymphocytic leukemia. *Cancer Chemother Pharmacol* 1984; 12:125–130.
42. Robert J, Monnier A, Poutignat N, Herait P. A pharmacokinetic and pharmacodynamic study of the new anthracycline pirarubicin in breast cancer patients. *Cancer Chemother Pharmacol* 1991; 29:75–79.
43. Robert J, Illiadis A, Hoerni B, et al. Pharmacokinetics of adriamycin in patients with breast cancer: correlation between pharmacokinetic parameters and clinical short-term response. *Eur J Cancer Clin Oncol* 1982; 18:739–745.
44. Robert J. Use of pharmacokinetic-pharmacodynamic relationships in the development of new anthracyclines. *Cancer Chemother Pharmacol* 1993; 32:99–102.
45. DeValeriola DL, Ross DD, Forrest A, et al. Use of plasma cytotoxic activity to model cytotoxic pharmacodynamics of anticancer drugs. *Cancer Chemother Pharmacol* 1991; 29:133–140.
46. Robert J, Giani L. Pharmacokinetics and metabolism of anthracyclines. *Cancer Surveys* 1993; 17:219–252.
47. Gabizon AA. Liposomal anthracyclines. *Hematol Oncol Clin North Am* 1994; 8:431–450.
48. Huang SK, Mayhew E, Giliani S, et al. Pharmacokinetics and therapeutics of sterically stabilized liposomes in mice bearing C-26 colon carcinoma. *Cancer Res* 1992; 52:6774–6781.
49. Mayhew EG, Lasic DD, Babbar S, et al. Pharmacokinetics and antitumor activity of epirubicin encapsulated in long-circulating liposomes. *Int J Cancer* 1992; 51:302–309.
50. Papahadjopoulos D, Allen TM, Gabizon AA, et al. Sterically stabilized liposomes: Improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc Natl Acad Sci* 1991; 88: 11,460–11,464.
51. Gabizon AA, Peretz T, Ben-Yosef R, et al. Phase I study of liposome-associated Adriamycin: Preliminary report. *Proc Am Soc Clin Oncol* 1986; 5:43.
52. Gill PS, Espina BM, Muggia F, et al. Phase I/II clinical and pharmacokinetic evaluation of liposomal daunorubicin. *J. Clin Oncol* 1995; 13:996–1003.
53. Gill PS, Wernz J, Scadden DT, et al. A randomized trial of liposomal daunorubicin (DX) vs Adriamycin, bleomycin and vincristine (ABV) in 232 patients with advanced AIDS-related Kaposi's sarcoma. *Proc Am Assoc Cancer Res* 1995; 14:A830.
54. Law TM, Mencel P, Motzer RJ. Phase II trial of liposomal encapsulated doxorubicin in patients with advanced renal cell carcinoma. *Invest New Drugs* 1994; 12:323–325.
55. Speyer JL, Freedberg R. In: Abeloff MD, et al. eds. *Clinical Oncology*. New York: Churchhill Livingstone. 1995:811.
56. Rajagopalan S, Politi PM, Sinha BK, Myers CE. Adriamycin-induced free radical formation in the perfused rat heart: implications for cardiotoxicity. *Cancer Res* 1988; 48:4766–4769.
57. Buss JL, Hasinoff BB. Ferrous iron strongly promotes the ring opening of the hydrolysis intermediates of the antioxidant cardioprotective agent dexrazoxane (ICR-187). *Arch Biochem Biophys* 1995; 317:121–127.
58. Speyer JL, Green MD, Zeleniuch-Jacquotte A, et al. ICRF-187 permits longer treatment with doxorubicin in women with breast cancer. *J Clin Oncol* 1992; 10:117–127.
59. Jelic S, Radulovic S, Neskovic-Konstantinovic Z, et al. Cardioprotection with ICRF-187 (Cardioxane) in patients with advanced breast cancer having cardiac risk factors for doxorubicin cardiotoxicity, treated with the FDC regimen. *Support Cancer Care* 1995; 3:176–182.
60. Kolaric K, Bradamante V, Cervek J, et al. A phase II trial of cardioprotection with Cardioxane (ICRF-187) in patients with advanced breast cancer receiving 5-fluorouracil, doxorubicin and cyclophosphamide. *Oncology* 1995; 52:251–255.
61. Monte E, Sinha BK. Potentiation of doxorubicin cytotoxicity by (+)-1,2-bis (3,5-dioxopipera-

zinyl-1-yl) propane (ICRF-187) in human leukemic HL-60 cells. *Cancer Commun* 1990; 2: 145–149.
62. Sehested M, Jensen PB, Sorensen BS, et al. Antagonistic effects of the cardioprotector (+)-1,2-bis (3,5-dioxopiperazinyl-1-yl) propane (ICRF-187) on DNA breaks and cytotoxicity induced by the topoisomerase II-directed drugs daunorubicin and etoposide (VP-16). *Biochem Pharmacol* 1993; 46:389–393.
63. Abramowicz M, Ed. *Med Lett Drugs Ther* 1995; 37:110,111.
64. Kessel D, Woo ES, Michalska AE, et al. Uptake and retention of daunomycin by mouse leukemic cells as factors in drug response. *Cancer Res* 1968; 28:938–941.
65. Biedler JL, Riehm H. Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic and cytogenetic studies. *Cancer Res* 1970; 30:1174–1184.
66. Roninson IB, Chin JE, Choi K, et al. Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proc Natl Acad Sci USA* 1986; 83:4538–4542.
67. Ueda K, Cardarelli C, Gottesman MM, Pastan I. Expression of a full length cDNA for the human "MDR 1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci USA* 1987; 84:3004–3008.
68. List AF. Multidrug resistance: clinical relevance in acute leukemia. *Oncology* 1993; 7:23–38.
69. Cole SPC, Bhardwaj G, Gerlach JH, et al. Overexpression of a transporter gene in multidrug-resistant human lung cancer cell line. *Science* 1992; 258:1650–1654.
70. Cole SPC, Sparks KE, Fraser K, et al. Pharmacologic characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* 1994; 54:5902–5910.
71. Cole SPC, Downes HF, Slovak ML. Effect of calcium antagonists on the chemosensitivity of two multidrug-resistant human tumor cell lines which do not overexpress P-glycoprotein. *Br J Cancer* 1989; 59:42–46.
72. Barrand MA, Rhodes T, Center MS, Twentyman PR. Chemosensitization and drug accumulation effects of cyclosporin A, PSC-833, and verapamil in human MDR large cell lung cancer cells expressing a 190k membrane protein distinct from P-glycoprotein. *Eur J Cancer* 1993; 29:408–415.
73. Kuss BJ, Deeley RG, Cole SPC, et al. Deletion of gene for multidrug resistance in acute myeloid leukemia with inversion in chromosome 16: prognostic implications. *Lancet* 1994; 343: 1531–1534.
74. Bordow SB, Haber M, Madafiglio J, et al. Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. *Cancer Res* 1994; 54:5036–5040.
75. Liu LF. DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* 1989; 58:351–375.
76. Osheroff N. Effect of antineoplastic agents on the DNA cleavage/religation reaction of eukaryotic topoisomerase II: inhibition of DNA religation by etoposide. *Biochemistry* 1989; 28: 6157–6160.
77. Beck WT, Danks MK, Wolverton JS, et al. Resistance of mammalian tumor cells to inhibitors of DNA topoisomerase II. In: Liu LF, ed. Topoisomerase-Targeting Drugs. New York: Academic. 1994:145–169.
78. Akimoto H, Bruno NA, Slate DL, et al. Effect of verapamil on doxorubicin cardiotoxicity: altered muscle gene expression in cultured neonatal rat cardiomyocytes. *Cancer Res* 1993; 53: 4658–4664.
79. Weiss RB. The anthracyclines: will we ever find a better doxorubicin? *Semin Oncol* 1992; 19: 670–686.
80. Priebe W. Mechanism of action-governed design of anthracycline antibiotics: "a turn off/turn on" approach. *Current Drug Design* 1995; 1:73–96.
81. Miller AA, Salewski E. Prospects for Pirarubicin. *Med Pediatr Oncol* 1994; 22:261–268.
82. Goldin A, Venditti JM, Geran R. The effectiveness of the anthracycline analogue 4'-epidoxorubicin in the treatment of experimental tumors: a review. *Invest New Drugs* 1985; 3:3–21.
83. Hurteloup P, for the French Epirubicin Study Group: A prospective randomized Phase III trial comparing combination chemotherapy with cyclophosphamide, fluorouracil, and either doxorubicin or epirubicin. *J Clin Oncol* 1988; 6:679–688.
84. Intimi C, for the Italian Multicentre Breast Study with Epirubicin: Phase III randomized study of fluorouracil, epirubicin and cyclophosphamide v. fluorouracil, doxorubicin and cyclophosphamide in advanced breast cancer: An Italian multicentre trial. *J Clin Oncol* 1988; 6:976–982.

85. Weenen H, Van Maanan JMS, De Planque MM, et al. Metabolism of 4′ modified analogs of doxorubicin. Unique glucuronidation pathway for 4′ epidoxorubicin. *Eur J Cancer Clin Oncol* 1984; 20:919–926.
86. Perez DJ, Harvey VJ, Robinson BA, et al. A randomized comparison of single-agent doxorubicin and epirubicin as first-line cytotoxic therapy in advanced breast cancer. *J Clin Oncol* 1991; 9:2148–2152.
87. Nielsen D, Jensen JB, Dombernowsky P, et al. Epirubicin cardiotoxicity: A study of 135 patients with advanced breast cancer. *J Clin Oncol* 1990; 8:1806–1810.
88. Dardir MD, Ferrans VJ, Mikhael YS, et al. Cardiac morphologic and functional changes induced by epirubicin chemotherapy. *J Clin Oncol* 1989; 7:947–958.
89. Kuffel MJ, Reid JM, Ames MM. Anthracyclines and their C-13 metabolites: growth inhibition and DNA damage following incubation with human tumor cells in culture. *Cancer Chemother Pharmacol* 1992; 30:51–57.
90. Weiss RB. A third anthracycline for acute leukemia. *Contemp Oncol* 1992; 2:30–39.
91. Wiernik PH, Banks PLC, Case DC, et al. Cytarabine plus idarubicin or daunorubicin as induction and consolidation therapy for previously untreated adult patients with acute myeloid leukemia. *Blood* 1992; 79:313–319.
92. Stewart DJ, Grewaal D, Green RM, et al. Bioavailability and pharmacology of oral idarubicin. *Cancer Chemother Pharmacol* 1991; 27:308–314.
93. Lopez M, Contegiacomo A, Vici P, et al. A prospective randomized trial of doxorubicin versus idarubicin in the treatment of advanced breast cancer. *Cancer* 1989; 64:2431–2436.
94. Stuart NSA, Cullen MH, Priestman TJ, et al. A phase II study of oral idarubicin (4-demethoxydaunorubicin) in advanced breast cancer. *Cancer Chemother Pharmacol* 1988; 21:351–354.
95. Hurteloup P, Armand JP, Schneider M, et al. Phase II trial of idarubicin (4-demethoxydaunorubicin) in advanced breast cancer. *Eur J Cancer Clin Oncol* 1989; 25:423–428.
96. Michieli M, Michelutti A, Damiani D, et al. A comparative analysis of the sensitivity of multidrug resistant (MDR) and non-MDR cells to different anthracycline derivatives. *Leukemia Lymphoma* 1993; 9:255–264.
97. Klumper E, Pieters R, den Boer ML, et al. In vitro anthracycline cross-resistance pattern in childhood acute lymphoblastic leukaemia. *Br J Cancer* 1995; 71:1188–1193.
98. Bastholt L, Dalmark M. Phase II study of idarubicin given orally in the treatment of anthracycline-naive advanced breast cancer patients. *Cancer Treat Rep* 1987; 71:451–454.
99. Han G, Israel M, Seshadri R, Sweatman TW. Metabolism and elimination of *N,N*-di(*n*-butyl) adriamycin-14-valerate in the rat. *Cancer Chemother Pharmacol* 1996; 37:472–478.
100. Priebe W, Van NT, Burke TG, Perez-Soler R. Removal of the basic center from doxorubicin partially overcomes multidrug resistance and decreases cardiotoxicity. *Anticancer Drugs* 1993; 4:37–48.
101. Acton EM. Unresolved structure-activity relationships in anthracycline analogue development. In: Priebe W, ed. *Anthracycline Antibiotics. Novel Analogues, Methods of Delivery and Mechanisms of Action.* Washington DC: American Chemical Society. 1995:1–13.
102. Ogawa M, Ariyoshi Y. New anticancer drugs under clinical trials in Japan. *Hematol Oncol Clin North Am* 1994; 8:277–287.
103. Acton EM, Tong GL, Mosher CW, Wolgemuth RL. Intensely potent morpholinyl anthracyclines. *J Med Chem* 1984; 27:638–645.
104. Mosher CW, Wu HY, Fujiwara AN, Acton EM. Enhanced antitumor properties of 3′-(4-morpholinyl) and 3′-(4-methoxy-1-piperidinyl) derivatives of 3′-deamino-daunorubicin. *J Med Chem* 1982; 25:18–24.
105. Sikic BT, Ehsam MS, Harker WG, et al. Dissociation of antitumor potency from the anthracycline cardiotoxicity in a doxorubicin analogue. *Science* 1985; 228:1544–1546.
106. Streeter PG, Johl JS, Gordon GR, Peters JH. Uptake and retention of morpholinyl anthracyclines by adriamycin-sensitive and -resistant P388 cells. *Cancer Chemother Pharmacol* 1986; 16:247–252.
107. Westendorf J, Aydin M, Groth G, et al. Mechanistic aspects of DNA damage by morpholinyl and cyanomorpholinyl anthracyclines. *Cancer Res* 1989; 49:5262–5266.
108. Graham MA, Clugson CK, King LH, et al. Mechanistic studies with methoxymorpholinodoxorubicin: evidence for a novel covalently bound DNA adduct following activation by CYT P450 3A. *Proc Am Assoc Cancer Res* 1992; 33:513.

109. Lau DH, Duran GE, Sikic BI. Characterization of covalent DNA binding of morpholino and cyanomorpholino derivatives of doxorubicin. *J Natl Cancer Inst* 1992; 84:1587–1592.
110. Lau DH, Lewis AD, Sikic BI. Association of DNA cross-linking with potentiation of the morpholino derivative of doxorubicin by human liver microsomes. *J Natl Cancer Inst* 1989; 81: 1034–1038.
111. Wasserman K, Markovits J, Jaxel C, et al. Effects of morpholinyl doxorubicin, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II. *Mol Pharmacol* 1990; 38: 38–45.
112. Watanabe M, Komeshima N, Nakajima S, Tsuruo T. MX2, a morpholino anthracycline, as a new antitumor agent against drug-sensitive and multidrug-resistant human and murine tumor cells. *Cancer Res* 1988; 48:6653–6657.
113. Streeter DG, Taylor DL, Acton EM, Peters JH. Comparative cytotoxicities of various morpholinyl anthracyclines. *Cancer Chemother Pharmacol* 1985; 14:160–164.
114. Komeshima N, Tsuruo T., Umezawa H. Antitumor activity of new morpholino anthracyclines. *J Antibiot* 1988; 41: 548–553.
115. Koseki Y, Sweatman TW, Israel M. *N*-Trifluoroacetyladriamycin-14-valerate (AD 32) cellular pharmacology in human bladder tumor cell lines. *Proc Am Assoc Cancer Res* 1992; 33:428.
116. Israel M, Sweatman TW, Seshadri R, Koseki Y. Comparative uptake and retention of Adriamycin and *N*-benzyladriamycin-14-valerate in human CEM leukemia lymphocyte cell cultures. *Cancer Chemother Pharmacol* 1989; 25:177–183.
117. Israel M, Seshadri R, Sweatman TW, et al. Structure and biological activity of *N*-substituted anthracyclines: correlation with DNA interaction. First Conference on DNA Topoisomerases in Cancer Chemotherapy, New York, November 1988.
118. Israel M, Seshadri R, Koseki Y, et al. New anthracycline analogues directed against DNA topoisomerase II. In Tapiero H, Robert J, Lampidis TJ, eds. Interface on Clinical and Laboratory Responses to Anticancer Drugs. Montrouge France: John Libby Eurotext, 1989; 191:39–47.
119. Israel M, Khetarpal VK. Tissue distribution and metabolism of *N*-trifluorocetyladriamycin-14-valerate (AD 32) compared with adriamycin in mice bearing established Lewis Lung carcinoma. Proceedings of the 13th International Congress of Chemotherapy, Vienna, 1983.
120. Israel M, Modest EJ, Frei E III. *N*-Trifluoroacetyladriamycin-14-valerate, an analog with greater experimental antitumor activity and less toxicity than adriamycin. *Cancer Res* 1975; 35:1365–1368.
121. Vecchi A, Cairo M, Mantovani A, et al. Comparative antineoplastic activity of adriamycin and *N*-trifluoroacetyladriamycin-14-valerate. *Cancer Treat Rep* 1978; 62:111–117.
122. Blum RH, Garnick MB, Israel M, et al. An initial clinical evaluation of *N*-trifluoroacetyladriamycin-14-valerate (AD 32), an adriamycin analog. *Cancer Treat Rep* 1979; 63:919–923.
123. Blum RH, Garnick MB, Israel M, et al. Preclinical rationale and phase I clinical trial of the adriamycin analog, AD 32. In: Carter SK, Sakurai Y, Umezawa H, eds. *New Drugs in Cancer Chemotherapy, Recent Results in Cancer Research,* vol. 76. New York: Springer-Verlag. 1981: 7–15.
124. Garnick MB, Griffin JD, Sack MJ, et al. Phase II evaluation of N-trifluoroacetyladriamycin-14-valerate (AD 32). In: Muggia FM, Young CW, Carter SK, eds. *Anthracycline Antibiotics in Cancer Therapy.* The Hague: Martinus Nijhoff. 1982:7.
125. Sweatman TW, Payne C, Koseki Y, et al. Drug penetration into dog bladder wall following intravesical (ive) instillation of *N*-trifluoroacetyladriamycin-14-valerate (AD 32). *Proc Am Assoc Cancer Res* 1993; 34:364.
126. Koseki Y, Sweatman TW, Israel M. *N*-Trifluoroacetyladriamycin-14-valerate (AD 32) cellular pharmacology in human bladder tumor cell lines. *Proc Am Assoc Cancer Res* 1992; 33:428.
127. Sweatman TW, Payne C, Patterson L, et al. *N*-Trifluoroacetyladriamycin-14-valerate (AD 32) penetration into human urinary bladder tissue following intravesical instillation. *Proc Am Assoc Cancer Res* 1996; 37:182.
128. Sweatman TW, Parker RF, Israel M. Pharmacologic rationale for intravesical *N*-trifluoroacetyladriamycin-14-valerate (AD 32): A preclinical study. *Cancer Chemother Pharmacol* 1991; 28:1–6.

129. Sweatman TW, Payne C, Israel M, et al. Clinical pharmacology of intravesical (IVE) *N*-trifluoroacetyladriamycin-14-valerate (AD 32). *Proc Am Soc Clin Oncol* 1993; 12:162.
130. Giantonio B, Greenberg R, Bamberger M, et al. A Phase-I trial of AD 32 administered intravesically in patients with superficial transitional cell carcinoma of the bladder. *Proc Am Soc Clin Oncol* 1993; 12:251.
131. Israel M, Chengelis CP, Naas DJ, Sweatman TW. Absence of *N*-trifluoroacetyladriamycin-14-valerate (AD 32) toxicity following intraperitoneal (ip) injection in rats. *Proc Am Assoc Cancer Res* 1993; 34:427.
132. Sweatman TW, Payne C, Israel M, et al. Clinical pharmacology of intraperitoneal (ip) *N*-trifluoroacetyladriamycin-14-valerate. *Proc Am Assoc Cancer Res* 1994; 35:245.
133. Markman M, Homesley H, Norberts DA, et al. Phase I trial of intraperitoneal AD 32 in gynecologic malignancies. *Gynecol Oncol* 1996; 61:90–93.
134. Devlin S, Sweatman TW. Pharmacology of intrapleural (ipl) *N*-trifluoroacetyladriamycin-14-valerate (AD 32) in the rat. *Proc Am Assoc Cancer Res* 1994; 35:431.
135. Israel M, Bernacki RJ, Kanter PM. Pharmacologic rationale for intraprostatic AD 32 (*N*-trifluoroacetyladriamycin-14-valerate). *Proc Am Assoc Cancer Res* 1996; 37:372.
136. DiDomenico D, Brown J, Shaw M, et al. Intralesional AD 32 is efficacious in a prostate cancer model. *Proc Am Assoc Cancer Res* 1996; 37:303.
137. Israel M, Seshadri R. *N*-Alkyl and *N*-benzyladriamycin derivatives. US Patent 4,610,977, 1986. US Patent Office, Washington, DC.
138. Israel M, Seshadri R, Koseki Y, et al. Amelioration of adriamycin toxicity through modification of drug-DNA binding properties. *Cancer Treat Rev* 1987; 14:163.
139. Ganapathi R, Grabowski D, Sweatman TW, et al. *N*-Benzyladriamycin-14-valerate versus progressively doxorubicin-resistant murine tumours: cellular pharmacology and characterization of cross-resistance in vitro and in vivo. *Br J Cancer* 1989; 60:819–826.
140. Traganos F, Israel M, Seshadri R, et al. Effects of new N-alkyl analogues of adriamycin on in vitro survival and cell-cycle progression. *Cancer Res* 1985; 45:6273–6279.
141. Lameh J, Chuang LF, Israel M, Chuang RY. Mechanistic studies on *N*-benzyladriamycin-14-valerate (AD 198), a highly lipophilic alkyl adriamycin analogue. *Anticancer Res* 1988; 8: 689–694.
142. Bodley A, Liu LF, Israel M, et al. DNA topoisomerase II-mediated interaction of doxorubicin and daunorubicin congeners with DNA. *Cancer Res* 1989; 49:5969–5978.
143. Israel M, Sweatman TW, Seshadri R, Koseki Y. Comparative uptake and retention of adriamycin and *N*-benzyladriamycin-14-valerate in human leukemic lymphocyte cell cultures. *Cancer Chemother Pharmacol* 1989; 25:177–183.
144. Sweatman TW, Israel M, Seshadri R, et al. Cytotoxicity and cellular pharmacology of *N*-benzyladriamycin-14-valerate in mechanistically different multidrug-resistant human leukemic cells. *J Cell Pharmacol* 1990; 1:96–102.
145. Krishan A, Sauerteig A, Gordon K, Swinkin C. Flow cytometric monitoring of cellular anthracycline accumulation in murine leukemia cells. *Cancer Res* 1986; 46:1768–1773.
146. Maniar N, Krishan A, Israel M, Samy TSA. Anthracycline-induced DNA breaks and resealing in doxorubicin-resistant murine leukemia P388 cells. *Biochem Pharmacol* 1988; 37:1763–1772.
147. Lothstein L, Sweatman TW, Dockter M, Israel M. Resistance of *N*-benzyladriamycin-14-valerate in mouse J774.2 cells: P-glycoprotein expression without reduced *N*-benzyladriamycin-14-valerate accumulation. *Cancer Res* 1992; 52:3409–3417.
148. Lothstein L, Koseki Y, Sweatman TW. P-glycoprotein overexpression in mouse cells does not correlate with resistance to *N*-benzyladriamycin-14-valerate (AD 198). *Anticancer Drugs* 1994; 5:623–633.
149. Koseki Y, Lothstein L, Sweatman TW, Israel M. Absence of P-glycoprotein (P-gp) mechanism for the synergistic effect of *N*-benzyladriamycin-14-valerate (AD 198) on Adriamycin (ADR) cytotoxicity in human ovarian carcinoma cells. *Proc Am Assoc Cancer Res* 1993; 34:367.
150. Koseki Y, Sweatman TW, Israel M. Pharmacologic rationale for lung cancer therapy with *N*-benzyladriamycin-14-valerate (AD 198). *Proc Am Assoc Cancer Res* 1994; 35:411.
151. Lothstein L, Sweatman TW, Priebe W. Study of the effect of charge and lipophilicity on

anthracycline cytotoxicity in multidrug resistant murine J774.2 cells. *Proc Am Assoc Cancer Res* 1994; 35:342.

152. Tsuchiya T, Takagi Y, Umezawa S, et al. Synthesis and antitumor activities of 14-*O*-acyl derivatives of 7-*O*-(2,6-dideoxy-2-fluoro-a-L-talopyranosyl) adriamycin. *J Antibiotic* 1988; 41: 988–991.
153. Tsuruo T, Yusa K, Sudo Y, et al. A fluorine-containing anthracycline (ME2303) as a new antitumor agent against murine and human tumors and their multidrug resistant sublines. *Cancer Res* 1989; 49:5537–5542.
154. Priebe W, Perez-Soler R. Design and tumor targeting of anthracyclines able to overcome multidrug resistance: a double-advantage approach. *Pharmacol Ther* 1993; 60:215–234.
155. Zou Y, Priebe W, Ling YH, Perez-Soler R. Organ distribution and tumor uptake of annamycin, a new anthracycline derivative with high affinity for lipid membranes, entrapped in multilamellar vesicles. *Cancer Chemother Pharmacol* 1993; 32:190–196.
156. Zou Y, Ling YH, Van NT, et al. Antitumor activity of free and liposome-entrapped annamycin, a lipophilic anthracycline antibiotic with non-cross-resistance properties. *Cancer Res* 1994; 54: 1479–1484.
157. Israel M, Seshadri R. Hybrid nitrosoureidoanthracyclines having antitumor activity. U.S. Patent 4,973,675, 1990. US Patent Office, Washington, DC.
158. Pawlik C, Payne C, Lothstein L, et al. Tissue distribution of *N*-(2-[^{14}C]-chloroethyl)-*N*-nitrosoureidodaunorubicin in the rat. *Proc Am Assoc Cancer Res* 1994; 35:431.
159. Sweatman TW, Rodrigues P, Pawlik C, et al. Metabolism and elimination of *N*-(2-[^{14}C]-chloroethyl)-*N*-nitrosoureidodaunorubicin in the rat. *Proc Am Assoc Cancer Res* 1995; 36:364.
160. Bertazzoli C, Bellini O, Magrini U, Tozana M. Quantitative experimental evaluation of adriamycin cardiotoxicity in the mouse. *Cancer Treat Rep* 1979; 63:1877–1883.
161. Israel M, Chengelis CP. Lack of anthracycline-type cardiotoxicity associated with *N*-(2-chloroethyl)nitrosoureidodaunorubicin (AD 312). *Proc Am Assoc Cancer Res* 1994; 35:407.
162. Johnson RK, Chitnis MP, Embrey WM, Gregory EB. In vivo characteristics of resistance and cross resistance of an adriamycin-resistant subline of P388 leukemia. *Cancer Treat Rep* 1978; 62:1535–1547.
163. Israel M, Koseki Y, Seshadri R. Further in vivo antitumor studies with 2-chloroethylnitrosoureidodaunorubicin (AD 312) in murine P388 leukemia systems. *Proc Am Assoc Cancer Res* 1991; 32:2401.
164. Israel M, Koseki Y, Sweatman TW, Seshadri R. DNA topoisomerase II-directed nitrosoureidoanthracyclines: high in vivo antitumor activity with minimal myelosuppression. *J Cancer Res Clin Oncol* 1990; 116:468.
165. Glaves D, Rustum Y, Bernacki RJ, et al. Therapeutic activity of a nitrosourea: anthracycline hybrid (AD 312) against human bladder, lung, and ovarian xenografts. *Proc Am Assoc Cancer Res* 1995; 36:392.
166. Israel M, Krishan A, Wellham LL. Antitumor activity of 2-chloroethylnitrosoureidodaunorubicin (AD 312) in nude mice bearing xenografts of FCC-8 human lung adenocarcinoma. *Proc Am Assoc Cancer Res* 1995; 36:396.

6 Topoisomerase I Inhibitors

Beppino C. Giovanella, PhD

CONTENTS

1. INTRODUCTION

The helical structure of DNA was proposed in 1953 by Watson and Crick (*1*). Twelve years later, Vinograd and collaborators (*2–4*) found that the helix axis can also be coiled in circular DNA. This structure was called supercoiling. This finding was extended to linear DNA by Pettijohn and others (*5–8*). It is now known that practically all DNA in vivo is supercoiled (*9*). Because most of the functions of DNA require untwisting, the importance of the enzymes required for this is self-evident. The first such enzyme identified was called the protein ω (*10*). It is now called topoisomerase I (top1) because in untwisting the supercoiled DNA, it breaks only one of the two strands, whereas topoisomerase II (top2), initially called gyrase, breaks both. Topoisomerase type I enzymes are ubiquitous, having been found in every prokaryotic or eukaryotic cells investigated so far with the exception of sea urchin sperm (*11*).

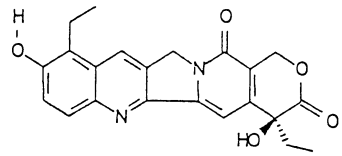
Eurkaryotic Topo I enzymes show extensive homology among themselves and form a group distinct from the bacterial topoisomerases I (*12*). Topoisomerase I inhibition became an important topic in cancer chemotherapy through the finding that Camptothecin (CAM), an alkaloid of plant origin, is the best known inhibitor of top1 and also a very potent anticancer agent. CAM is contained in a Chinese tree, *Camptotheca acuminata.* During a systemic search for natural anticancer products in plants, an alcoholic extract from such plant demonstrated high activity against L1210 mouse leukemia. Wall, who had supplied the alcoholic extract, proceeded to identify the active constituent of it (*13,14*) and called it Camptothecin. CAM rapidly reached

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

clinical testing (*15–19*). Unfortunately, in order to render the compound hydrosoluble, the sodium salt of CAM was prepared and used exclusively for iv injection in the clinical trials. Such sodium salt, which is produced by opening the lactone E ring of the CAM molecule, is practically devoided of anticancer activity (*14,20*). Nor surprisingly, the clinical trials resulted in failure and were abandoned. Contemporary with the clinical trials, research demonstrated that CAM inhibited DNA and RNA synthesis, and caused DNA fragmentation in cultured mammalian cells (*21–28*). Sometime afterward, in the late 1980s, the mechanism of action of CAM started to be clarified. Topoisomerase I was identified as the primary target of CAM (*29,30*) and of its derivatives (*31,32*). Topl, in order to act, has first to form a complex with DNA and then rolls along the DNA molecule releasing the supercoiling. At any moment, there is on one side of the topl a portion of DNA still supercoiled and on the other another portion uncoiled. CAM and derivatives stabilize and prolong this situation (*33*), rendering the cleavable complex (DNA + topl + CAM) vulnerable to degradation by other nucleases that first produce reversible, and then irreversible, DNA damage (*34–37*). In effect, cells in the S phase are about 1000 times more sensitive to CAM than cells in other phases of the cell cycle (*38*). Working on the premise that CAM and its derivatives are cytotoxic, by stabilizing the DNA–topl complex and finding higher content of topl in colon carcinomas than in the normal colon mucosa in 1989, Giovanella et al. (*39*) successfully treated xenografts of human colon cancer in nude mice with two water-insoluble CAM derivatives (9-amino,20[RS]Camptothecin-9AC and 10-11-Methylene-dioxy,20[RS]Camptothecin). These results were rapidly confirmed and extended to other malignancies (*20,40*). It was established that only the S form of these compounds is active. At about the same time, two water-soluble, but close lactone ring CAM derivatives were also developed, CPT-11 (Irinotecan) (*41*) and Hycamptamin (Topotecan) (*42*). Both these drugs were introduced into clinical research (*43,44*), as were the nonidrosoluble CAM and 9AC. Let us examine in detail the characteristics of the four camptothecin drugs that have reached clinical trials.

1. 20(S)-Camptothecin (NSC 94600)—CAM

CAM, the mother compound of this family, is contained in different parts of several plants, *C. acuminata, Mappia Foetida,* and so forth, and it is extracted from them by organic solvents. Chemically, CAM is 4-Ethyl-4-Hydroxy-1H-pyrano-[3′, 4′:6,7]Indolizino[1,2-b]Quinoline-3,14 (4H,12H)-Dione:



It has a mol wt of 348, and is water-insoluble, and poorly soluble in alcohols and chloroform. It is, however, readily dissolved in sodium hydroxyde with opening of the lactone ring and formation of the sodium salt.

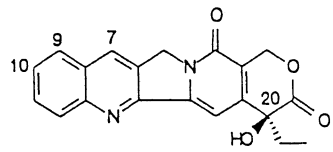
CAM is highly fluorescent and can be detected by fluorescence in nanogram quantities. Several syntheses of CAM have been described (*45–53*), but none of them has been used so far for industrial production. Availability, however, is good because the plants from which it is extracted, first among them *C. acuminata,* grow abundantly

and rapidly in tropical and semitropical climates. Extraction is relatively easy and simple, using several solvents. Interest in these compounds was raised first by the finding of antitumor activities in such extracts (*54–56*), and CAM was the first of them to be identified and isolated (*13,14*). After the failure of clinical trials owing to the mistaken belief that the sodium salt of CAM possesses the same anticancer activity as the mother compound—whereas it contains only about 10% of the same (*14,20*)—CAM remained unused for 20 years. After the finding in 1989 of the high antitumor activities of some of its nonwater-soluble derivatives, CAM itself was tested against xenografts of human tumors and found to be very active (*20*). Extensive toxicological and pharmacological studies in mice, dogs, and pigs determined that this compound is relatively well tolerated with a primary toxicity consisting of inflammatory ileitis totally reversible after treatment cessation (Giovanella et al., unpublished results). Secondary myelosuppression has also been observed under strenuous treatment in dogs. CAM was active when administered im, sc, and orally. It proved largely ineffective when administered iv by bolus injection (Giovanella et al., unpublished results). Because of its intense fluorescence, CAM can be easily detected in biological fluids in nanogram quantities. CAM is excreted mostly through the bile and feces, although a substantial amount is also eliminated in the urine. Plasma levels of CAM have different profiles according to the route of administration. Intramuscular injection supplies the most sustained levels, and oral administration give much shorter profiles. In mice, to maintain the same plasma levels, CAM was administered twice weekly im and daily orally (same dose). Phase I clinical trials of CAM were conducted at the Stehlin Foundation in Houston, Texas (*57*). Fifty-two patients were enrolled in the study. Thirteen of them had colon cancer, eight breast, seven melanoma, five lung, five prostate, three pancreas, two myeloma, two sarcoma, and one each of liver, kidney, larynx, rectum, cholahgiole cancer, Hodgkin's lymphoma, and non-Hodgkin's lymphoma. All of them had advanced disease refractory to available therapy. The schedule used was daily administration of oral CAM in gelatin capsule for 3 wk followed by 1 wk of rest, 3 wk of treatment, 1 wk of rest, and so on. Initial dose was 0.3 mg/m^2 escalated following a Fibonacci progression (*58–60*). Toxicity generally manifested itself around doses of 8–10 mg/m^2, and consisted mostly of diarrhea (40% of patients) and some episodes of cystitis (17% of patients). Significant drops of leukocyte counts were observed in only two cases, both of whom were heavily pretreated with other chemotherapies. All toxicities were totally reversible. The general policy has been of suspending treatment until normalization of patient condition and then restart of treatment at the lower dose level (toxicity observed at level 10, treatment restarted at level 9). With these precautions, the treatment has been well tolerated. The maximum tolerated dose (MTD) was established to be 8.7 mg/m^2/d. Responses have been observed in six patients. One complete response in a non-Hodgkin lymphoma remained in complete remission for 4 mo after discontinuation of CAM treatment, which had been given for 1 yr. The patient died following exploratory celiotomy for diverticulosis. No tumor was present. Two melanoma patients had major regression of multiple skin tumor nodules. Two breast cancer patients had disappearance of liver masses by CAT scan, confirmed in one case by autopsy. One patient with prostate cancer experienced loss of bone pain with fall of rapidly rising PSA from 63 to 29. Three additional patients, one with lung cancer, one with melanoma, and one with breast cancer, exhibited stable disease for more than 6 mo while

on the drug. In each case, the disease was in a rapidly escalating phase prior to the treatment. Pharmacokinetics in humans gave the best fit for second-order kinetics. The mean maximum concentration of CAM (total drug) in blood was calculated to be 39 ng/mL. Phase II trials are in progress at the Stehlin Foundation in Houston and at Dana Farber Center in Boston on breast cancer patients.

2. CPT 11 (IRINOTECAN)

CPT 11 is obtained by chemical modification of CAM introducing an ethyl group at the 7-position, then an hydroxyl group at the 10 position (*61,62*), and then binding a piperidinopiperidinocarbonyl group to the hydroxyl group (*62,63*). Chemically, CPT 11 is 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin and is water-soluble. Its mol wt is 586.

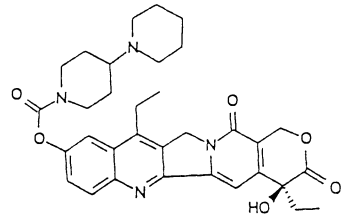


CPT 11 was found to have antitumor activity against murine transplantable tumors (*64,65*) when administered iv, ip, or orally. It also demonstrated activity against xenografts of human tumors (*66,67*). Several Phase I trials have been conducted with iv administration under various schedules in Japan, the US, and Europe (*68–72*). Mostly the drug was administered by 90-min iv infusion. When given once a week, repeated three times (*68*), the maximum tolerated dose (HTD) was 100 mg/m^2/infusion. Daily infusions for 5 d (*69*) had an MTD of 30 mg/m^2/infusion. Gastrointestinal toxicity and leukopenia were the dose-limiting factors. Phase II studies (*73–79*) were conducted on leukemias, lymphomas, cervical cancers, colon cancers, and lung cancer.

As in Phase I studies, gastrointestinal toxicities and myelosuppression were dose-limiting. Twenty to 30% partial responses were observed with some total responses, particularly in non-Hodgkin lymphomas. It is interesting to note that these total responses were observed with prolonged treatment, but not with single-dose schedules (*73*). CPT 11 has been combined with other anticancer agents, particularly cisplatinum (*80,81*) alone, and with granulocyte colony-stimulating factor (*82*). In advanced untreated nonsmall-cell lung cancers, 50% partial responses were observed with these regimens. With the use of the colony-stimulating factor, it was possible to increase the dose of CPT 11 by 33%, and diarrhea became the limiting factors. Cisplatinum was administered on day 1 at 80 mg/m^2. CPT 11 was given (90-min iv infusion) on days 1, 8, and 15 at the dose of 60 mg/m^2 without and 80 mg/m^2 with colony-stimulating factor.

3. TOPOTECAN-SKF104864 (NSC 609699)

Topotecan is prepared by chemical modification of 10 hydroxy CAM, which is a natural product present in small amounts in the tissues of *C. acuminata* and other plants. It is extracted together with CAM and can be separated later. Chemically, Topotecan is 9-dimethylaminomethyl-10-hydroxy-camptothecin and is water-soluble.



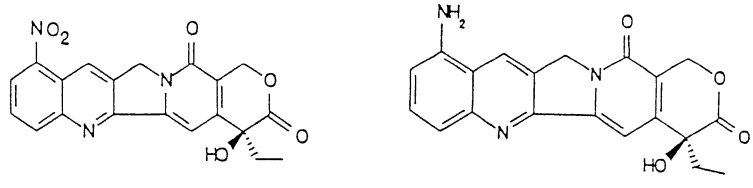
Its mol wt is 421. This compound was found to have anticancer activity in vivo and in vitro (*83–87*) against murine and human tumors.

Shortly afterward, Topotecan entered Phase I clinical trials in the US and in Europe with various schedules iv bolus or infusions of various durations (*88–96*). Dose-limiting toxicity was mostly neutropenia. However, thrombocytopenia was also observed in some continuous infusion regimens (*91–93*). The median MTDs varied depending on the schedule. For 21-d continuous infusion repeated every 4 wk, MTD was 0.53 mg/m²/d. For a 24-h infusion every 3 wk, it was 8.4 mg/m²/d.

Partial responses were observed in patients with nonsmall-cell lung cancer, cancer of the ovary, esophagus, and colon carcinoma (*90–92,97,98*). A complete remission was observed in a patient with nonsmall-cell lung cancer. Another complete remission together with several partial ones were observed in acute leukemias treated with 120-h continuous infusions every 3 wk (*95*). A Phase II trial of nonpretreated metastatic melanomas treated with two 30-min iv infusions at 5-d distance and at the dose of 1.5 mg/m² has given 1 response in 17 treated patients (*99*).

Another Phase II trial in pretreated small-cell lung cancer patients given Topotecan 1.5 mg/m² DX5Q3 for 2 weeks until progression or excessive toxicity results is in progress (*100*). Toxicity so far observed was mostly hematological.

4. 9-AMINO-CAMPTOTHECIN—(NSC 603071); 9-NITRO-CAMPTOTHECIN—(9NC)



These two derivatives of CAM should be treated as one since the finding by Hinz et al. (*101*) that 9NC is converted in the body of humans and other mammals into 9AC, presumably by reductases that are apparently quite ubiquitous, the same phenomenon having been observed in many tissues (*101,102*). The observation in 1989 (*39*) that 9AC was a potent inhibitor of human colon cancer xenografts was one of the findings that triggered the revival of interest in CAMs as anticancer agents. This compound and 9NC had been synthesized (*103,104*) by nitration of CAM (which produces 9NC and the inactive 12-nitrocamptothecin), separation of 9NC, and reduction of the same to 9AC. Both 9NC and 9AC were found to be very active against L1210 leukemia with the same t/c, 348 (*104*). The amino compound was found to be the most active derivative of CAM in inducing Topo I-mediated cleavage of linear DNA (*32*)—however, 9NC was not tested on this occasion.

After the finding of potent antitumor activity against xenografts by 9AC, several other papers confirmed and extended it, utilizing both in vivo and in vitro systems, and both 9NC and 9AC (*105–111*). Both of these compounds proved to be very potent anticancer agents against a very large spectrum of human tumors. After toxicology studies in mice and dogs, 9AC entered Phase I clinical trials at Dana-Farber Cancer Center in Boston and at the Naval Hospital in Bethesda in 1994, and 9NC in 1995 at the Stehlin Foundation in Houston, TX.

5. PHARMACOLOGY AND PHARMACOKINETICS

Studies with iv injected H^3 generally labeled CAM demonstrated widespread distribution of the drug in the various tissues and organs of the mouse and excretion mostly through bile and feces (*112*). Analytical methods for the determination of CAM and derivatives in biological fluids by HPLC were developed and used to determine the pharmacokinetics of CAMs in the biological fluids of humans and animals (*113–116*). Such methods allow for the determination of the closed and opened lactone forms of CAM and its derivatives at very low concentrations. Using HPLC methods, numerous studies were conducted on the pharmacokinetics of CAMs (*90,92–94,117–126*).

Wide variations have been observed according to route of administration, derivative, and animal species. In humans, terminal half-life, for the closed lactone form, appears to be 30–36 h for oral CPT and iv 9AC, respectively, which are both nonhydrosoluble (our observations; *126*). Hydrosoluble derivatives (CPT 11 and Topotecan) are eliminated more rapidly in the 2–14 h range (*90,94,120,122,123*).

A most imporant finding was that serum albumin binds preferentially to the open lactone form of CAM and its derivatives. This binding alters the equilibrium between the closed and open lactone forms in the serum in favor of the last and because it catalyzes the opening of the lactone ring (*127–129*). Because albumin binds with various avidities to different CAM derivatives, their lactone ring are opened with an efficiency directly proportional to the affinity of albumin for a given derivative. If it is remembered that the opening of the lactone ring completely inactivates these compounds as anticancer agents, the importance of this finding cannot be overemphasized. The same authors found also that albumins of different species are more or less efficient in catalyzing the opening of the lactone ring with the mouse being the less efficient of the species studied and humans having the dubious distinction of being the most effective (*130,131*). Fortunately, it was also found that CAMs bind to lipids and lipoproteins. The lactone ring of CAMs in these complexes remains closed (*130,132–134*).

Data on metabolism of CAMs are scanty, and still not much is known about their excretion, which is accomplished through both urine and bile.

6. RESISTANCE

CAMs, both water-soluble and nonwater-soluble, show little or no crossresistance with other anticancer drugs and appear not to be substrates of the P-glycoprotein multidrug transporter (*135–138*). Only Topotecan seems to be affected by overexpression of the MDR1 resistance gene (*136,139*) and even there, resistance is very low. Cells can become resistant to CAMs, however, both in vitro and in vivo under treatment. Resistant lines contain mutated topl genes or display a loss of topl activity more or less pronounced (*140–156*). Interestingly, some of these resistant cells exhibit an increased sensitivity to top2 inhibitors (*157,158*).

7. COMBINATION THERAPY

Combination of Topotecan (*159*) and of CPT 11 (*160*) with cisplatinum has been tried in vitro based on the rationale of interfering with repair of cisplatinum-induced DNA damage (*161*). Clinical trials have been conducted (*80*) and are being run now. It is too soon to draw conclusions.

Attempts to associate CAMs and ionizing radiations have brought very exciting results in vitro and in xenografts (*162–167*). The rationale for such association is that topl will attach to any fresh break in the DNA. CAMs will block the enzyme-DNA complex, and the irreversible degradation of DNA following will be multiplied by the number of DNA breaks in presence of CAMs. In animal models, doses of ionizing radiations and of 9AC, totally ineffective by themselves, produced total eradication of artificial brain metastases of human lung adenocarcinoma in nude mice (*162,164,176*).

8. CONCLUSIONS

The CAMs have already established themselves as very promising anticancer agents. Other topl inhibitors have appeared and are under investigation, but it is too soon to evaluate them. Many others will certainly appear in the near future, considering the interest in this type of compound existing now in many laboratories. Although this field is relatively young, the amount of published literature is already considerable. It was obviously already impossible to cover all the papers in existence. For more detailed treatment, consult some recent reviews (*168–175*) where subjects covered in this chapter receive a more comprehensive treatment.

At the present moment, CAMs are undergoing intensive development, and various members of this family of compounds are in clinical trials more or less advanced, and others probably will reach this stage soon.

Summarizing what we know today, we can list the following relatively well-established findings:

1. CAMs can be divided in two categories, water-soluble (Irinotecan, Topotecan, and so on) and water-insoluble (CPT, 9NC, and so forth). The first category is easy to administer, especially iv but appears to have less anticancer activity than the insoluble compound in xenograft models of human tumors.
2. Prolonged administration appears to be more effective than high doses. Scheduling seems to be crucial for these drugs. In our experience, high doses for short periods are totally ineffective. In order to obtain eradication of human cancer xenotransplants in nude mice, sustained treatment for at least 3–4 wk is necessary, no matter what the route of administration.
3. Only the close lactone ring form of CAMs has anticancer activity. Fortunately, such substances are very powerful. In vitro, 10–20 ng/mL will selectively kill tumor cells in 72–96 h, leaving alive nontumor cells. (They stop proliferating, but resume division on removal of the drug.)
4. In the serum, at pH of 7.4 and in the presence of albumin, the lactone ring of CAMs is rapidly open (below pH 7.0, the ring does not open). Every effort is now made to overcome this difficulty, rendered more serious by the finding that human serum albumin is much more effective in catalyzing the opening of the lactone ring than any other albumin tested. Fortunately, it appears that whereas albumin catalyzes the opening of the lactone ring by binding preferentially to the carboxylate form of the CAMs, lipids

and lipoproteins tend to bind to the closed lactone form and to afford a degree of protection to it.

5. Any agent that will cause DNA breaks appears to be a candidate for combination therapy with the CAMs. The most obvious is ionizing radiation, which has already given exciting results in preliminary experiments.
6. Under optimal conditions, against human tumor xenografts, the CAMs have demonstrated an astounding spectrum of activity, proving very effective against all malignant tumors studied. In vitro they have demonstrated a not less astounding degree of selectivity against malignant cells (defined functionally as cells capable of producing a malignant tumor when inoculated into nude mice). CAMs are always cytocidal for tumorigenic cells and cytostatic for nontumorigenic cells at equal doses. Viability of the nontumorigenic cells is preserved at the end of the experiments.

REFERENCES

1. Watson JD, Crick L. A structure for deoxyribose nucleic acid. *Nature* 1953; 171:737,738.
2. Vinograd J, Lebowitz J, Radloff R, Watson R, Laipis P. The twisted circular form of polyoma viral DNA. *Proc Natl Acad Sci* 1965; 53:1104–1111.
3. Vinograd J, Lebowitz J. Physical and topological properties of circular DNA. *J Gen Physiol* 1966; 49:103–125.
4. Vinograd J, Lebowitz J, Watson R. Early and late helix-coil transitions in closed circular DNA: The number of superhelical turns in polyoma DNA. *J Mol Biol* 1968; 33:173–197.
5. Stonington OG, Pettijohn DE. The folded genome of *Escherichia coli* isolated in a protein-DNA-RNA complex. *Proc Natl Acad Sci* 1971; 68:6–9.
6. Worcel A, Burgi E. On the structure of the folded chromosome of *Escherichia coli*. *J Mol Biol* 1972; 72:127–147.
7. Pettijohn DE, Hecht R. RNA molecules bound to the folded bacterial genome stabilize DNA folds and segregate domains of supercoiling. *Cold Spring Harbor Symp Quant Biol* 1974; 38: 31–42.
8. Drlica K, Worcel A. Conformational transitions in the *Escherichia coli* chromosome: Analysis by viscometry and sedimentation. *J Mol Biol* 1975; 98:393–411.
9. Bauer W. Structure and reactions of closed duplex DNA. *Annu Rev Biophys Bioeng* 1978; 7:287–313.
10. Wang JC. Interaction between DNA and an *Escherichia coli* protein ω. *J Mol Biol* 1971; 55:523–533.
11. Poccia DL, Le Vine D, Wang JC. Activity of a DNA topoisomerase (nicking-closing enzyme) during sea urchin development and the cell cycle. *Dev Biol* 1978; 64: 273–283.
12. Hsieh TS. *DNA Topoisomerases in Nucleases,* 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1993:209–234.
13. Wall ME, Wani MC, Cook CE, Palmer KH, McPhail AT, Sim GA. Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. *J Am Chem Soc* 1966; 88:3888–3890.
14. Wall ME. Alkaloids with antitumor activity. In: Mothes K, Schreiber K, Schutte HR, eds., *International Symposium on Biochemistry and Physiology of the Alkaloids.* Berlin: Academic-Verlag. 1969:77–87.
15. Gottlieb JA, Guarino AM, Call JB, Oliverio VT, Block JB. Preliminary pharmacologic and clinical evaluation of camptothecin sodium (NSC-100880). *Cancer Chemother Rep* 1970; 54: 461–470.
16. Gottlieb JA, Luce JK. Treatment of malignant melanoma with camptothecin (NSC-100880). *Cancer Chemother Rep* 1972; 56:103–105.
17. Moertel CG, Reitemeier RJ, Schutt AJ. A phase II study of camptothecin (NSC-100880) in gastrointestinal cancer. *Proc Am Assoc Cancer Res* 1971; 12:18.
18. Moertel CG, Schutt AJ, Reitemeier RJ, Hahn RG. Phase II study of camptothecin (NSC-100880) in the treatment of advanced gastrointestinal cancer. *Cancer Chemother Rep* 1972; 56: 95.

19. Muggia FM, Creavan PJ, Hansen HH, Cohen MH, Selawry OS. Phase I clinical trial of weekly and daily treatment with camptothecin (NSC-100880): Correlation with preclinical studies. *Cancer Chemother Rep* 1972; 56:515–521.
20. Giovanella BC, Hinz HR, Kozielski AJ, Stehlin JS Jr, Silber R, Potmesil M. Complete growth inhibition of human cancer xenografts in nude mice by treatment with 20-(S)-camptothecin. *Cancer Res* 1991; 51:3052–3055.
21. Bosmann HB. Camptothecin inhibits macromolecular synthesis in mammalian cells but not in isolated mitochondria of *E. coli*. *Biochem Biophys Res Commun* 1970; 41:1412–1420.
22. Horwitz SB, Chang C-K, Grollman AP. Studies on comptothecin. 1. Effects on nucleic acid and protein synthesis. *Mol Pharmacol* 1971; 7:632–644.
23. Horwitz MS, Horwitz SB. Intracellular degradation of HeLa and adenovirus type DNA induced by camptothecin. *Biochem Biophys Res Commun* 1971; 45:723–727.
24. Horwitz SB, Horwitz MS. Effects of camptothecin on the breakage and repair of DNA during the cell cycle. *Cancer Res* 1973; 33:2834–2836.
25. Kessel D. Effects of camptothecin on RNA synthesis in leukemia L1210 cells. *Biochem Biophys Acta* 1971; 246:225–232.
26. Kessel D, Bosmann HB, Lohr K. Camptothecin effects on DNA synthesis on murine leukemia cells. *Biochim Biophys Acta* 1972; 269:210–216.
27. Wu RS, Kumar A, Warner JR. Ribosomal formation is blocked by camptothecin, a reversible inhibitor of RNA synthesis. *Proc Natl Acad Sci USA* 1971; 68:3009–3014.
28. Abelson HT, Penman S. Selective interruption of high molecular weight RNA synthesis in HeLa cells by camptothecin. *Nature (Lond) New Biol* 1972; 237:144–146.
29. Hsiang Y-H, Hertzberg R, Hecht S, Liu LF. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerases I. *J Biol Chem* 1985; 260:14,873–14,878.
30. Hsiang Y-H, Liu LF. Identification of mammalian topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res* 1988; 8:1722–1726.
31. Jaxel C, Kohn KW, Wani MC, Wall ME, Pommeir Y. Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I. Evidence for a specific receptor site and for a relation to antitumor activity. *Cancer Res* 1989; 49:1465–1469.
32. Hsiang Y-H, Liu LF, Wall ME, Wani MC, Kirshenbaum S, Silber R, Potmesil M. DNA topoisomerase I-mediated DNA cleavage and cytotoxicity of camptothecin analogs. *Cancer Res* 1989; 49:4385–4389.
33. Liu LF, DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* 1989; 58:351–375.
34. Avemann K, Knippers R, Koller T, Sogo JM. Camptothecin, a specific inhibitor of type I DNA topoisomerase, induces DNA breakage at replication forks. *Mol Cell Biol* 1988; 8:3026–3034.
35. Holm C, Covey JM, Kerrigan D, Pommier Y. Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I and II inhibitors in Chinese hamster DC3F cells. *Cancer Res* 1989; 49:6365–6368.
36. Hsiang Y-H, Lihou MG, Liu LF. Mechanism of cell killing by camptothecin: arrest of replication forks by drug-stabilized topoisomerase 1-DNA cleavable complexes. *Cancer Res* 1989; 49:5077–5082.
37. Ryan AJ, Squires S, Strutt HL, Johnson RT. Camptothecin cytotoxicity in mammalian cells is associated with the induction of persistent double strand breaks in replicating DNA. *Nucleic Acids Res* 1991; 19:3295–3300.
38. Li LH, Fraser TJ, Olin EJ, Bhuyan BK. Action of camptothecin on mammalian cells in culture. *Cancer Res* 1972; 32:2643–2650.
39. Giovanella BC, Stehlin JS, Wall ME, Wani M, Nicholas AW, Liu LF, Silber R, Potmesil M. DNA topoisomerase I-targeted chemotherapy of human colon cancer in xenografts. *Science* 1989; 246:1046–1048.
40. Giovanella BC, Wall ME, Wani MC, Silber R, Stehlin JS, Hochster H, Potmesil M. Efficacy of camptothecin [NSC 94600, CA], 9-amino-camptothecin [NSC 603071, 9-AC] and 10,11-methylenedioxycamptothecin [NSC 606174, 10-11-MDC] in the human cancer xenograft model. *Proc Am Assoc Cancer Res* 1992; 3:432.
41. Kunimoto T, Nitta K, Tanaka T, Uehara N, Baba H, Takeuchi M, Yokokura T, Sawada S, Miyasaka T, Mutai M. Antitumor activity of 7-ethyl-10-[4-(1-piperidino) carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin against murine tumors. *Cancer Res* 1987; 47:5944–5947.

42. Kingsbury WD, Boehm JC, Jakes DR, Holden KG, Hecht SM, Gallagher G, Caranfa MJ, McCabe FL, Faucette LF, Johnson RK, Hertzberg RP. Synthesis of water-soluble (aminoalkyl) camptothecin analogues; Inhibition of topoisomerase I and antitumor activity. *J Med Chem* 1991; 34:98-107.
43. Negoro S, Fukuoka M, Masuda N, Takada M, Kusunoki Y, Matsui K, Takifuji N, Kudoh S, Niitani H, Taguchi T. Phase I study of weekly intravenous infusion of CPT-11, a new derivative of camptothecin, in the treatment of advanced non-small-cell lung cancer. *J Natl Cancer Inst* 1991; 83:1164-1168.
44. Rowinsky E, Grochow L, Hendricks C, Sartorius S, Ettinger D, McGuire W, Forastiere A, Hurowitz L, Easter V, Donehower R. Phase I and pharmacologic study of topotecan (SK&F 104864): A novel topoisomerase I inhibitor. *Proc Am Soc Clin Oncol* 1991; 10:93.
45. Stork G, Schultz AG. The total synthesis of dl-Camptothecin. *J Am Chem Soc* 1971; 93: 4074-4075.
46. Volkmann R, Danishesky S, Eggler J, Solomon DM. A total synthesis of dl Camptothecin. *J Am Chem Soc* 1971; 93:5576,5577.
47. Winterfeldt E, Korth T, Pike D, Boch M. Biogenetically oriented total synthesis of camptothecin and 7 chlorocamptothecin. *Angew Chem Int Ed Englies* 1972; 11:289,290.
48. Corey EJ, Crouse DN, Anderson JE. A total synthesis of natural 20(S)-camptothecin. *J Org Chem* 1972; 40:2140,2141.
49. Cyril SF, Tang GJ, Morrow J, Rapoport H. A total synthesis of dl-Camptothecin. *J Am Chem* 1975; 97:159-167.
50. Wani MC, Ronman PE, Lindley JT, Wall ME. Plant antitumor agents: 18 Synthesis and biological activity of camptothecin analogues. *J Med Chem* 1980; 23:534-560.
51. Shanghai No 5 and No 1 Pharmaceutical Plant. Shanghai Institute of Pharmaceutical Industrial Research, Shanghai Institute of Materia Medica. The total synthesis dl Camptothecin. *Scientia Sinica* (*Engl. Ed.*) 1978; 21:27-98.
52. Wani MC, Nicholas W, Wall ME. Plant antitumor agents 28 Resolution of a key tricyclic synthon, 5′(RS)-1,5Dioxo-5′-ethyl-5′-hydroxy-2′H,5′H,6″h-6′-oxopyrana[3′,4-F]6,8-tetrahydro-indolizine: total synthesis and antitumor activity of 20(S)- and 20(R)-camptothecin. *J Med Chem* 1987; 30:2317-2319.
53. Akio E, Hirofumi T, Masamicni S, Hiroaki T. Antitumor agents: Part 2. Asymmetric synthesis of (S) camptothecin. *J Chem Soc Perkin Trans* 1990; 1:27-32.
54. Abbott BJ, Leiter J, Hartwell JL, Perdue RE Jr, Schepartz SA. Screening data from the cancer chemotherapy national service screening laboratory. XXX Plant Extracts. *Cancer Res* 1966; 26 part 2:34-45, 429-470.
55. Perdue RE, Jr, Wall ME, Hartwell JL, Abbott BJ. Comparison of the activity of crude *Camptotheca acuminata* ethanolic extracts against lymphoid leukemia L-1210. *Lloydia* 1968; 31: 229-236.
56. Wall ME, Wani MC. Camptothecin and taxol: discovery to clinic. *Cancer Res* 1995; 55: 753-760.
57. Stehlin JS, Natelson EA, Hinz HR, Giovanella BC, de Ipolyi P, Fehir KM, Trezona TP, Vardeman DM, Harris NJ, Marcee AK, Kozielski AJ, Ruiz-Razura A. Phase I clinical trial and pharmacokinetic results with oral administration of 20(S)-Camptothecin. In: Potmesil M, Pinedo H, eds. *Camptothecins: New Anticancer Agents.* Boca Raton, FL: CRC. 1995:59-65.
58. Goldsmith MA, Slavik M, Carter SK. Quantitative prediction of drug toxicity in humans from toxicology in small and large animals. *Cancer Res* 1975; 35:1354-1364.
59. Lowe Michael C. Large animal toxicological studies of anticancer drugs. In: Hellman K, Carter SK, eds. *Fundamentals of Cancer Chemotherapy.* New York: McGraw-Hill. 1987: 236-247.
60. Carter SK. The Phase I study. In: Hellman K, Carter SK, eds. *Fundamentals of Cancer Chemotherapy.* New York: McGraw-Hill. 1987:285-300.
61. Matsuzono H. Japan Patent; Kokai, 56-158786; 1981.
62. Miyasaka T, Sawada S, Nokata K, Sugino E, Mutai M. US Patent 4604463; 1986.
63. Miyasaka T, Sawada S, Nokata K, Sugino E, Mutai M. Japan Patent, Kowai, 60-17970; 1985.
64. Nitta K, Yokokura T, Sawada S, Takeuchi M, Tanaka T, Uehara N, Baba H, Kunimoto T, Miyasaka T, Mutai M. Antitumor activity of a new derivative of camptothecin. In *Recent Ad-*

vances in Chemotherapy (Proc. 14th Int. Cong. Chemotherapy). Tokyo: University of Tokyo Press. 1985:28–30.

65. Kunimoto T, Nitta K, Tanaka T, Uehara N, Baba H, Takeuchi M, Yokokura T, Sawada S, Miyasaka, T, Muta M. Antitumor activity of 7-ethyl-10[4-(1-Piperidino)-1-Piperidino]carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin, against murine tumors. *Cancer Res* 1987; 47:5944–5947.
66. Kawato Y, Furuta T, Aonuma M, Yasuoka M, Yokokura T, Matsumoto K. Antitumor activity of a camptothecin derivative, CPT 11 against human tumor xenografts in nude mice. *Cancer Chemother Pharmacol* 1991; 28:192–198.
67. Houghton PJ, Cheshire PJ, Hallman JC, Bissery MC, Mathiew-Boue' A, Houghton JA. Therapeutic efficacy of the topoisomerase I inhibitor 7-ethyl-10-[4[1-Piperidino]-1-Piperidinocarbonyloxy-camptothecin against human tumor xenografts:lack of cross-resistance in vivo in tumors with acquired resistance to the topoisomerase I inhibitor. 9-dimethylaminomethyl-10-hydroxy-camptothecin. *Cancer Res* 1993; 53:2823–2389.
68. Negoro S, Fukuoka M, Masuda N, Takada M, Kasunoki Y, Matsui K, Takifuji N, Kudoh S, Niitani H, Taguchi T. Phase I study of weekly intravenous infusions of CPT 11, a new derivative of camptothecin in the treatment of advanced non-small cell lung cancer. *J Natl Cancer Inst* 1991; 83:1164–1168.
69. Ohe Y, Sasaki Y, Shinkai T, Eguchi K, Tamura T, Kojima A, Kunikane H, Okamoto H, Karato A, Ohmatsu H, Kanzawa F, Saijo N. Phase I study and pharmacokinetics of CPT 11 with 5-day continuous infusion. *J Natl Cancer Inst* 1992; 84:972–974.
70. Clavel M, Mathiew-Boue A, Dumortier A, Chabot GG, Bissery MC, Marty M. Phase I study of CPT-11 administered as a daily infusion for 3 consecutive days. *Proc Am Assoc Cancer Res* 1992; 33:262.
71. Culine S, De Forni M, Extra JM, Chabot GG, Madelaine I, Herait P, Bugat R, Marty M, Mathieu-Boue A. Phase I study of the camptothecin analogue CPT 11 using a weekly schedule. *Proc Am Soc Clin Oncol* 1992; 11:110.
72. Rowinsky E, Grochow L, Ettinger D, Hendricks L, Lubejko B, Sartorius S, Hurowitz L, McGuire W, Rock M, Donehower R. Phase I and pharmacologic study of CPT 11, a semisynthetic topoisomerase I-targeting agent, on a single dose schedule. *Proc Am Soc Clin Oncol* 1992; 11:115.
73. Ohno R, Okada K, Masaoka T, Kuramoto A, Arima T, Yoshida Y, Ariyoshi H, Ichimaru M, Sakai Y, Oguro M, Ito Y, Morishima Y, Yokomaku S, Ota K. An early phase II study of CPT 11: A new derivative of camptothecin for the treatment of leukemia and lymphoma. *J Clin Oncol* 1990; 8:1907–1912.
74. Tsuda H, Takatsuki K, Ohno R, Masaoki T, Okada K, Shirakawa S, Ohashi Y, Oma K, Taguchi T. A late phase II trial of a potent topoisomerase I inhibitor, CPT 11, in malignant lymphoma. *Proc Am Soc Clin Oncol* 1992; 11:1316.
75. Takeuchi S, Dobashi K, Fujimoto S, Tanaka K, Suzuki M, Terashima Y, Hasumi K, Akiya K, Negishi Y, Tamaya T, Tanizawa O, Sugawa T, Umesaka N, Sekiba, K, Aono T, Nakano H, Noda K, Shiota M, Yakushiji M, Sugiyama T, Hashimoto M, Yajima A, Takamizawa H, Sonoda T, Takeda Y, Tomoda Y, Ohta M, Ozaki M, Hirabayashi K, Hiura M, Hate M, Nishigaki K, Taguchi T. A late phase II study of CPT-11 in uterine cervical cancer and ovarian cancer. *Jpn J. Cancer Chemother* 1991; 18:1681–1689.
76. Takeuchi S, Noda K, Yakushiji M. CPT-11 study group on gynecologic malignancy. Late phase II study of CPT-11, topoisomerase I inhibitor, in advanced cervical carcinoma (CC). *Proc Am Soc Clin Oncol* 1992; 22:224 (abstr).
77. Masuda N, Fukuoka M, Kusunoki Y. CPT-11: A new derivative of camptothecin for the treatment of refractory or relapsed small-cell lung cancer. *J Clin Oncol* 1992; 10:1225–1229.
78. Fukuoka M, Niitani H, Suzuki A, Motomiya M, Hasegawa K, Nishiwaki Y, Kuriyama T, Ariyoshi Y, Negoro S, Masuda N, Nakajima S, and Taguchi T. A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small-cell lung cancer. *J Clin Oncol* 1992; 10:16–20.
79. Shimada Y, Yoshino M, Wakui A, Nakao I, Futatasuki K, Sakata Y, Kambe M, Taguchi T, Ogawa N. CPT-11 Gastrointestinal Cancer Study Group. Phase II study of CPT-11, new camptothecin derivative, in metastatic colorectal cancer. *J Clin Oncol* 1993; 11:909–913.

80. Masuda N, Fukuoka M, Takada M, Kusunoki Y, Negoro S, Matsui K, Kudoh S, Takifuji N, Nakagawa K, Kishimoto S. CPT-11 in combination with cisplatin for advanced non-small-cell lung cancer. *J Clin Oncol* 1992; 10:1775–1780.
81. Kudoh S, Kusunoki Y, Matsui K, Nakagawa K, Hirashima T, Tamaoki M, Nitta T, Yana T, Negoro S, Takifuji N, Takada M. Phase I study of irinotecan and cisplatin with granulocyte colony-stimulating factor support for advanced non-small-cell lung cancer. *J Clin Oncol* 1994; 12:90–96.
82. Johnson RK, McCabe FL, Faucette LF, Hertzberg RP, Kingsbury WD, Boehm JC, Caranfa MJ, Holden KG. SKF 104864, a water-soluble analog of camptothecin with broad spectrum activity in preclinical tumor models. *Proc Am Assoc Cancer Res* 1989; 30:623.
83. Tong W, Saltz L, Sirott K, Tookasi F, Cuisoh B. Rapid HPLC assay for topotecan. *Proc Am Assoc Cancer Res* 1991; 32:433.
84. Kingsbury WD, Boehm JC, Jakas DR, Holden KG, Hecht SM, Gallagher GG, Caranfa MJ, McCabe FL, Faucett LF, Johnson RK, Hertzberg RP. Synthesis of water soluble (aminoalkyl) camptothecin analogues: inhibition of topoisomerase I and antitumor activity. *J Med Chem* 1991; 34:98–107.
85. Johnson RK, Herzberg RP, Kingsbury WD, Boehm JC, Caranfa MJ, Faucett LF, McCabe FL, Holden KG. Preclinical profile of SKF104864, a water-soluble analog of camptothecin. Proc. NCI-EORTC 6th Symp. *New Drugs Cancer Ther* 1991:301.
86. Burris HA, Hanauske AR, Johnson RK. Activity of Topotecan, a new topoisomerase I inhibitor, against human tumor colony-forming units *in vitro*. *J Natl Cancer Inst* 1992; 84:1816–1820.
87. Houghton PJ, Cheshire PJ, Myers L, Stewart CF, Synold TW, Houghton JA. Evaluation of 9-dimethylaminomethyl-10-hydroxycamptothecin against xenografts derived from adult and childhood solid tumors. *Cancer Chemother & Pharmacol* 1992; 31:229–239.
88. Recondo G, Abbruzzese J, Newman B, Newman R, Kuhn J, Von Hoff D, Gartiez D, Raber M. A phase I trial of topotecan (TOPO) administered by a 24-hour infusion. *Proc Am Assoc Cancer Res* 1991; 32:206.
89. Wall JG, Burris HA, III, Von Hoff DD, Rodriguez G, Kneuper-Hall R, Shaffer D, O'Rourke T, Brown T, Weiss G, Clark G, McVea S, Brown J, Johnson R, Friedman C, Smith B, Mann WS, Kuhn J. A phase I clinical and pharmacokinetic study of the topoisomerase I inhibitor topotecan (SK&F 104864) given as an intravenous bolus every 21 days. *Anticancer Drugs* 1992; 3:337–345.
90. Rowinsky EK, Grochow LB, Hendricks CB, Ettinger DS, Forastiere AA, Hurowitz LA, McGuire WP, Sartorius SE, Lubejko BG, Kaufmann SH, Donehower RC. Phase I and pharmacologic study of topotecan: A novel topoisomerase I inhibitor. *J Clin Oncol* 1992; 10: 647–656.
91. ten Bokkel Huinink WW, Rodenhuis S, Beijnen J, Dubbelman R, Koier I. Phase I study of the topoisomerase I inhibitor topotecan (SK&F 104864-A). *Proc Am Soc Clin Oncol* 1992; 11:110.
92. Haas NB, LaCreta FP, Walczak J, Hudes GR, Brennan J, Ozols RF, O'Dwyer PJ. Phase I/pharmacokinetic trial of topotecan on a weekly 24-hour infusional schedule. *Proc Am Assoc Cancer Res* 1992; 33:523.
93. Eckardt J, Burris H, Kuhn J, Smith S, Rodriguez G, Weiss G, Smith L, Shaffer D, Johnson R, Von Hoff D. Phase I and pharmacokinetic trial of continuous infusion topotecan in patients with refractory solid tumors. *Proc Am Soc Clin Oncol* 1992; 1:138.
94. Blaney SM, Balis FM, Cole DE, Craig C, Reid JM, Ames MM, Krailo M, Reaman G, Hammond D, Poplack DG. Pediatric phase I trial and pharmacokinetic study of topotecan administered as a 24-hour continuous infusion. *Cancer Res* 1993; 53:1032–1036.
95. Kantarjian HM, Beran M, Ellis A, Zwelling L, O'Brien S, Cazenave L, Koller C, Rios MB, Plunkett W, Keating MJ, Estey EH. Phase I study of topotecan, a new topoisomerase I inhibitor, in patients with refractory or relapsed acute leukemia. *Blood* 1993; 81:146–151.
96. Hochster H, Speyer J, Oratz R, Meyers M, Wernz J, Chachoua A, Raphael B, Lee R, Sorich J, Taubes B, Liebs L, Fry D, Blum R. Topotecan 21 day continuous infusion-Excellent tolerance of a novel schedule. *Proc Am Soc Clin Oncol* 1993; 12:139.
97. Sirott MN, Saltz L, Young C, Tong W, Trochanowski B, Niedzwiecki D, Toomasi F, Kelsen D. Phase I and clinical pharmacologic study of intravenous topotecan. *Proc Am Soc Clin Oncol* 1991; 10:104.

98. Verweij J, Lund B, Beynen J, de Boer M, Koier I, Hansen HH. Clinical studies with topotecan: The EORTC experience, Proc. NCI-EORTC Symp. *New Drugs Cancer Ther* 1992; 7:118 (abstr).
99. Kraut EH, Staubus A, Majernick D, King G, Balcerzak SP. Phase II trial of topotecan in metastatic malignant melanoma. *Proc Am Assoc Cancer Res* 1995; 36:238.
100. Wanders J, Ardizzoni A, Hansen HH, Domernowsky P, Postmus PE, Buitenhuis M, McDonald M, Giacone G, Verweij J. Phase II study of topotecan in refractory and sensitivity small cell lung cancer (SCLC). *Proc Am Assoc Cancer Res* 1995; 36:237.
101. Hinz HR, Harris NJ, Natelson EA, Giovanella BC. Pharmacokinetics of the *in vivo* and *in vitro* conversion of 9-nitro-20(S)-camptothecin to 9-amino-20(S)-camptothecin in humans, dogs and mice. *Cancer Res* 1994; 52:3096–3100.
102. Pantazis P, Harris N, Mendoza J, Giovanella B. Conversion of 9-nitro-camptothecin to 9-aminocamptothecin by human blood cells *in vitro*. *Eur J. Haematol* 1994; 53:246–248.
103. Yakult Honsha CO., Ltd., Japan Patent 59-51288, 1984. Chem. Abstr. 1984, 101, 91319d, 91320x, 91322 2 (9NC and NH).
104. Wani MC, Nicholas AW, Wall ME. Plant antitumor agents, 23 synthesis and antileukemic activity of camptothecin analogues. *J Med Chem* 1986; 29:2358–2363.
105. Potmesil M, Giovanella BC, Wall ME, Liu LF, Silber R, Stehlin JS, Wani MC, Hochster H. Preclinical and clinical development of DNA topoisomerase I inhibitors in the United States. In: Andoh T, Ikeda H, Oguro M, eds. *Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy*. Nagoya, Japan: CRC. 1993:301–311.
106. Potmesil M, Hinz HR, Marcee A, Vardeman D, Stehlin JS, Wall ME, Wani MC, Silber R, Giovanella BC. Growth inhibition of human cancer metastases in the xenograft model by camptothecin [NSC 94600, CAM], 9-amino-[NSC 603071, 9-AC], and 9-nitrocamptothecin. *Proc Am Asoc Cancer Res* 1992; 33:432.
107. Giovanella BC, Wall ME, Wani MC, Silber R, Stehlin JS, Hochster H, Potmesil M. Efficacy of camptothecin (NSC 94600, CAM], 9-aminocamptothecin [NSC 603071, 9-AC], and 10,11-methylenedioxycamptothecin [NSC 606174, 10-11-MDC] in human cancer xenograft model. *Proc Am Assoc Cancer Res* 1992; 33:432.
108. Pantazis P, Early JA, Kozielski AJ, Mendoza JT, Hinz HR, Giovanella BC. Regression of human breast carcinoma tumors in immunodeficient mice treated with 9-nitrocamptothecin: Differential response of nontumorigenic and tumorigenic human breast cells *in vitro*. *Cancer Res* 1993; 53:1577–1582.
109. Pantazis P, Kozielski AJ, Mendoza JT, Early JA, Hinz HR, Giovanella BC. Campothecin derivatives induce regression of human ovarian carcinomas grown in nude mice and distinguish between non-tumorigenic and tumorigenic cells *in vitro*. *Int J Cancer* 1993; 53:863–871.
110. Pantazis P, Mendoza JT, Kozielski AJ, Natelson EA, Giovanella BC. 9-nitro-camptothecin delays growth of U-937 leukemia tumors in nude mice and is cytotoxic or cytostatic for human myelomonocytic leukemia lines *in vitro*. *Eur J Haematol* 1993; 50:81–89.
111. Pantazis P, Early JA, Mendoza JT, DeJesus AR, Giovanella BC. Cytotoxic efficacy of 9-nitro-camptothecin in the treatment of human malignant melanoma cells *in vitro*. *Cancer Res* 1994; 54:771–776.
112. Smith PL, Liehr JG, Ahmed AE, Hinz HR, Mendoza J, Kozielski A, Stehlin JS, Giovanella BC. Pharmacokinetics of tritium labeled camptothecin in nude mice. *Proc Am Assoc Cancer Res* 1992; 33:432.
113. Loh JR, Ahmed AE. Determination of camptothecin in biological fluids using reversed-phase high-performance liquid chromatography with fluorescence detection. *J Chromatogr* 1990; 53: 367–376.
114. Beijnen JH, Smith BR, Keijer WJ, Van Gijn R, ten Bokkel Huinink WW, Vlasveld LT, Rodenuhuis S, Underberg WJM. High-performance liquid chromatographic analysis of the new antitumor drug SK&F 104864-A (NSC 609699) in plasma. *J Pharmacol Biomed Anal* 1990; 8:789–794.
115. Supko JG, Malspeis L. A reversed-phase HPLC method for determining camptothecin in plasma with specificity for the intact lactone form of the drug. *J Liquid Chromatogr* 1991; 14: 1779–1803.

116. Supko JG, Malspeis L. Liquid chromatographic analyses of 9-aminocamptothecin in plasma monitored by fluorescence induced upon postcolumn acidification. *J Liquid Chromatogr* 1992; 15:3261-3283.
117. Supko JG, Plowman J, Dykes DJ, Zaharko DS. Relationship between the schedule dependence of 9-amino-20(S)-camptothecin (AC; NSC 603071) antitumor activity in mice and its plasma pharmacokinetics. *Proc Am Assoc Cancer Res* 1992; 33:432.
118. Supko JG, Malspeis L. Pharmacokinetics of the 9-amino and 10,11-methylenedioxy derivatives of camptothecin in mice. *Cancer Res* 1993; 53:3062-3069.
119. Beijnen JH, Rosing H, ten Bokkel Huinink WW, Pinedo HM. High-performance liquid chromatographic analysis of the antitumor drug camptothecin and its lactone ring-opened form in rat plasma. *Cancer Res* 1993; 53:3062-3069.
120. Rowinsky EK, Grochow LB, Ettinger DS, Sartorius SE, Lubjeko BG, Chen T, Rock MK, Donehower RC. Phase I and pharmacological study of the novel topoisomerase I inhibitor 7-ethyl-10[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) administered as a ninety-minute infusion every 3 weeks. *Cancer Res* 1994; 54:427-436.
121. Kaneda N, Yokokura T. Nonlinear pharmacokinetics of CPT 11 in rats. *Cancer Res* 1990; 50: 4187-4191.
122. Rothenberg ML, Kuhn JG, Burris HA, Nelson J, Eckhardt JR, Tristan-Morales M, Hilsenbeck SG, Weiss GR, Smith LS, Rodriguez GI, Rock MK, Von Hoff DD. Phase I and pharmacokinetic trial of weekly CPT-11. *J Clin Oncol* 1993; 11:2194-2204.
123. Saltz L, Sirott M, Young C, Tong W, Niedzwiecki D, Tzy-Jyun Y, Tao Y, Trochanowski B, Wright P, Barbosa K, Toomasi F, Kelsen D. Phase I clinical and pharmacology study of topotecan given daily for 5 consecutive days to patients with advanced solid tumors, with attempt at dose intensification using recombinant granulocyte colony-stimulating factor. *J Natl Cancer Inst* 1993; 85:1499-1507.
124. Scott DO, Bindra DS, Stella VJ. Plasma pharmacokinetics of the lactone and carboxylate forms of 20(S)-camptothecin in anesthetized rats. *Pharmacol Res* 1993; 10:1451-1457.
125. de Forni M, Bugat R, Chabot GG, Culine S, Extra J, Couyette A, Madelaine I, Marty ME, Mathiew-Boue A. Phase I and pharmacokinetic study of the camptothecin derivative irinotecan, administered on a weekly schedule in cancer patients. *Cancer Res* 1994; 54:4347-4354.
126. Rubin E, Wood V, Bharti A, Trites D, Lynch C, Hurwitz S, Bartel S, Levy S, Rosowsky A, Toppmeyer D, Kufe D. A phase I and pharmacokinetic study of a new camptothecin derivative, 9-aminocamptothecin. *Clin Cancer Res* 1995; 269:269-276.
127. Burke TG, Mi Z. Preferential binding of the carboxylate form of camptothecin by human serum albumin. *Anal Biochem* 1993; 212:285-287.
128. Burke TG, Mi Z. The structural basis of camptothecin interactions with human serum albumin: Impact on drug stability. *J Med Chem* 1994; 37:40-46.
129. Burke TG, Munshi CB, Mi Z, Jiang Y. The important role of albumin in determining the relative human blood stabilities of the camptothecin anticancer drugs. *J Pharm Sci.* 1995; 84:518,519.
130. Mi Z, Burke TG. Marked interspecies variations concerning interactions of camptothecin with serum albumin. *Biochemistry* 1994; 33:12,540-12,545.
131. Mi Z, Burke TG. Marked interspecies variations concerning interactions of camptothecin analogues with serum albumins. *Proc Am Assoc Cancer Res* 1995; 36:444(2647).
132. Burke TG, Staubus AE, Mishra AK, Malak H. Liposomal stabilization of camptothecin lactone ring. *J Am Chem Soc* 199; 114:8318,8319.
133. Burke TG, Mishra AK, Wan M, Wall ME. Lipid bilayer partitioning and stability of camptothecin drugs. *Biochemistry* 1992; 32:5352-5354.
134. Mi Z, Burke TG. Differential interactions of camptothecin lactone and carboxylate forms with human blood components. *Biochemistry* 1994; 33:10,325-10,336.
135. Tsuruo T, Matsuzaki T, Matsushita M, Saito H, Yokokura T. Antitumor effect of CPT-11, a new derivative of camptothecin, against pleiotropic drug-resistant tumors *in vitro* and *in vivo*. *Cancer Chemother Pharmacol* 1988; 21:71-74.
136. Chen AY, Yu C, Potmesil M, Wall ME, Wani MC, Liu LF. Camptothecin overcomes MDR1-mediated resistance in human KB carcinoma cells. *Cancer Res* 1991; 51:6039-6044.
137. Potmesil M, Giovanella BC, Liu LF, Wall ME, Silber R, Stehlin JS Jr, Hsiang Y-H, Wani MC. Preclinical studies of DNA topoisomerase I-targeted 9-amino and 10,11-methylenedioxy camp-

tothecins. In: Potmesil M, Kohn KW, eds. *DNA Topoisomerases in Cancer.* New York: Oxford University Press. 1991:299.
138. Potmesil M, Giovanella BC, Wall ME, Liu LF, Silber R, Stehlin JS, Wani MC, Hochseer H. Preclinical and clinical development of DNA topoisomerase I inhibitors in the United States. In: Andoh T, Ikeda H, Oguro M, eds. *Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy.* Nagoya, Japan: CRC. 1993:301–311.
139. Hendricks CB, Rowinsky ER, Grochow LB, Donehower RC, Kaufmann SH. Effect of P-glycoprotein expression on the accumulation and cytotoxicity of topotecan (SK&F 104864), a new camptothecin analogue. *Cancer Res* 1992; 52:2268–2278.
140. Kessel D. Some determinants of camptothecin responsiveness in leukemia L1210 cells. *Cancer Res* 1971; 31:1883–1887.
141. Andoh T, Ishii K, Suzuki Y, Ikegami Y, Kusunki Y, Takemoto Y, Okada K. Characterization of a mammalian mutant with a camptothecin-resistant DNA topoisomerase I. *Proc Natl Acad Sci USA* 1987; 94:5565–5569.
142. Gupta RS, Gupta R, Eng B, Lock RB, Ross WE, Hertzberg RP, Caranfa MJ, Johnson RK. Camptothecin-resistant mutants of Chinese hamster ovary cells containing a resistant form of topoisomerase I. *Cancer Res* 1988; 48:6404–6410.
143. Tan KB, Mattern MR, Eng W-K, McCabe FL, Johnson RK. Nonproductive rearrangement of DNA topoisomerase I and II genes: correlation with resistance to topoisomerase inhibitors. *J Natl Cancer Inst* 1989; 81:1732–1735.
144. Eng WK, McCabe FL, Tan KB, Mattern MR, Hofmann GA, Woessner RD, Hertzberg RP, Johnson RK. Development of stable camptothecin-resistant subline of P388 leukemia with reduced topoisomerase I content. *Mol Pharmacol* 1990; 38:471–480.
145. Kanzawa F, Sugimoto Y, Minato K, Kasahara K, Bungo M, Nakagawa K, Fujiwara Y, Liu LF, Saijo N. Establishment of a camptothecin analogue (CPT-11)-resistant cell line of human non-small cell lung cancer: characterization and mechanism of resistance. *Cancer Res* 1990; 50: 5919–5924.
146. Oguro M, Seki Y, Okada K, Andoh T. Collateral drug sensitivity induced in CPT-11 (a novel derivative of camptothecin)-resistant cell lines. *Biomed & Pharmacother* 1990; 44:209–216.
147. Sugimoto Y, Tsukahara S, Oh-hara T, Isoe T, Tsuruo T. Decreased expression of DNA topoisomerase I in camptothecin-resistant tumor cell lines as determined by a monoclonal antibody. *Cancer Res* 1990; 50:6925–6930.
148. Tamura H-O, Kohichi C, Yamada R, Ikeda T, Koiwa O, Patterson E, Keene JD, Okada K, Kjeldsen E, Nishikawa K, Andoh T. Molecular cloning of a cDNA of a camptothecin-resistant human DNA topoisomerase I and identification of mutation sites. *Nucleic Acids Res* 1991; 19: 69–75.
149. Ishimi Y, Nishizawa M, Andoh T. Characterization of a camptothecin-resistant human DNA topoisomerase I in an *in vitro* system for simian virus 40 DNA replication. *Eur J Biochem* 1992; 202:835–839.
150. Tanizawa A, Pommier Y. Topoisomerase I alterations in a camptothecin-resistant cell line derived from Chinese hamster DC3F cells in culture. *Cancer Res* 1992; 52:1848–1854.
151. Chang J-Y, Dethlefsen LA, Barley LR, Zhou BS, Cheng Y-C. Characterization of camptothecin-resistant Chinese hamster lung cells. *Biochem Pharmacol* 1992; 43:1732–1735.
152. Tanizawa A, Tabuchi A, Bertrand R, Pommier Y. Cloning of Chinese hamster DNA topoisomerase I cDNA and identification of a single-point mutation responsible for camptothecin resistance. *J Biol Chem* 1993; 268:25,463–25,468.
153. Andoh T, Koiwa O, Okada K. Molecular basis of resistance to CPT-11, specific inhibitor of DNA topoisomerase I. In: Miyazaki T, Takaku F, Sakuraba K, eds. *The Mechanisms and New Approach on Drug Resistance of Cancer Cells.* Amsterdam: Elsevier Science Publishers B.U. 1993:95–101.
154. Fujimori A, Harker WG, Hoki Y, Kohlhagen G, Pommier Y. Mutation at the catalytic site of topoisomerase I in a human leukemia cell line resistant to camptothecin. *Proc Am Assoc Cancer Res* 1994; 35:363.
155. Rubin E, Pantazis P, Bharti T, Toppmeyer D, Giovanella BC, Kufe D. Identification of a mutant human topoisomerase I with intact catalytic activity and resistance to 9-nitrocamptothecin. *J Biol Chem* 1994; 269:2433–2439.

156. Pantazia P, Mendoza JT, DeJesus A, Rubin E, Kufe D, Giovanella BC. Partial characterization of human leukemia U-937 cell sublines resistant to 9-nitro-camptothecin. *Eur J Haematol* 1994; 53:135–144.
157. Pantazis P, Mendoza J, DeJesus A, Early J, Shaw M, Giovanella B. Development of resistance to 9-nitro-camptothecin by human leukemia U-937 cells *in vitro* correlates with altered sensitivities to several anticancer drugs. *Anti-Cancer Drugs* 1994; 5:473–479.
158. Pantazis P, Vardeman D, Mendoza J, Early J, Kozielski A, DeJesus A, Giovanella BC. Sensitivity of camptothecin-resistant human leukemia cells and tumors to anticancer drugs with diverse mechanisms of action. *Leuk Res* 1995; 19:45–55.
159. Katz EJ, Vick JS, Kling KM, Andrews PA, Howell SB. Effect of topoisomerase modulators on cisplatin cytotoxicity in human ovarian carcinoma cells. *Eur J Cancer* 1990; 26:724–727.
160. Kano Y, Suzuki K, Akutsu M, Suda K, Inoue Y, Yoshida M, Sakamoto S, Miura Y. Effects of CPT-11 in combination with other anticancer agents in culture. *Int J Cancer* 1992; 50:604–610.
161. Johnson RK, Mattern MR, McCabe FL. Use of topotecan, a topoisomerase I inhibitor, in combination with other anticancer agents to circumvent resistance. Conf. DNA Topoisomerases Ther., 1992, 4th p. 33.
162. Kim JH, Kim SH, Kolozsvary A, Khil MS. Potentiation of radiation response in human carcinoma cells *in vitro* and murine fibrosarcoma *in vivo* by topotecan, an inhibitor of DNA topoisomerase I. *Int J Radiat Oncol Biol Phys* 1992; 22:515–518.
163. Mattern MR, Hofmann GA, McCabe FL, Johnson RK. Synergistic cell killing by ionizing radiation and topoisomerase I inhibitor topotecan (SK&F 104864). *Cancer Res* 1991; 51:5813–5816.
164. Giovanella B, Potmesil M, Wall ME, Stehlin JS, Hinz HR, Vardeman D, Mendoza J. Treatment of human cancer xenografts with camptothecin analogues in combination with cytotoxan or x irradiation. *Proc Am Assoc Cancer Res* 1994; 35:454(2711).
165. Lamond JP, Kirwin R, Wall ME, Boothman DA. Topoisomerase I inhibitors potentiate radiation lethality *in vitro*. *Proc Am Assoc Cancer Res* 1995; 36:605(3599).
166. Tamura K, Tacada M, Masuda N, Fukuoka M. Radiosensitization effect of CPT 11 against human lung tumor xenografts. *Proc Am Assoc Cancer Res* 1995; 605(3599).
167. Giovanella BC, Vardeman DM, Kozieski AK, Stehlin JS, Potmesil M. Enhanced antitumor effectiveness of camptothecin (cpt) and derivatives administered by continuous infusion through scheduling and combination with DNA damaging agents. *Proc Am Assoc Cancer Res* 1995; 36:391(2328).
168. Cozzarelli NR, Wand JC, eds. *DNA Topology and Its Biological Effects.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1990.
169. Li S, Adar KT. *Camptotheca Acuminata Decaisne.* Henry A and Rockwell M. Monographs Stephen F. Austin State University, Nacogdoches, TX 1994.
170. Schneider E, Hsiang Y-H, Liu LF. DNA topoisomerase as anticancer drug targets. In: August T, Anders MW, Murad F, Ikes A, eds. *Advances in Pharmacology,* vol. 21. New York: Academic, 1990:149–183.
171. Andoh T, Ikeda N, Oguro M, eds. *Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy.* Boca Raton, FL: CRC, 1993.
172. Liu L, ed. *Advances in Pharmacology,* vol. 29A. *DNA Topoisomerases: Biochemistry and Molecular Biology.* San Diego, CA: Academic, 1994.
173. Liu L, ed. *Advances in Pharmacology,* vol. 29B, *DNA Topoisomerases: Topoisomerase-Targeting Drugs.* San Diego, CA: Academic, 1994.
174. Potmesil M, Pinedo H, eds. *Camptothecins: New Anticancer Agents.* Boca Raton, FL: CRC, 1995.
175. Potmesil M. Camptothecins: from bench research to hospital wards. *Cancer Res* 1994; 54:1432–1439.
176. Potmesil M, Vardehar D, Kozielski AI, Mendoza I, Stehlin IS, Giovanella BC. Growth inhibition of human cancer metastases by camptothecins in heavy developed xenograft models. *Cancer Res* 1995; 55:5637–5641.

7 DNA Topoisomerase II Inhibitors

Yves Pommier, MD, PhD

CONTENTS

1. INTRODUCTION

DNA topoisomerases represent a major focus of research not only for cancer chemotherapy, but also for gene regulation, cell cycle, mitosis, and chromosome structure. A number of reviews have been written on the subject (*1–8*).

Several of the most active anticancer drugs, including etoposide (VP-16) and anthracyclines, poison cellular DNA topoisomerases II (top2). The genomic sites of top2 inhibition differ for each class of drug, thereby providing a rationale for the molecular interactions of drugs with top2 ("stacking model") and for the differential activity of top2 inhibitors in the clinic. More recently, another class of top2 inhibitors with a different mechanism of action has been identified. Bis(2,6)-dioxo-piperazines block the catalytic cycle of top2 by preventing the formation of cleavable complexes. This chapter will describe the enzymatic and biological properties of top2 and the peculiarities of each class of inhibitor. Top2 inhibitors will be classified in two groups: top2 poisons, which trap cleavable complexes, and top2 suppressors, which prevent cleavable complexes from forming.

1. DNA TOP2: ENZYMOLOGY AND CELL BIOLOGY

DNA topoisomerases catalyze the unlinking of DNA strands by making transient DNA strand breaks and allowing another DNA to traverse through these breaks. Two types of topoisomerases have been described in mammalian cells, type 1 and type 2 topoisomerases (top1 and top2), and three types in yeast, including topoisomerases III (*3,8–13*). DNA gyrase is the bacterial equivalent of top2. Antibacterial quinolones (nalidixic acid, ciprofloxacin, norfloxacin, and derivatives) are DNA gyrase inhibitors with no or very limited effect on the host human top2. Topoisomerase-mediated DNA breaks correspond to transesterification reactions where a DNA phosphoester bond is transferred to a specific enzyme tyrosine residue.

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

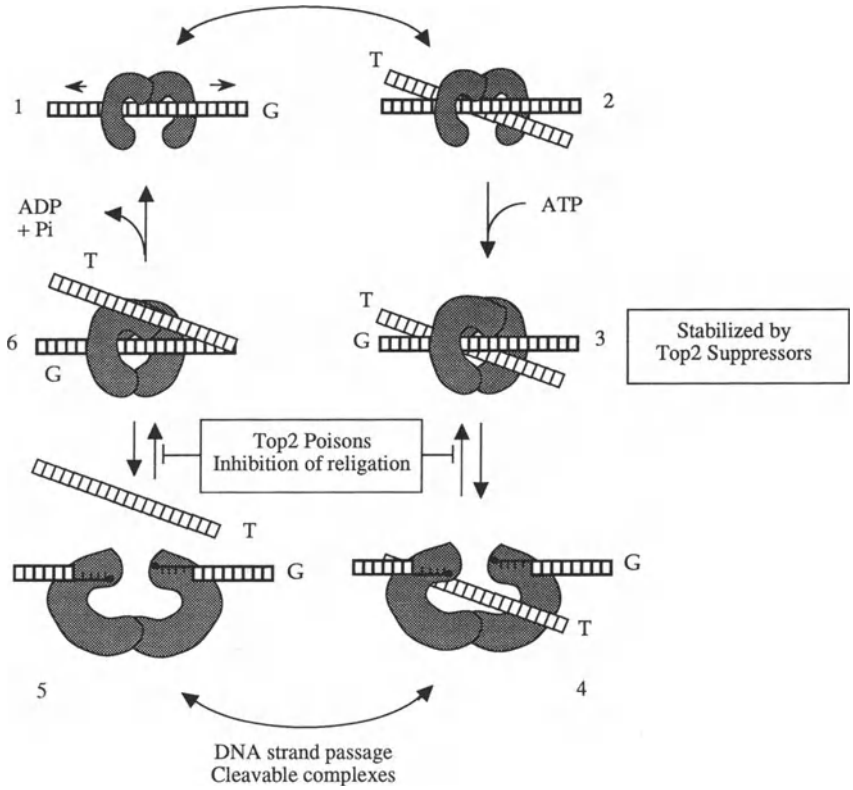


Fig. 1. Top2 catalytic cycle. (1): Noncovalent binding of the top2 dimer to the Gate DNA duplex. (2): DNA recognition and preferential binding to crossovers with G and Transported duplexes. (3): ATP binding promotes the formation of a topological complex on the G duplex; 2-6-dioxopiperazines stabilize this intermediate. (4): DNA cleavage with covalent linking of each top2 monomer to the 5′-DNA terminus of the break on the G duplex (DNA-enzyme transesterification); (5): Poststrand passage cleavable complex; the transported (T) duplex comes out on the other side of the enzyme complex. (6): Religation of the G duplex (DNA–DNA transesterification) followed by ATP hydrolysis and enzyme turnover. Top2 poisons block religation and trap cleavable complexes.

Mammalian DNA top1 and top2 differ in at least two ways. First, top1 relaxes DNA by forming a covalent bond with the 3′-terminus of a DNA single-strand break (for recent review, *see* Gupta et al. [*4*]), whereas top2 functions as a dimer and forms a double-strand break with each top2 molecule covalently bound to the 5′-terminus of the DNA double-strand break (*see* Fig. 1 and caption). Second, the top1 poisons, camptothecin and its derivatives, do not affect top2 (*4,14*). Conversely, top2 poisons (etoposides, doxorubicin, amsacrine, and so on) do not trap top1 cleavable complexes with the exception of a few DNA intercalators that trap cleavable complexes of both top1 and top2: actinomycin D (*15,16*), intoplicine (*17*), and saintopin (*18,19*).

Through its ability to open both strands of a DNA duplex (like a Gate; *see* Fig. 1) and to catalyze strand passage (Transport of another duplex through the gate; *see* Fig. 1 [*20*]), top2 can perform a variety of DNA topoisomerization reactions. Although DNA relaxation is common to top1, conversion of circular DNA to knotted forms, and removal of pre-existing knots are specific to top2. Top2-catalyzed strand passage between independent DNA rings is vital, because it allows decatenation of mitotic

and meiotic chromosomes (*see* next paragraph). These biochemical reactions are commonly used to assay topoisomerase activities in vitro: relaxation of supercoiled plasmid DNA in the absence of ATP and Mg^{2+} in the case of top1; and decatenation of kinetoplast DNA (kDNA) and unknotting of P4 DNA in the case of top2 (*12*).

Top1 and top2 can complement each other in yeast, where the absence of one can be compensated by the presence of the other topoisomerase. However, yeast top2 mutants are not viable and die at mitosis, because top2 is essential for chromosome condensation and structure (*3,8–13,21–24*), and for the proper segregation of mitotic (*21–27*) and meiotic (*28,29*) chromosomes. This is because, in addition to its DNA relaxing activity, top2 is essential for the separation of chromatin loops (decatenation of replicated DNA) and condensation of chromosomes, as well as proper segregation of sister chromatids (*25–27,30–34*). The accumulation of top2 at the end of S phase and during G2, and its concentration in the chromosome scaffold are consistent with the enzyme's roles during mitosis. The role of top2 in maintaining the structural integrity of mitotic chromosomes has recently been disputed (*24*). However, a fraction of top2 remains selectively associated with the telophase chromosomes, indicative of an important function during mitosis (*35*). A relationship is possible between top2 and cell-cycle-associated kinases/cyclins, since top2 phosphorylation increases during G2/M (*36*), resulting in enhanced catalytic activity (*see* next paragraph). Both top2α and β proteins are recognized by the monoclonal mitotic phosphoprotein antibody MPM-2 (*37*). The epitope for MPM-2 is conserved for a number of nuclear proteins and is dependent on phosphorylation during or just before M phase. Thus, both top2α and β are probably regulated by mitotic kinase(s). One of the kinases generating immunoreactivity with MPM-2 is MAP kinase (*38*), Today, evidence indicates, however, that top2 phosphorylation during G2/M in yeast is carried out by casein kinase II rather than cdk1-cyclin B (*39,40*).

Phosphorylation at serine/threonines by casein kinase II or protein kinase C enhances top2 catalytic activity (*41,42*), but attenuates the effects of the top2 poisons, etoposide and amsacrine (*43*). Together with the result of Takano et al. (*44*) showing increased phosphorylation in a human KB cell line resistant to etoposide, these observations suggest that top2 phosphorylation is important for determining the enzyme sensitivity to top2 inhibitors. Increased phosphorylation may activate the enzyme catalytic activity while reducing its sensitivity to top2 poisons.

Two top2 isoenzymes have been isolated, top2α and top2β. They differ in their molecular mass (170 dDa for top2α and 180 kDa for top2β), enzymatic properties (*45*), cell-cycle regulation (*46–48*), and cellular distribution (*49,50*). They are encoded by different genes (*45,51–54*). The top2α gene is on chromosome 17q21-22 (*51*), and the top2β gene on chromosome 3p24 (*52,54*). The top2 poison, teniposide (VM-26), and the top2 suppressor, merbarone, have been reported to be three- to eightfold more active on the top2α isoform (*45*). Recently, top2β has been cloned, expressed as a recombinant protein (*55*), and shown to form heterodimers with top2α (Westergaard et al., personal communication).

The top2 catalytic cycle is described in Fig. 1. Top2 dimers first bind reversibly to DNA [step 1 with a preference for DNA crossover regions (step 2)] (*56*). Hence, top2 interaction with DNA is determined both by DNA superstructure (DNA crossovers, bends, and so on) (*56–58*) and local sequence (*59–62*). Although top2 interacts with preferred sequences, its specificity is far less stringent than restriction endonucleases.

This lack of stringency probably allows the enzyme to act at multiple sites of the genome in order to perform its vital functions. On ATP binding, the top2 dimer forms a topological complex with the two DNA duplexes inside the enzyme (step 3) (*20,63,64*). DNA cleavage (step 4) requires divalent cation (Mg^{2+} in vivo) and corresponds to a nucleophilic attack from the catalytic tyrosine hydroxyl residue (Tyr 804 for human top2 [*9*]) toward the 5′-side of a DNA phosphodiester bond. This results in the formation of a DNA break associated with the covalent linkage of the 5′-DNA terminus to the catalytic tyrosine (DNA-enzyme transesterification reaction). The other DNA terminus is a 3′-hydroxyl. Each top2 subunit of the homodimer cleaves one strand of the duplex with a stagger of 4 overhanging bases at the 5′-DNA termini (steps 4–5, Fig. 1). The resulting double-strand break opens a gap in the first DNA duplex through which the other duplex can pass (strand passage reaction: steps 4–5). This strand passage reaction is completely dependent on the binding of Mg·ATP, but does not require ATP hydrolysis. Following strand passage, top2 religates the DNA by a reverse transesterification reaction (step 6). In this case, the enzyme catalyzes the nucleophilic attack of the enzyme tyrosine-DNA ester bond by a 3′-DNA hydroxyl terminus. Finally, top2 hydrolyzes ATP and can enter a new catalytic cycle.

Top2 suppressors (bis[2,5]-dioxopiperazines) inhibit the DNA cleavage step (steps 3–4, Fig. 1) by trapping the enzyme in the form of a closed protein clamp (*65*). Top2 poisons can trap cleavable complexes by inhibiting their religation either before or after DNA strand passage depending on the inhibitor (*66,67*) (Fig. 1). Formally, the possibility also exists that top2 poisons trap cleavable complexes without a passing DNA duplex inside (not shown in Fig. 1).

2. TOP2 POISONS

Anthracyclines and epipodophyllotoxins (Fig. 2) play key roles in the cancer chemotherapy armamentarium. Formation of cleavable complexes, rather than inhibition of top2 catalytic activity is responsible for activity.

Drug-induced cleavable complexes can be detected in cells as protein-linked DNA breaks by alkaline elution (*68,69*) and by sodium dodecyl sulfate (SDS)-KCl precipitation assays (*70,71*) (for review, *see 12,72*). However, these methods are limited to experimentally growing cells, since they require DNA readiolabeling. There is presently no convenient method to measure DNA cleavage in tumor samples.

Work by Osheroff and coworkers indicates that drugs may act differently in the top2 catalytic cycle (*66,73–75*). Although etoposide (VP-16) severely inhibits cleavable complex religation and has little effect on strand passage and ATP hydrolysis, genistein and quinolones have little effect on top2-mediated religation, but impair the ability of top2 to carry out its strand passage event and ATP hydrolysis. Amsacrine is unique because it inhibits similarly religation, strand passage, and ATP hydrolysis. These observations strongly suggest that the drugs interact with different top2 protein domains, which is consistent with the finding that some drug-resistant top2 mutant enzymes are not crossresistant to all inhibitors (*76–79*). Enzyme deletion mutants may prove useful in delineating the top2 domain(s) that interact with the drugs (*80*), and other cellular proteins involved in the subcellular distribution and phosphorylation of top2 (*81,82*).

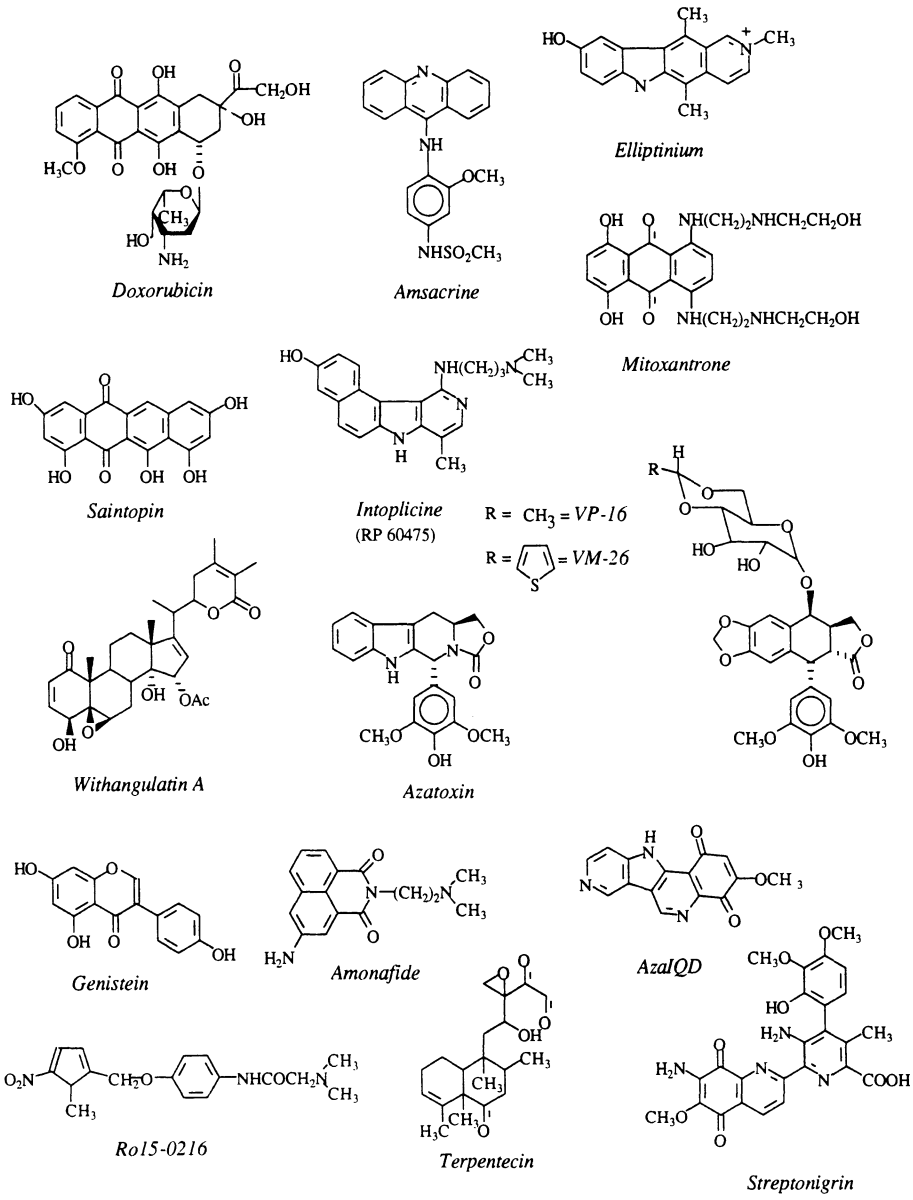


Fig. 2. Chemical structure of top2 poisons.

The molecular interactions of top2 poisons are not known precisely, since the structure of the drug–enzyme–DNA ternary complexes have not been resolved. Drug-induced cleavages do not occur randomly, and each class of inhibitor tends to act at top2 sites with different base sequence preference, either at the 3′- or 5′-terminus of the one of the cleavage sites of the DNA double-strand break (*19,83–92*). These strong drug-selective preferences for certain bases immediately flanking the cleavage sites suggest that the drugs interact directly with these bases. Top2 inhibitors (whether intercalator or not) have a planar aromatic portion that in some cases mimics a base

pair (Fig. 2). Hence, the simplest interpretation is that the drugs stack (intercalate) inside the cleavage sites. Depending on the drug structure, the preferential stacking would take place either at the 3′- or the 5′-terminus with a specific base. This hypothesis implies that topoisomerases first cleave the DNA at many sites, and that the drugs bind specifically to some sites and prevent their religation (*74,75*). The base sequence analyses also imply that stacking at one site is sufficient for the creation of a DNA double-strand break, a view consistent with the concerted action of both enzyme subunits during catalysis (*see* Fig. 1). Recent support for this hypothesis has been provided by Freudenreich and Kreuzer, who showed that an amsacrine derivative alkylates the + 1 base of top2 cleavable complexes (*92*). The same type of molecular interaction appears also to account for top1 poisoning by camptothecins (*4,93–95*).

Most clincial antitumor top2 inhibitors are substrates for the P-glycoprotein responsible for the classic multidrug resistance (MDR) phenotype: doxorubicin and analogs, mitoxantrone, anthrapyrazoles, ellipticines, and VP-16 (*96*), and to a lesser extent m-AMSA analogs (*97*). Cells overexpressing the P-glycoprotein are resistant to top2 inhibitors, because the drugs are actively extruded from the cells. In addition, non-*P*-glycoprotein-associated resistance with reduced drug uptake has recently been attributed to overexpression of a 190-kDa protein expressed by the multidrug resistance-associated protein (MRP) gene (*98–102*).

2.1. Demethylepipodophyllotoxins

Extracts from the mayapple or mandrake plant have long been used as a source of folk medicine (*103*). The active principle in this plant, podophyllotoxin, acts as an antimitotic agent that binds to tubulin at a site distinct from that occupied by the vinca alkaloids. A number of semisynthetic derivatives of podophyllotoxin have been made. Two glycosidic derivatives, VM-26 (teniposide) and VP-16 (etoposide) (Fig. 2), are active against a number of human malignancies. Etoposide (VP-16) was introduced in clinical trials in 1971 and approved by the FDA for marketing by Bristol Laboratories under the trade name Vepesid in early 1984. Teniposide (VM-26) has been used in Europe for several years and was approved by the FDA in 1992 for refractory childhood leukemia. More recently, etoposide phosphate has been designed as a prodrug of etoposide in order to obtain a water-soluble compound that could be specifically activated at the tumor sites using antibody–alkaline phosphatase conjugates (*104,105*). In fact, etoposides phosphate (Etopofos®) is almost immediately converted to etoposide in plasma by host endogenous phosphatases, and represents a water-soluble prodrug of etoposide.

The methyl group in *para* of the podophyllotoxin pendant ring is crucial for selectivity toward tubulin, and demethylation at this position is required for top2 poisoning (*106–108*). Demethylepipodophyllotoxins do not bind to DNA significantly in the absence of top2 and can be considered specific top2 poisons. This is in contrast to anthracyclines, which have other cellular targets (*see* below). Top2 cleavable complexes exhibit preference for DNA sites with cytosine at the 3′-DNA terminus opposite to the top2-linked terminus (position -1) (*83*). VP-16 or VM-26 induces similar cleavage patterns (*109,110*), and the cleavage frequency by nucleotide is one of the highest among top2 inhibitors (*61,111*). VM-26 is approximately 10-fold more potent than VP-16 at inducing cleavable complexes both in the presence of purified top2 (*109,110*) and in cells (*107,112*). In cells, cleavable complexes reverse rapidly after drug removal, and consist of a mixture of single- and double-strand breaks (*112,113*).

2.2. Anthracyclines

In contrast to demethylepipodophyllotoxins, anthracyclines (Fig. 2) exert other effects in addition to top2 inhibition: DNA intercalation (*114*) and inhibition of other DNA processing enzymes, such as helicases (*115*), and production of free radicals (*116*) (for recent review, *see 117*). New anthracyclines have also been introduced in the clinics (4-demethoxydaunorubicin [Idarubicin], 4′-epidoxorubicin [Epirubicin]). 4-Demethoxydaunorubicin [Idarubicin] exhibits greater potency against purified top2 than daunorubicin, indicating that removal of the 4-methoxy group yields better top2 inhibitors (*114*). Another approach has been to synthesize analogs with modified sugar to limit the drug recognition by the P-glycoprotein^{MDR}. The amino group on the daunosamine sugar is probably involved in the recognition of the drug by the P-glycoprotein, since the deamino derivative, hydroxyrubicin, is less subject to drug resistance while retaining top2 inhibitory activity (*118*).

The DNA sequence selective inhibition of top2 is common to all anthracyclines (*114, 119*), and the frequency of top2 cleavage per nucleotide is markedly less than in the case of epipodophyllotoxins (*61,120*). Thus, anthracyclines exhibit the highest DNA sequence selectivity among top2 poisons. DNA sequence analyses of the top2-induced cleavage sites indicate a requirement for adenine at the 3′-terminus of one of the two break sites of the top2-induced DNA double-strand break (*85*). In addition, anthracycline-induced cleavable complexes decrease with increasing concentration, probably as a result of DNA intercalation (*114*). Hence, anthracyclines are top2 poisons at low concentrations and suppressors of cleavable complexes at higher concentrations.

The cleavable complexes induced in cells by anthracyclines are also relatively infrequent at a given cytotoxicity level and consist mostly of DNA double-strand breaks. Another difference with the top2 cleavable complexes induced by demethylepipodophyllotoxins and amsacrine is the relatively slow reversibility of the anthracycline-induced top2 cleavable complexes (*121,122*).

2.3. Anthraquinones—Mitoxantrone and Derivatives

Mitoxantrone and derivatives are primarily used in the treatment of breast cancers. They are top2 poisons and DNA intercalators (*123–126*). Hence, they exhibit the dual top2 effects: poisoning at low doses and suppression of cleavable complexes at higher doses. Their pattern of DNA cleavage is unique, although they show similar preference for trapping top2 cleavable complexes with C-1 as in the case of demethylepipodophyllotoxins (*87*). That their top2-induced DNA cleavage patterns are different from those of demethylepipodophyllotoxins implies that other DNA base sequence requirements or structures determine their specificity (*87,126–128*).

MDR cells are usually crossresistant to mitoxantrone, and mitoxantrone can induce P-glycoprotein^{MDR}-mediated (*123,125,129,130*) as well as MRP-mediated (*131*) multidrug resistance. Interestingly, a mitoxantrone-resistant human leukemia HL60 cell line has been shown to exhibit selective alteration of the top2β gene, suggesting that mitoxantrone might preferentially poison top2β (*129,132*).

2.4. Amsacrine

Amsacrine (4′[9-acridinylamino]-methanesulfon-*m*-aniside) (*m*-AMSA) (Fig. 2) was designed and synthesized in 1974 by Cain and Atwell (*133*). It first entered clinical evaluation under National Cancer Institute (NCI) sponsorship in 1976, and its clinical profile is now established. Amsacrine is primarily used in the treatment of

hematological malignancies, with emphasis on pediatric and adult acute leukemias, and some activity in lymphomas (*134*). A variety of Phase II trials demonstrated no useful activity against human solid tumors (*135*).

The *m*-AMSA analog, 4-methyl-5-methylcarboxamide (CI-921) (*136,137*), exhibits significantly greater activity against solid tumors both in vitro and in vivo. It has recently entered Phase I and II clinical trials (*138,139*).

m-AMSA is a potent top2 poison; it induces high frequency of top2 cleavable complexes at relatively low concentrations (*121,122*). *m*-AMSA is a relatively weak DNA intercalator (*140*), and the top2 suppressive effect is only observed at high concentrations (generally above 50 μM in the presence of purified top2 and above 100 μM in cells) (*122,141*). These properties turned out to be crucial during the identification of top2 as the target of DNA intercalators, since *m*-AMSA was the first drug used successfully to show top2 poisoning (*142*), and to identify and purify top2 as the nuclear protein forming the protein-linked DNA breaks (*143*). Another interesting feature of *m*-AMSA is that its stereoisomer, *o*-AMSA, which has a methoxygroup in position *ortho* instead of *meta,* is almost inactive as a top2 poison (*122,142*), while being a slightly better DNA intercalator (*140*). This demonstrates a lack of correlation between DNA intercalation and top2 poisoning for acridines (*144*) and probably for most top2 poisons, since the same holds true for anthracyclines (*114*). It has been suggested that the differential anti-top2 activity of *m*-AMSA and *o*-AMSA reflects selective interaction of the *m*-AMSA side chain with the enzyme in the top2–DNA ternary complex (*128,137*).

The frequency of *m*-AMSA-induced top2 cleavable complexes per nucleotide is intermediate between that of anthracyclines and demethylepipodophyllotoxins (*61*). It is comparable to mitoxantrone. The base sequence selectivity of *m*-AMSA-induced top2 cleavable complexes is unique among top2 poisons, since the pattern of cleavage differs from those of other top2 poisons. *m*-AMSA-induced cleavable complexes exhibit preference for an adenine at the 5′-DNA termini of top2 cleavable complexes (*86*). A recent study from Freudenreich and Kreuzer strongly supports the drug stacking model by showing that an *m*-AMSA derivative selectively alkylates the +1 base of top2 cleavable complexes (*92*).

m-AMSA is less susceptible to P-glycoprotein-mediated MDR than epipodophyllotoxins, anthracyclines, and mitoxantrones (*96,97*). Its cellular uptake and distribution exhibit unique characteristics (*145,146*).

2.5. Ellipticines

Ellipticine is an alkaloid derived from the Apocynaceae family, including *Ochrosia, Bleekeria vitensis,* and *Aspidosperma subincanum* (*103*). Despite its promising preclinical activity, severe toxic effects observed in animal studies hampered the progress of ellipticine toward clinical trials. The semisynthetic derivative, 2-*N*-methyl-9-hydroxyellipticinium acetate (NMHE = elliptinium) (Fig. 2), however, exhibited good activity in vivo in various mouse and rat systems, and also lacked hematopoietic toxicity (*147*). Clinical trials of elliptinium were initiated in 1977. Presently, it is used in the treatment of advanced breast cancer in Europe.

Ellipticines are potent DNA intercalators. Hence, top2 cleavable complexes tend to be suppressed at high drug concentrations, and this property, common to all intercalators, was observed early on for ellipticines (*141,148,149*). The DNA sequence selectiv-

ity for ellipticines is T-1 (*90*). In cells, the ellipticine-induced DNA breaks have the unique characteristic to be almost exclusively double-stranded (*150,151*). A Chinese hamster lung fibroblast cell line made resistant to ellipticine was used first to show that top2 resistance is associated with reduction of top2 cleavable complexes (*152,153*).

2.6. Other Top2 Poisons

A large number of top2 poisons from diverse chemical families have been identified (amonafide [*154–157*], flavones, genistein and derivatives [*158–160*], the nitroimidazole Ro 15-0216 [*161*], withangulatin [*162*], streptonigrin [*88,89,163*], terpenoids [*164,165*], azatoxins [*110,166,167*], quinolones [*73,168–171*], anthraquinones [*172*], menogaril [*173*], naphtoquinones [*174*], the polyaromatic quinone antibiotic UCE6 [*175*]), and NSC 665517, a compound recently identified from the NCI Anticancer Drug Screen using the COMPARE algorithm (*176*).

Azatoxin was rationally designed from a pharmacophore analysis of top2 poisons (*110,177*). It turned out to be a dual inhibitor of tubulin polymerization and of top2 religation without detectable DNA intercalation (*110,167*). Analogs have recently been made that act only as top2 poisons or tubulin inhibitors (*166*). Some of the azatoxins with top2 poisoning activity are among the most potent top2 inhibitors without being P-glycoprotein^{MDR} substrates (*166,178*).

Each class of drug exhibits specific DNA cleavage patterns in the presence of top2. Streptonigrin has the unique property to select top2 cleavable complexes with base preference in the middle of the 4-bp stagger (T + 2 and A + 3) (*88,89*). Also some, such as terpenoids (*164,165*) and anthraquinones with alkylating groups (*172*), produce irreversible cleavable complexes. This last class of compounds might be useful in determining the drug binding sites on top2 (and/or DNA).

3. TOP2 SUPPRESSORS

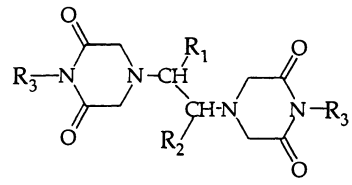
Inhibition of top2 catalytic activity without trapping of cleavable complexes can also be observed (Fig. 3). This is the case for strong DNA intercalators, such as anthracyclines and ellipticines (*120,141,149,179–181*). DNA minor groove binders, such as distamycin, have more complex effects with global enhancement of top2 cleavable complexes at low concentrations (< 0.1 μM in purified systems), and DNA sequence-specific redistribution of cleavable complexes at higher concentrations (*181*).

Hence, three types of dose–response curves can be observed for top2 inhibitors:

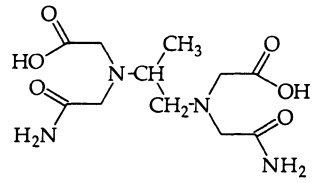
1. A monotonal increase of cleavable complexes with drug concentration in the case of weak (or non-) DNA binders (VP-16, VM-26, azatoxins, amsacrine);
2. A bell-shaped curve in the case of anthracyclines, mitoxantrones, ellipticines, and other DNA intercalators; and
3. A monotonal decrease of cleavable complexes in the case of bulky intercalators (ethidium bromide, ditercalinium, aclarubicin) (*179,180,189*) or non-DNA binders that inhibit the catalytic activity without trapping cleavable complexes (2,6-dioxopiperazines, merbarone, and fostriecin).

Some non-DNA binders can also suppress cleavable complexes and will be described in more details below. They are merbarone (*182*), bis(dioxopiperazine) derivatives (*183,184*), suramin (*185*), and fostriecin, for which contradictory results have been published (*186–188*) (Fig. 3).

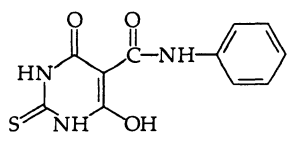
2,6-DIOXOPIPERAZINES



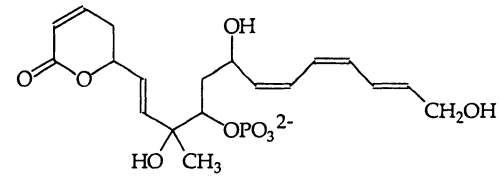
ICRF	R1	R2	R3	
154	H	H	H	
159	CH3	H	H	racemic = Razoxane
187	CH3	H	H	R+ = ADR-529 = Dexrazoxane
186	CH3	H	H	R-
192	CH2-CH3	H	H	
193	CH3	CH3	H	mesoisomer
196	CH3	CH3	H	diisomer
MST-16	H	H	CH_2-O-C(=O)-O-CH_2-CH(CH_3)$_2$	



ICRF-198 = ADR-925



Merbarone



Fostriecin

Fig. 3. Chemical structure of suppressors of top2 cleavable complexes.

3.1. 2,6-Dioxopiperazines

Bis(dioxopiperazines) were originally synthesized as potential intracellular chelating agents, and their biochemical and pharmacological properties, including antitumor activity, were studied extensively well before top2 was identified as their cellular target (*190*). The structures of several 2,6-dioxopiperazines are shown in Fig. 3. ICRF-159 (razoxane) is a racemic mixture, and ICRF-187 (dexrazoxane = ADR529 = NSC 169780) and ICRF-186 (levrazoxane) are its *R* + and *R* − stereoisomers, respectively. In 1991, Tanabe and coworkers reported that the ICRF derivatives inhibit top2 catalytic activity without inducing cleavable complexes or binding to DNA (*184*). ICRF-193 was the most potent compound with an IC_{50} around 2 μM, ICRF-154 and ICRF-159 were approximately equipotent with IC_{50}s around 20 μM, and MST-16 was approx 10-fold less potent with an IC_{50} around 300 μM (*184*). The same group also reported that the cellular effects were consistent with inhibition of top2 catalytic activity, since

etoposide-induced cleavable complexes were greatly inhibited both in the presence of purified top2 (*184*) and in cells (*183*). Drug-treated cells are arrested in G2 and early M phase with fewer condensed and entangled chromosomes and with multilobed nuclei (*183*). More recent analysis showed that, in the presence ICRF-193, cells traverse many rounds of the cell cycle with their genome replicated, but not segregated, resulting in polyploidization (*191*). These effects are analogous to those observed in *top2* ts mutants of yeast at nonpermissive temperature (*27*). 2,6-Dioxopiperazines are presently used to study the cellular functions of top2 (*192*).

The molecular interactions of ICRF-193 with top2 have been examined, and appear to result from trapping the enzyme in the form of a closed protein clamp as a result of blocking the interconversion between the open- and closed-clamp forms of the enzyme (Fig. 1) (*65*). Although no cleavable complexes are induced by 2,6-dioxopiperazines, chromosome breakage may be generated as a result of disruption of segregation as in the case of *Saccharomyces cerevisiae top2* mutants (*26*).

MST-16, a bis(dioxopiperazine) (Fig. 3) derivative developed (*193*) before knowing its effects on top2 (*184*), has recently been shown to exhibit significant antitumor activity and selective inhibition of cell proliferation at the G2/M transition (*193,194*). Clinical trials of orally administered MST-16 in adult T-cell leukemia-lymphoma, which has no standard therapy, showed the drug to be effective (*195–197*).

ICRF-187 (ADR 529 = dexrazoxane) is very effective in protecting against doxorubicin-induced cardiotoxicity both in animal and clinical studies (*198*). Anthracyclines produce cumulative, dose-dependent, irreversible cardiomyopathy that can lead to congestive heart failure. Through its semiquinone metabolite, doxorubicin can generate superoxide anion and superhydroxide free radicals with iron as a cofactor. These free radicals cause extensive lipid peroxidation and mitochondrial destruction. Dexrazoxane is hydrolyzed to its active one or two rings-opened (ADR925) metal chelating forms intracellularly in the presence of iron (*199*. The active ring-opened metabolites displace Fe^{3+} from its complex with doxorubicin, daunorubicin, epirubicin, and idarubicin within minutes (*200*) to prevent formation of superhydroxide radicals. The protection against anthracyclines effect may also be related to the suppression of anthracycline-induced cleavable complexes by the ring-closed form (ICRF-187, dexrazoxane), an effect that may not be so desirable, because it would also reduce the antitumor activity of the anthracyclines.

3.2. Merbarone

Drake et al. first reported that merbarone (5-[*N*-phenylcarboxamido]-2-thiobarbituric acid [NSC 336628]) (Fig. 3) (*201*) inhibits top2 catalytic activity without inducing cleavable complexes (*182*), and that merbarone exhibited some selectivity for top2α (p170) (*45*). More recently, Chen and Beck reported that merbarone, like ICRF-193, inhibits chromosome condensation and separation without preventing cells from exiting mitosis. It causes S-phase retardation G2 arrest, and polyploidy in synchronized cultures of HeLa cells (*202*) and in human leukemic CEM/VM-1 cells (*203*). Interestingly, the VM-26-resistant cells CEM/VM-1 are not crossresistant to merbarone, indicating that the merbarone and VM-26 interact on different enzyme sites.

Merbarone is in Phase II clinical trials, but so far does not appear active in a variety of solid tumors including renal cell (*204*), hepatocellular (*205*), pancreatic (*206,207*), or gastric (*208*) carcinomas, or small-cell lung cancer (*209*).

3.3. *Fostriecin*

Fostriecin (CI-920) is a phosphate-containing polyene lactone antitumor agent (Fig. 3) isolated from a previously undescribed subspecies of *Streptomyces pulveraceus* cultured from a Brazilian soil sample. Fostriecin is active on a variety of tumor models (*210–212*) and causes an accumulation of cells in the G2 + M phase of the cell cycle (*188,210*).

Fostriecin inhibits the catalytic activity of top2 and the cleavable complexes induced by *m*-AMSA (*188*). As in the case of merbarone, teniposide (VM-26)-resistant cells are not crossresistant to fostriecin (*203*). Recent work by Roberge and coworkers has challenged top2 inhibition as the most relevant cellular effect of fostriecin. Roberge et al. found that fostriecin is 10- to 100-fold more potent against protein phosphatases 1 and 2A, respectively, than against top2 (*213*). They proposed that fostriecin interferes with the mitotic control and induces cycling cells to enter mitosis prematurely. Cells that are in the division cycle by treatment with the DNA replication inhibitor aphidicolin or with the DNA-damaging agents teniposide (VP-16) or camptothecin (*213*) are forced into mitosis by fostriecin. A more recent publication makes an analogy between the cell-cycle effects of fostriecin and another protein phosphatase inhibitor, okadaic acid (*214*). Chromosome condensation induced by fostriecin does not require p34cdc2 kinase activity and histone H1 hyperphosphorylation, but is associated with enhanced histone 2A and H3 phosphorylation (*214*).

4. CONCLUSIONS

DNA topoisomerase poisons are presently a major element of the anticancer armamentarium. Their clinical differences suggest that novel top2 poisons may provide additional progress for cancer chemotherapy. Suppressors top2 cleavable complexes represent another class of drugs with interesting biological, pharmacological, and possibly therapeutic properties.

ACKNOWLEDGMENTS

The author wishes to thank Kurt W. Kohn, Laboratory of Molecular Pharmacology, NCI, for his continuous support and exciting discussions, and Malini Gupta for suggestions on the manuscript.

REFERENCES

1. Wang JC. DNA topoisomerases. *Annu Rev Biochem* 1985; 54:665–697.
2. Wang JC. Recent studies of DNA topoisomerases. *Biochim Biophys Acta* 1987; 909:1–9.
3. Wang JC. DNA topoisomerases: why so many? *J Biol Chem* 1991; 266:6659–6662.
4. Gupta M, Fujimori A, Pommier Y. Eukaryotic DNA topoisomerases I. *Biochim Biophys Acta* 1995; 1262:1–14.
5. Pommier Y, Leteurtre F, Fesen M, Fujimori A, Bertrand R, Solary E, Kohlhagen G, Kohn KW. Cellular determinants of sensitivity and resistance to DNA topoisomerase inhibitors. *Cancer Invest* 1994; 12:530–542.
6. Pommier Y. DNA topoisomerases I and II in cancer chemotherapy: update and perspectives. *Cancer Chemother Pharmacol* 1993; 32:103–108.
7. Watt PM, Hickson ID. Structure and function of type II DNA topoisomerases. *Biochem J* 1994; 303:681–695.

8. Osheroff N, Zechierich EL, Gale KC. Catalytic function of DNA topoisomerase II. *BioEssays* 1991; 13:269-275.
9. Caron PR, Wang JC. DNA topoisomerases as target of therapeutics: a structural overview. In: Andoh T, Ikeda H, Oguro M, eds. *Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy*. Boca Raton, FL: CRC. 1993:1-18.
10. Champoux J. Mechanistic aspects of type-I topoisomerases. In: Wang JC, Cozarelli NR, eds. DNA topology and its biological effects. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1990:217-242.
11. Kohn KW, Pommier Y, Kerrigan D, Markovits J, Covey JM. Topoisomerase II as a target of anticancer drug action in mammalian cells. *NCI Monographs* 1987; 4:61-71.
12. Pommier Y, Kohn KW. Topoisomerase II inhibition by antitumor intercalators and demethylepipodophyllotoxins. In: Glazer RI, eds. *Developments in Cancer Chemotherapy*. Boca Raton, FL: CRC. 1989:175-196.
13. Pommier Y, Tanizawa A. Mammalian DNA topoisomerase I and its inhibitors. In: Hickman J, Tritton T, eds. *Cancer Chemotherapy*. Oxford: Blackwell Scientific Publications. 1993: 214-250.
14. Hsiang YH, Hertzberg R, Hecht S, Liu LF. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* 1985; 260:14,873-14,878.
15. Trask DK, Muller MT. Stabilization of type I topoisomerase-DNA covalent complexes by actinomycin D. *Proc Natl Acad Sci USA* 1988; 85:1417-1421.
16. Wasserman K, Markovits J, Jaxel C, Capranico G, Kohn KW, Pommier Y. Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerases I and II. *Mol Pharmacol* 1990; 38:38-45.
17. Poddevin B, Riou J-F, Lavelle F, Pommier Y. Dual topoisomerase I and II inhibition by intoplicine (RP-60475) a new antitumor agent in early clinical trials. *Mol Pharmacol* 1993; 44: 767-774.
18. Yamashita Y, Kawada S-Z, Fujii N, Nakano H. Induction of mammalian topoisomerase I and II mediated DNA cleavage by saintopin, a new antitumor agent from fungus. *Biochemistry* 1991; 30:5838-5845.
19. Leteurtre F, Fujimori A, Tanizawa A, Chhabra A, Mazumder A, Kohlhagen G, Nakano H, Pommier Y. Saintopin, a dual inhibitor of DNA topoisomerases I and II, as a probe for drug-enzyme interactions. *J Biol Chem* 1994; 269:28,702-28,707.
20. Roca J, Wang JC. DNA transport by a type II DNA topoisomerase: evidence in favor of a two-gate mechanism. *Cell* 1994; 77:609-616.
21. Adachi Y, Luke M, Laemmli UK. Chromosome assembly in vitro: topoisomerase II is required for condensation. *Cell* 1991; 64:137-148.
22. Earnshaw WC, Heck MM. Localization of topoisomerase II in mitotic chromosomes. *J Cell Biol* 1985; 100:1716-1725.
23. Gasser SM, Laroche T, Falquet J, Boy de la Tour E, Laemmli UK. Metaphase chromosome structure. Involvement of topoisomerase II. *J Mol Biol* 1986; 188:613-629.
24. Hirano T, Mitchison TJ. Topoisomerase II does not play a scaffolding role in the organization of mitotic chromosomes assembled in *Xenopus* egg extracts. *J Cell Biol* 1993; 120:601-612.
25. DiNardo S, Voelkel K, Sternglanz R. DNA topoisomerase II mutant of Saccharomyces cerevisiae: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. *Proc Natl Acad Sci USA* 1984; 81:2616-2620.
26. Holm C, Stearns T, Botstein D. DNA topoisomerase II must act at mitosis to prevent nondisjunction and chromosome breakage. *Mol Cell Biol* 1989; 9:159-168.
27. Uemura T, Ohkura H, Adachi Y, Morino K, Shiozaki K, Yanagida M. DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*. *Cell* 1987; 50:917-925.
28. Rose D, Holm C. Meiosis-specific arrest revealed in DNA topoisomerase II mutants. *Mol Cell Biol* 1993; 13:3445-3455.
29. Moens PB, Earnshaw WC. Anti-topoisomerase II recognizes meiotic chromosome cores. *Chromosoma* 1990; 98:317-322.
30. Shamu CE, Murray AM. Sister chromatid separation in frog egg extracts requires DNA topoisomerase II activity during anaphase. *J Cell Biol* 1992; 117:921-934.

31. Wood ER, Earnshaw WC. Mitotic chromatin condensation in vitro using somatic cell extracts and nuclei with variable levels of endogenous topoisomerase II. *J Cell Biol* 1990; 111:2839–2850.
32. Newport J. Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. *Cell* 1987; 48:205–217.
33. Newport J, Spann T. Disassembly of the nucleus in the mitotic extracts: membrane vesiculization, lamin disassembly, and chromosome condensation are independent processes. *Cell* 1987; 48:219–230.
34. Downes CS, Mullinger AM, Johnson RT. Inhibitors of DNA topoisomerase II prevent chromatid separation in mammalian cells but do not prevent exit from mitosis. *Proc Natl Acad Sci USA* 1991; 88:8895–8899.
35. Swedlow JR, Sedat JW, Agard DA. Multiple chromosomal populations of topoisomerase II detected in vivo by time-lapse, three-dimensional wide-field microscopy. *Cell* 1993; 73:97–108.
36. Heck MMS, Hittelman WN, Earnshaw WC. In vivo phosphorylation of the 170-kDa form of eukaryotic DNA topoisomerase II. *J Biol Chem* 1989; 264:15,161–15,164.
37. Taagepera S, Rao PN, Drake FH, Gorbsky GJ. DNA topoisomerase II-alpha is the major chromosome protein recognized by the mitotic phosphoprotein antibody MPM-2. *Proc Natl Acad Sci USA* 1993; 90:8407–8411.
38. Kuang J, Ashorn CL. At least two kinases phosphorylate the MPM-2 epitope during *Xenopus oocyte* maturation. *J Cell Biol* 1993; 123:859–868.
39. Cardenas ME, Dang Q, Glover CVC, Gasser SM. Casein kinase II phosphorylates the eukaryote-specific C-terminal domain of topoisomerase II in vivo. *EMBO J* 1992; 11:1785–1796.
40. Bojanowski K, Filhol O, Cochet C, Chambaz EM, Larsen AK. DNA topoisomerase II and casein kinase II associate in a molecular complex that is catalytically active. *J Biol Chem* 1994; 268:22,920–22,926.
41. Ackerman P, Glover CV, Osheroff N. Phosphorylation of DNA topoisomerase II in vivo and in total homogenates of Drosophila Kc cells. The role of casein kinsae II. *J Biol Chem* 1988; 263:12,653–12,660.
42. Corbett AH, Fernald AW, Osheroff N. Protein kinase C modulates the catalytic activity of topoisomerase II by enhancing the rate of ATP hydrolysis: evidence for a common mechanism by regulation by phosphorylation. *J Biol Chem* 1993; 32:2090–2097.
43. De Vore RF, Corbett AH, Osheroff N. Phosphorylation of topoisomerase II by casein kinase II and protein kinase C: effects on enzyme-mediated DNA cleavage/religation and sensitivity to the antineoplastic drugs etoposide and 4′-(9-acridinylamino)methane-sulfon-*m*-anisidide. *Cancer Res* 1992; 52:2156–2161.
44. Takano H, Kohno K, Ono M, Uchida Y, Kuwano M. Increased phosphorylation of DNA topoisomerase II in etoposide-resistant mutants of human KB carcinoma cells. *Cancer Res* 1991; 51:3951–3957.
45. Drake FH, Hofmann GA, Bartus HF, Mattern MR, Crooke ST, Mirabelli CK. Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry* 1989; 28:8154–8160.
46. Tsutsui K, Tsutsui K, Okada S, Watanabe M, Shohmori T, Seki S, Inoue Y. Molecular cloning of partial cDNAs for rat DNA topoisomerase II isoforms and their differential expression in brain development. *J Biol Chem* 1993; 268:19,076–19,083.
47. Woessner RD, Mattern MR, Mirabelli CK, Johnson RK, Drake FH. Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. *Cell Growth Differ* 1991; 2:209–214.
48. Capranico G, Tinelli S, Austin CA, Fisher ML, Zunino F. Different patterns of gene expression of topoisomerase II isoforms in differentiated tissues during murine development. *Biochim Biophys Acta* 1992; 1132:43–48.
49. Negri C, Scovassi AI, Braghetti A, Guano F, Astaldi Ricotti GC. DNA topoisomerase II beta: stability and distribution in different animal cells in comparison to DNA topoisomerase I and II alpha. *Exp Cell Res* 1993; 206:128–133.
50. Petrov P, Drake F, Loranger A, Huang W, Hancock R. Localization of DNA topoisomerase II in chinese hamster fibroblasts by confocal and electron microscopy. *Exp Cell Res* 1993; 204:73–81.
51. Tsai-Pflugfelder M, Liu LF, Liu AA, Tewey KM, Whang-Peng J, Knutsen T, Huebner K, Croce CM, Wang JC. Cloning and sequencing of cDNA encoding human DNA topoisomerase

II and localization of the gene to chromosome region 17q21-22. *Proc Natl. Acad Sci USA* 1988; 85:7177-7181.
52. Jenkins JR, Ayton P, Jones T, Davies SL, Simmons DL, Harris AL, Sheer D, Hickson ID. Isolation of cDNA clones encoding the beta isozyme of human DNA topoisomerase II and localisation of the gene to chromosome 3p24. *Nucleic Acids Res* 1992; 20:5587-5592.
53. Patel S, Fisher LM. Novel HeLa topoisomerase II is the II beta isoform: complete coding sequence and homology with other type II topoisomerases. *Br J Cancer* 1993; 67:456-463.
54. Tan KB, Dorman TE, Falls KM, Chung TDY, Mirabelli CK, Crooke ST, Mao J-I. Topoisomerase II-alpha and topoisomerase II-beta genes: characterization and mapping to human chromosomes 17 and 3, respectively. *Cancer Res* 1992; 52:231-234.
55. Austin CA, Marsh KL, Wasserman RA, Willmore E, Sayer PJ, Wang JC, Fisher LM. Expression, domain structure, and enzymatic properties of an active recombinant human DNA topoisomerase II beta. *J Biol Chem* 1995; 270:15,739-15,746.
56. Zechiedrich EL, Osheroff N. Eukaryotic topoisomerases recognize nucleic acid topology by preferentially interacting with DNA crossovers. *EMBO J* 1990; 9:4555-4562.
57. Hsieh C-H, Griffith JD. The terminus of SV40 DNA replication and transcription contains a sharp sequence-directed curve. *Cell* 1988; 52:535-544.
58. Howard MT, Lee MP, Hsieh T-S, Griffith JD. Drosophila topoisomerase II-DNA interactions are affected by DNA structure. *J Mol Biol* 1991; 217:53-62.
59. Sander M, Hsieh TS. Drosophilia topoisomerase II double-strand DNA cleavage: analysis of DNA sequence homology at the cleavage site. *Nucleic Acids Res* 1985; 13:1057-1072.
60. Spitzner JR, Muller MT. A consensus sequence for cleavage by vertebrate DNA topoisomerase I. *Nucleic Acids Res* 1988; 16:5533-5556.
61. Pommier Y, Capranico G, Orr A, Kohn KW. Distribution of topoisomerase II cleavage sites in SV40 DNA and the effects of drugs. *J Mol Biol* 1991; 222:909-924.
62. Pommier Y, Capranico G, Orr A, Kohn KW. Local base sequence preferences for DNA cleavage by mammalian topoisomerase II in the presence of amsacrine and teniposide. *Nucleic Acids Res* 1991; 19:5973-5980.
63. Pommier Y, Kerrigan D, Kohn D. Topological complexes between DNA and topoisomerase II and effects of polyamines. *Biochemistry* 1989; 28:995-1002.
64. Corbett AH, Zechiedrich EL, Osheroff N. A role for the passage helix in the DNA cleavage reaction of eukaryotic topoisomerase II. *J Biol Chem* 1992; 267:683-686.
65. Roca J, Ishida R, Berger JM, Andoh T, Wang JC. Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp. *Proc Natl Acad Sci USA* 1994; 91:1781-1785.
66. Corbett AH, Osheroff N. When good enzymes go bad: conversion of topoisomerase II to a cellular toxin by antineoplastic drugs. *Chem Res Toxicol* 1993; 6:585-597.
67. Froelich-Ammon SJ, Osheroff N. Topoisomerase poisons: harnessing the dark side of enzyme mechanism. *J Biol Chem* 1995; 270:21,429-21,432.
68. Kohn KW. Principles and practice of DNA filter elution. *Pharmacol Ther* 1991; 49:55-77.
69. Bertrand R, Pommier Y. Assessment of DNA damage in mammalian cells by DNA filtration methods. In: Studzinski G, Ed. *Cell Growth and Apoptosis: A Practical Approach.* Oxford: IRL, Oxford University Press. 1995:96-117.
70. Trask DK, DiDonato JA, Muller MT. Rapid detection and isolation of covalent DNA/protein complexes: application to topoisomerase I and II. *EMBO J* 1984; 3:671-676.
71. Rowe TC, Chen GL, Hsiang YH, Liu LF. DNA damage by antitumor acridines mediated by mammalian DNA topoisomerase II. *Cancer Res* 1986; 46:2021-2026.
72. Liu LF. DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* 1989; 58:351-375.
73. Robinson MJ, Martin BA, Gootz TD, McGuirk PR, Moynihan M, Sutcliffe JA, Osheroff N. Effects of quinolone derivatives on eukaryotic topoisomerase II. A novel mechanism for enhancement of enzyme mediated DNA cleavage. *J Biol Chem* 1991; 266:14,585-14,592.
74. Robinson MJ, Corbett AH, Osheroff N. Effects of topoisomerase II-targeted drugs on enzyme-mediated DNA cleavage and ATP hydrolysis: evidence for distinct drug interaction domains on topoisomerase II. *Biochemistry* 1993; 32:3638-3643.
75. Robinson MJ, Osheroff N. Effects of antineoplastic drugs on the post-strand passage DNA cleavage/religation equilibrium of topoisomerase II. *Biochemistry* 1991; 30:1807-1813.

76. Zwelling LA, Hinds M, Chan D, Mayes J, Sie KL, Parder E, Silberman L, Radcliffe A, Beran M, Blick M. Characterization of an amsacrine-resistant line of human leukemia cells. *J Biol Chem* 1989; 264:16,411-16,420.
77. Mayes J, Hinds M, Soares L, Altschuler E, Kim P, Zwelling LA. Further characterization of an amsacrine-resistant line of HL-60 human leukemia cells and its topoisomerase II. *Biochem. Pharmacol* 1993; 46:599-607.
78. Huff AC, Ward RE, Kreuzer KN. Mutational alterations of the breakage/resealing subunits of bacteriophage T4 DNA topoisomerase confers resistance to antitumor agent m-AMSA. *Mol Gen Genet* 1990; 221:27-32.
79. Huff AC, Kreuzer KN. Evidence for a common mechanism of action of antitumor and antibacterial agents that inhibit type II DNA topoisomerases. *J Biol Chem* 1990; 265:20,496-20,505.
80. Crenshaw DG, Hsieh T-S. Function of the hydrophilic carboxyl terminus of type II DNA topoisomerase from *Drosophila melanogaster*. *J Biol Chem* 1993; 28:21,328-21,334.
81. Harker WG, Slade DL, Parr RL, Holguin MH. Selective use of an alternative stop codon and polyadenylation signal within intron sequences leads to a truncated topoisomerase IIα messenger RNA and protein in human HL-60 leukemai cells selected for resistance to mitoxantrone. *Cancer Res* 1995; 55:4962-4971.
82. Mirski SEL, Cole SPC. Cytoplasmic localization of a mutant Mr 160,000 topoisomerase II alpha is associated with the loss of putative bipartite nuclear localization signals in a drug-resistant human lung cancer cell line. *Cancer Res* 1995; 55:2129-2134.
83. Pommier Y, Capranico G, Kohn KW. Local DNA sequence requirements for topoisomerase II-induced DNA cleavage produced by amsacrine and teniposide. *Proc Am Assoc Cancer Res* 1991; 32:335.
84. Pommier Y, Kohn KW, Capranico G, Jaxel C. Base sequence selectivity of topoisomerase inhibitors suggests a common model for drug action. In: Andoh T, Ikeda H, Oguro M, eds. *Molecular Biology of DNA Topoisomerase and Its Application to Chemotherapy.* Boca Raton, FL: CRC. 1993:215-227.
85. Capranico G, Kohn KW, Pommier Y. Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin. *Nucleic Acids Res* 1990; 18: 6611-6619.
86. Capranico G, Tinelli S, Zunino F, Kohn KW, Pommier Y. Effects of base mutations on topoisomerase II DNA cleavage stimulated by mAMSA in short DNA oligomers. *Biochemistry* 1993; 32:145-152.
87. Capranico G, De Isabella T, Tinelli S, Bigioni S, Zunino F. Similar sequence specificity of mitoxantrone and VM-26 stimulation of in vitro DNA cleavage by mammalian DNA topoisomerase II. *Biochemistry* 1993; 32:3032-3048.
88. Capranico G, Palumbo M, Tinelli S, Zunino F. Unique sequence specificity by topoisomerase II DNA cleavage stimulation and DNA binding mode of streptonigrin. *J Biol Chem* 1994; 40:25,004-25,009.
89. Leteurtre F, Kohlhagen G, Pommier Y. Streptonigrin-induced topoisomerase II sites exhibit base preference in the middle of the enzyme stagger. *Biochem Biophys Res Commun* 1994; 203:1259-1267.
90. Fosse P, Rene B, Le Bret M, Paoletti C, Saucier J-M. Sequence requirements for mammalian topoisomerase II mediated DNA cleavage stimulated by an ellipticine derivative. *Nucleic Acids Res* 1991; 19:2861-2868.
91. Freudenreich CH, Kreuzer KN. Mutational analysis of a type II topoisomerase cleavage site: distinct requirements for enzyme and inhibitors. *EMBO J* 1993; 12:2085-2097.
92. Freudenreich CH, Kreuzer KN. Localization of an aminoacridine antitumor agent in a type II topoisomerase-DNA complex. *Proc Natl Acad Sci USA* 1994; 91:11,007-11,011.
93. Jaxel C, Capranico G, Kerrigan D, Kohn KW, Pommier Y. Effect of local DNA sequence on topoisomerase I cleavage in the presence or absence of camptothecin. *J Biol Chem* 1991; 266: 20,418-20,423.
94. Tanizawa A, Kohn KW, Pommier Y. Induction of cleavage in topoisomerase I cDNA by topoisomerase I enzymes from calf thymus and wheat germ in the presence and absence of camptothecin. *Nucleic Acids Res* 1993; 21:5157-5166.
95. Pommier Y, Kohlhagen G, Kohn F, Leteurtre F, Wani MC, Wall ME. Interaction of an

alkylating camptothecin derivative with a DNA base at topoisomerase I-DNA cleavage sites. *Proc Natl Acad Sci USA* 1995; 92:8861–8865.
96. Beck WT, Danks MK. Mechanisms of resistance to drugs that inhibit DNA topoisomerases. *Semin Cancer Biol* 1991; 2:235–244.
97. Granzen B, Graves DE, Baguley BC, Danks MK, Beck WT. Structure-activity studies of amsacrine analogs in drug resistant human leukemia cell lines expressing either altered DNA topoisomerase II or P-glycoprotein. *Oncol Res* 1993; 4:489–496.
98. Marquardt D, McCrone S, Center MS. Mechanisms of multidrug resistance in HL60 cells: detection of resistance-associated proteins with antibodies against synthetic peptides that correspond to the deduced sequence of P-glycoprotein. *Cancer Res* 1990; 50:1426–1430.
99. Barrand MA, Heppell-Parton AC, Wright KA, Rabbitts PH, Twentyman PR. A 190-kilodalton protein overexpressed in non-P-glycoprotein-containing multidrug-resistant cells and its relationship to the MRP gene. *J Natl Cancer Inst* 1994; 86:110–117.
100. Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line (*see* comments). *Science* (*Washington, DC*) 1992; 258:1650–1654.
101. Schneider E, Horton JK, Yang CH, Nakagawa M, Cowan KH. Multidrug resistance-associated protein gene overexpression and reduced drug sensitivity of topoisomerase II in a human breast carcinoma MCF7 cell line selected for etoposide resistance. *Cancer Res* 1994; 54:152–158.
102. Binaschi M, Supino R, Gambetta RA, Giaccone G, Prosperi E, Capranico G, Cataldo I, Zunino F. MRP gene overexpression in a human doxorubicin-resistant SCLC cell line: alterations in cellular pharmacokinetics and in pattern of cross-resistance. *Int J Cancer* 1995; 62:84–89.
103. Cragg G, Suffness M. Metabolism of plant-derived anticancer agents. *Pharmacol Ther* 1988; 37:425–461.
104. Bendixen C, Thomsen B, Alsner J, Westergaard O. Camptothecin-stabilized topoisomerase I-DNA adducts cause premature termination of transcription. *Biochemistry* 1990; 29:5613–5619.
105. Been MD, Burgess RR, Champoux JJ. Nucleotide sequence preference at rat liver and wheat germ type 1 DNA topoisomerase breakage sites in duplex SV40 DNA. *Nucleic Acids Res* 1984; 12:3097–3114.
106. Loike JD, Brewer CF, Sternlicht H, Gensler WJ, Horwitz SB. Structure–activity study of the inhibition of microtubule assembly in vitro by podophyllotoxin and its congeners. *Cancer Res* 1978; 38:2688–2693.
107. Long BH, Musial ST, Brattain MG. Comparison of cytotoxicity and DNA breakage activity of congeners of podophyllotoxin including VP16-213 and VM26: a quantitative structure-activity relationship. *Biochemistry* 1984; 23:1183–1188.
108. Sinha BK, Polita PM, Eliot HM, Kerrigan D, Pommier Y. Structure–activity relations, cytotoxicity and topoisomerase II dependent DNA cleavage induced by pendulum ring analogues of etoposide. *Eur J Cancer* 1990; 26:590–593.
109. Chen GL, Yang L, Rowe TC, Halligan BD, Tewey KM, Liu LF. Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J Biol Chem* 1984; 259:13,560–13,566.
110. Leteurtre F, Madalengoitia J, Orr A, Guzi TJ, Lehnert E, Macdonald T, Pommier Y. Rational design and molecular effects of a new topoisomerase II inhibitor, azatoxin. *Cancer Res* 1992; 52:4478–4483.
111. Pommier Y, Orr A, Kohn KW, Riou JF. Differential effects of amsacrine and epipodophyllotoxins on topoisomerase II cleavage in the human c-*myc* proto-oncogene. *Cancer Res* 1992; 52: 3125–3130.
112. Kerrigan D, Pommier Y, Kohn KW. Protein-linked DNA strand breaks produced by etoposide and teniposide in mouse L1210 and human VA-13 and HT-29 cell lines: relationship to cytotoxicity. *NCI Monographs* 1987; 4:117–121.
113. Long BH, Musial ST, Brattain MG. Single- and double-strand DNA breakage and repair in human lung adenocarcinoma cells exposed to etoposide and teniposide. *Cancer Res* 1985; 45: 3106–3112.
114. Capranico G, Zunino F, Kohn KW, Pommier Y. Sequence-selective topoisomerase II inhibition by anthracycline derivatives in SV40 DNA: relationship with DNA affinity and cytotoxicity. *Biochemistry* 1990; 29:562–569.

115. Bachur NR, Johnson R, Yu F, Hickey R, Applegreen N, Malkas L. Antihelicase activity of DNA-binding anticancer agents: relationship to guanosine-cytidine intercalator binding. *Mol Pharmacol* 1993; 44:1064–1069.
116. Zunino F, Capranico G. DNA topoisomerase II as the primary target of anti-tumor anthracyclines. *Anticancer Drug Design* 1990; 5:307–317.
117. Pommier Y. DNA topoisomerases and their inhibition by anthracyclines. In: Priebe W, eds. *Antracycline Antibiotics.* Washington, DC: American Chemical Society. 1995:183–203.
118. Solary E, Ling Y-H, Perez-Soler R, Priebe W, Pommier Y. Hydroxyrubicin, a deaminated derivative of doxorubicin, inhibits mammalian DNA topoisomerase II and partially circumvents multidrug resistance. *Int J Cancer* 1994; 57:1–10.
119. Capranico G, Butelli E, Zunino F. Change of the sequence specificity of daunorubicin-stimulated topoisomerase II DNA cleavage by epimerization of the amino group of the sugar moiety. *Cancer Res* 1995; 55:312–317.
120. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* 1984; 226:466–468.
121. Zwelling LA, Kerrigan D, Michaels S. Cytotoxicity and DNA strand breaks by 5-iminodaunorubicin in mouse leukemia L1210 cells: comparison with adriamycin and 4′-(9-acridinylamino) methanesulfon-*m*-anisidide. *Cancer Res* 1982; 42:2687–2691.
122. Zwelling LA, Michaels S, Erickson LC, Ungerleider RS, Nichols M, Kohn KW. Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4′-(9-acridinylamino) methanesulfon-m-anisidide and adriamycin. *Biochemistry* 1981; 20:6553–6563.
123. Capolongo L, Belvedere G, D'Incalci M. DNA damage and cytotoxicity of mitoxantrone and doxorubicin in doxorubicin-sensitive and -resistant human colon carcinoma cells. *Cancer Chemother Pharmacol* 1990; 25:430–434.
124. Neidle S, Jenkins TC. Molecular modeling to study DNA intercalation by antitumor drugs. *Methods Enzymol* 1991; 203:433–458.
125. Smith PJ, Morgan SA, Fox ME, Watson JV. Mitoxantrone-DNA binding and the induction of topoisomerase II associated DNA damage in multi-drug resistant small cell lung cancer cells. *Biochem Pharmacol* 1990; 40:2069–2078.
126. Leteurtre F, Kohlhagen G, Paull KD, Pommier Y. Topoisomerase II inhibition by anthrapyrazoles, DuP 937 & DuP 941 (Losoxanthrone) and cytotoxicity in the NCI cell screen. *J Natl Cancer Inst* 1994; 86:1239–1244.
127. De Isabella P, Capranico G, Palumbo M, Sissi C, Krapcho AP, Zunino F. Sequence selectivity of topoisomerase II DNA cleavage stimulated by mitoxantrone derivatives: relationship to drug DNA binding and cellular effects. *Mol Pharmacol* 1993; 43:715–721.
128. Capranico G, Palumbo M, Tinelli S, Mabilia M, Pozzan A, Zunino F. Conformational drug determinants of the sequence specificity of drug-stimulated topoisomerase II DNA cleavage. *J Mol Biol* 1994; 235:1218–1230.
129. Harker WG, Slade DL, Drake FH, Parr RL. Mitoxantrone resistance in HL-60 leukemia cells: reduced nuclear topoisomerase II catalytic actvity and drug-induced DNA cleavage in association with reduced expression of the topoisomerase IIb isoform. *Biochemistry* 1991; 30: 9953–9961.
130. Kamath N, Grabowski D, Ford J, Kerrigan D, Pommier Y, Ganapathi R. Overexpression of P-glycoprotein and alterations in topoisomerase II in P388 mouse leukemia cells selected in vivo for resistance to mitoxantrone. *Biochem Pharmacol* 1992; 44:937–945.
131. Yang C-HJ, Horton JK, Cowan KH, Schneider E. Cross-resistance to camptothecin analogues in mitoxantrone-resistant human breast carcinoma cell line is not due to DNA topoisomerase I alterations. *Cancer Res* 1995; 55:4004–4009.
132. Harker WG, Slade KL, Parr RL, Feldhoff PW, Sullivan DM, Holguin MH. Alterations in the topoisomerase IIα gene, messenger RNA, and subcellular protein distribution as well as reduced expression of the DNA topoisomerase IIβ enzyme in a mitoxantrone-resistant HL-60 human leukemia cell line. *Cancer Res* 1995; 55:1707–1716.
133. Cain BF, Atwell GJ. The experimental antitumor properties of three congeners of the acridylmethanesulphonanisilide(AMSA)series. *Eur J Cancer* 1974; 10:539–549.
134. Hayat M, Ostronoff M, Gilles G, Zambon E, Baume D, Moran A. Carde P, Droz J, Pico J. Salvage therapy with methyl-gag,high-dose ara-C,m-amsa, and ifosfamide(MAMI) for recur-

rent or refractory lymphoma. *Cancer Invest* 1990; 8:1-5.
135. Louie AC, Issel BF. Amsacrine (AMSA)—a clinical review. *J Clin Oncol* 1985; 3:562-592.
136. Covey JM, Kohn KW, Kerrigan D, Tilchen EJ, Pommier Y. Topoisomerase II-mediated DNA damage produced by 4′-(9-acridinylamino)methanesulfon-m-anisidide and related acridines in L1210 cells and isolated nuclei: relation to cytotoxicity. *Cancer Res* 1988; 48:860-865.
137. Zwelling LA, Mitchell MJ, Satitpunwaycha P, Mayes J, Altschuler E, Hinds M, Baguley BC. Relative activity of structural analogues of amsacrine against human leukemia cell lines containing amsacrine-sensitive or -resistant forms of topoisomerase II: use of computer simulation in new drug development. *Cancer Res* 1992; 52:209-217.
138. Harvey VJ, Hardy JR, Smith S, Grove W, Baguley BC. Phase III study of the amsacrine analogue CI-921(NSC343499) in non-small cell lung cancer. *Eur J Cancer* 1991; 27:1617-1620.
139. Sklarin NT, Wiernik PH, Grove WR, Benson L. A phase II trial of CI-921 in advanced malignancies. *Invest New Drugs* 1992; 10:309-312.
140. Pommier Y. Covey JM, Kerrigan D, Markovits J, Pham R. DNA unwinding and inhibition of mouse leukemia L1210 DNA topoisomerase I by intercalators. *Nucleic Acids Res* 1987; 15: 6713-6731.
141. Pommier Y, Minford JK, Schwartz RE, Zwelling LA, Kohn KW. Effects of the DNA intercalators 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and 2-methyl-9-hydroxyellipticinium on topoisomerase II mediated DNA strand cleavage and strand passage. *Biochemistry* 1985; 24:6410-6416.
142. Nelson EM, Tewey KM, Liu LF. Mechanism of antitumor drug action: poisoning of mammalian DNA topoisomerase II on DNA by 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide. *Proc Natl Acad Sci USA* 1984; 81:1361-1365.
143. Minford J, Pommier Y, Filipski J, Kohn KW, Kerrigan D, Mattern M, Michaels S, Schwartz R, Zwelling LA. Isolation of intercalator-dependent protein-linked DNA strand cleavage activity for cell nuclei and identification as topoisomerase II. *Biochemistry* 1986; 25:9-16.
144. Pommier Y, Covey J, Kerrigan D, Mattes W, Markovits J, Kohn KW. Role of DNA intercalation in the inhibition of purified mouse leukemia (L1210) DNA topoisomerase II by 9-aminoacridines. *Biochem Pharmacol* 1987; 36:3477-3486.
145. Darkin S, Ralph RK. Transport of AMSA drugs into cells. *FEBS Lett* 1985; 190:349-353.
146. Zwelling LA, Kerrigan D, Michaels S, Kohn KW. Cooperative sequestration of m-AMSA in L1210 cells. *Biochem Pharmacol* 1982; 31:3269-3277.
147. Paoletti C, Le Pecq JB, Dat Xuong N, Juret P, Garnier H, Amiel JL, Rouessé J. Antitumor activity, pharmacology, and toxicity of ellipticines, ellipticinium and 9-hydroxy-derivatives: preliminary clinical trials of 2-methyl-9-hydroxy-ellipticinium(NSC-264-137). *Recent Res Cancer Res* 1980; 74:107-123.
148. Tewey KM, Chen GL, Nelson EM, Liu LF. Intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J Biol Chem* 1984; 259:9182-9187.
149. Pommier Y, Schwartz RE, Zwelling LA, Kohn KW. Effects of DNA intercalating agents on topoisomerase II induced DNA strand cleavage in isolated mammalian cell nuclei. *Biochemistry* 1985; 24:6406-6410.
150. Zwelling LA, Michaels S, Kerrigan D, Pommier Y, Kohn KW. Protein-associated deoxyribonucleic acid strand breaks produced in mouse leukemia L1210 cells by ellipticine and 2-methyl-9-hydroxyellipticinium. *Biochem Pharmacol* 1982; 31:3261-3267.
151. Pommier Y, Schwartz RE, Kohn KW, Zwelling LA. Formation and rejoining of deoxyribonucleic acid double-strand breaks induced in isolated cell nuclei by antineoplastic intercalating agents. *Biochemistry* 1984; 23:3194-3201.
152. Pommier Y, Kerrigan D, Schwartz RE, Swack JA, McCurdy A. Altered DNA topoisomerase II activity in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res* 1986; 46:3075-3081.
153. Pommier Y, Schwartz RE, Zwelling LA, Kerrigan D, Mattern MR, Charcosset JY, Jacquemin-Sablon A, Kohn KW. Reduced formation of protein-associated DNA strand breaks in Chinese hamster cells resistant to topoisomerase II inhibitors. *Cancer Res* 1986; 46:611-616.
154. Andersson BS, Beran M, Bakic M, Silberman LE, Newman RA, Zwelling LA. *In vitro* toxicity and DNA cleaving capacity of benzisoquinolinedione (nafidimide; NSC 308847) in human leukemia. *Cancer Res* 1987; 47:1040-1044.

155. Hsiang Y-H, Jiang JB, Liu LF. Topoisomerase II-mediated DNA cleavage by amonafide and structural analogs. *Mol Pharmacol* 1989; 36:371-376.
156. Earnshaw WC, Honda BM, Laskey RA, Thomas JO. Assembly of nucleosomes: the reaction involving *X. laevis* nucleoplasmin. *Cell* 1980; 21:373-383.
157. De Isabella P, Zunino F, Capranico G. Base sequence determinants of amonafide stimulation of topoisomerase II DNA cleavage. *Nucleic Acids Res* 1995; 23:223-229.
158. Markovits J, Linassier C, Fosse P, Couprie J, Pierre J, Jacquemin-Sablon A, Saucier J-M, Le Pecq JB, Larsen AK. Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. *Cancer Res* 1989; 49:5111-5117.
159. Austin CA, Patel S, Ono K, Nakane H, Fisher LM. Site-specific DNA cleavage by mammalian DNA topoisomerase II induced by novel flavone and catechin derivatives. *Biochem J* 1992; 282:883-889.
160. Yamashita Y, Kawada S-Z, Nakano H. Induction of mammalian topoisomerase II dependent DNA cleavage by nonintercalative flavonoids, genistein and orobol. *Biochem Pharmacol* 1990; 39:737-744.
161. Sorensen BS, Jensen PS, Andersen AH, Christiansen K, Alsner J, Thomsen B, Westergaard O. Stimulation of topoisomerase II mediated DNA cleavage at specific sequence elements by the 2-nitroimidazole Ro 15-0216. *Biochemistry* 1990; 29:9507-9515.
162. Juang J-K, Huang HW, Chen C-M, Liu HJ. A new compound, withangulatin A, promotes type II DNA topoisomerase-mediated DNA damage. *Biochem Biophys Res Commun* 1989; 159:1128-1134.
163. Yamashita Y, Saitoh Y, Ando K, Takahashi K, Ohno H, Nakano H. Saintopin, a new antitumor antibiotic with topoisomerase II dependent DNA cleavage activity, from Paecilomyces. *J Antibiotics* 1990; 43:1344-1346.
164. Kawada S-Z, Yamashita Y, Fujii N, Nakano H. Induction of heat-stable topoisomerase II-DNA cleavable complex by nonintercalative terpenoides, terpentecin and clerocidin. *Cancer Res* 1991; 51:2922-2925.
165. Kawada S-Z, Yamashita Y, Uosaki Y, Gomi K, Iwasaki T, Takiguchi T, Nakano H. UCT48, a new antitumor antibiotic with topoisomerase II mediated DNA cleavage activity from *streptomyces sp. J Antibiot* 1992; 45:1182-1184.
166. Leteurtre F, Sackett DL, Madalengoitia J, Kohlhagen G, Macdonald T, Hamel E, Paull KD, Pommier Y. Azatoxin derivatives with potent and selective action on topoisomerase II. *Biochem Pharmacol* 1995; 49:1283-1290.
167. Solary E, Leteurtre F, Paull KD, Scudiero D, Hamel E, Pommier Y. Dual inhibition of topoisomerase II and tubulin polymerization by azatoxin, a novel cytotoxic agent. *Biochem Pharmacol* 1993; 45:2449-2456.
168. Yamasahita Y, Ashizawa T, Marimoto M, Hosomi J, Nakano H. Antitumor quinolones with mammalian topoisomerase II mediated DNA cleavage activity. *Cancer Res* 1992; 52:2818-2822.
169. Elsea SH, Osheroff N, Nitiss JL. Cytotoxicity of Quinolones toward eukaryotic cells. *J Biol Chem* 1992; 267:13,150-13,153.
170. Froelich-Ammon SJ, McGuirk PR, Gootz TD, Jefson MR, Osheroff N. Novel 1-8-bridged chiral quinolones with activity against topoisomerase II: stereospecificity of the eukaryotic enzyme. *Antimicrob Agent Chemother* 1993; 37:646-651.
171. Yoshinari T, Mano E, Arakawa H, Kurama M, Iguchi T, Nakagawa S, Tanaka N, Okura A. Stereo(C7)-dependent topoisomerase II inhibition and tumor growth suppression by a new quinolone, BO-2367. *Jpn J Cancer Res* 1993; 84:800-806.
172. Kong XB, Rubin L, Chen LI, Ciszewska G, Watanabe KA, Tong WP, Sirotnak FM, Chou TC. Topoisomerase II-mediated DNA cleavage activity and irreversibility of cleavable complex formation induced by DNA intercalator with alkylating capability. *Mol Pharmacol* 1992; 41:237-244.
173. Ono K, Ikegami Y, Nishizawa M, Andoh T. Menogaril, an anthracycline derivative, inhibits DNA topoisomerase II by stabilizing cleavable complexes. *Jpn J Cancer Res* 1992; 83:1018-1023.
174. Fujii N, Yamashita Y, Arima Y, Nagashima M, Nakano H. Induction of topoisomerase II-mediated DNA cleavage by the plant naphthoquinones plumbagin and shikonin. *Antimicrob Agents Chemother* 1992; 36:2589-2594.
175. Fujii N, Yamashita Y, Chiba S, Uosaki Y, Saitoh Y, Tuji Y, Nakano H. UCE6, a new antitumor antibiotic with topoisomerase I mediated DNA cleavage activity, from actinomycetes

(letter). *J Antibiot* 1993; 46:1173,1174.
176. Gupta M, Abdel-Megeed M, Hoki Y, Kohlhagen G. Paull K, Pommier Y. Eukaryotic DNA topoisomerase mediated DNA cleavage induced by a new inhibitor: NSC 665517. *Mol Pharmacol* 1995; 48:658.
177. Macdonald TL, Lehnert EK, Loper JT, Chow K-C, Ross WE. On the mechanism of interaction of DNA topoisomerase II with chemotherapeutic agents. In: Potmesil M, Kohn KW, eds. *DNA Topoisomerase in Cancer.* New York: Oxford University Press. 1991:199–214.
178. Eymin B, Solary E, Chevillard S, Dubrez L, Goldwasser F, Duchamp O, Genne P, Leteurtre F, Pommier Y. Cellular pharmacology of azatoxins (topoisomerase II and tubulin inhibitors) in P-glycoprotein-positive and negative cell lines. *Int J. Cancer* 1995; 63:268–275.
179. Rowe T, Kupfer G, Ross W. Inhibition of epipodophyllotoxin cytotoxicity by interference with topoisomerase-mediated DNA cleavage. *Biochem Pharmacol* 1985; 34:2483–2487.
180. Markovits J, Pommier Y, Mattern MR, Esnault C, Roques BP, Le Pecq JB, Kohn KW. Effects of the bifunctional antitumor intercalator ditercalinium on DNA in mouse leukemia L1210 cells and DNA topoisomerase II. *Cancer Res* 1986; 46:5821–5826.
181. Fesen M, Pommier Y. Mammalian topoisomerase II activity is modulated by the DNA minor groove binder distamyhcin in simian virus 40 DNA. *J Biol Chem* 1989; 19:11,354–11,359.
182. Drake FH, Hofmann GA, Mong SM, Bartus JO, Hertzberg RP, Johnson RK, Mattern MR, Mirabelli CK. In vitro and intracellular inhibition of topoisomerase II by the antitumor agent merbarone. *Cancer Res* 1989; 49:2578–2583.
183. Ishida R, Miki T, Narita T, Yui R, Sato M, Utsumi KR, Tanabe K, Andoh T. Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: modes of cell growth inhibition distinct from that of cleavable complex-forming inhibitors. *Cancer Res* 1991; 51:4909–4916.
184. Tanabe K, Ikegami R, Andoh T. Inhibition of topoisomerase II by antitumor agent bis(2,6-dioxopiperazine). *Cancer Res* 1991; 51:4903–4908.
185. Bojanowski K, Lelievre S, Markovits J, Couprie J, Jacquemin-Sablon A, Larsen AK. Suramin is an inhibitor of DNA topoisomerase II in vitro and in chinese hamster fibrosarcoma cells. *Proc Natl Acad Sci USA* 1992; 89:3025–3029.
186. Gedik CM, Collins AR. Comparison of effects of fostriecin, novobiocin, and camptothecin, inhibitors of DNA topoisomerases, on DNA replication and repair in human cells. *Nucleic Acids Res* 1990; 18:1007–1013.
187. Frosina G, Rossi O. Effect of topoisomerase poisoning by antitumor drugs VM 26, fostriecin and camptothecin on DNA repair replication by mammalian cell extracts. *Carcinogenesis* 1992; 13:1371–1377.
188. Boritzki TJ, Wolfard TS, Besserer JA, Jackson RC, Fry DW. Inhibition of type II topoisomerase by fostriecin. *Biochem Pharmacol* 1988; 37:4063–4068.
189. Jensen PB, Jensen PS, Demant EJF, Friche E, Sorensen BS, Sehested M, Wassermann K, Vindelov L, Westergaard O, Hensen HH. Antagonistic effect of aclarubicin on daunorubicin-induced cytotoxicity in human small cell lung cancer cells: relationship to DNA integrity and topoisomerase II. *Cancer Res* 1991; 51:5093–5099.
190. Herman EH, Witiak K, Hellman K, Waravdekar VS. Biological properties of ICRF-159 and related bis(dioxopiperazine) compounds. In: Garattini A, Goldin F, Hawking F, Kopin IJ, eds. *Advances in Pharmacology and Chemotherapy.* New York: Academic. 1982:249–291.
191. Ishida R, Sato M, Narita T, Utsumi KR, Nishimoto T, Morita T, Nagata H, Andoh T. Inhibition of DNA topoisomerase II by ICRF-193 induces polyploidization by uncoupling chromosome dynamics from other cell cycle events. *J Biol Chem* 1994; 26:1341–1351.
192. Takasuga Y, Andoh T, Yamashita J, Yagura T. ICRF-193, an inhibitor of topoisomerase II, demonstrates that DNA replication in sperm nuclei reconstituted in Xenopus egg extracts does not require chromatin decondensation. *Exp Cell Res* 1995; 217:378–384.
193. Narita T, Yaguchi S, Komatsu T, Takase M, Hoshino A, Inaba M, Tsukagoshi S. Antitumor activity of MST-16, a novel derivative of bis(2,6-dioxopiperazine), in murine models. *Cancer Chemother Pharmacol* 1990; 26:193–197.
194. Narita T, Koide Y, Yaguchi S, Kimura S, Izumisawa Y, Takase M, Inaba M, Tsukagoshi S. Antitumor activities and schedule dependence of orally administered MST-16, a novel derivative of bis(2,6-dioxopiperazine). *Cancer Chemother Pharmacol* 1991; 28:235–240.

195. Ohno R, Yamada K, Hirano M, Shirakawa S, Tanaka M, Oguri T, Kodera Y, Mitomo Y, Ikeda Y, Yokomaku S, et al. Phase II study: treatment of non-Hodgkin's lymphoma with an oral antitumor derivative of bis(2,6-dioxopiperazine). *J Natl Cancer Inst* 1992; 84:435–438.
196. Ichihashi T, Kiyoi H, Fukutani H, Kubo K, Yamauchi T, Naoe T, Yamada K, Ohno R. Effective treatment of adult T cell leukemia/lymphoma with a novel oral antitumor agent, MST-16. *Oncology* 1992; 49:333–335.
197. Ohno R, Masaoka T, Shirakawa S, Sakamoto S, Hirano M, Hanada S, Yasunage K, Yokomaku S, Mitomo Y, Nagai K, et al. Treatment of adult T-cell leukemia/lymphoma with MST-16, a new oral antitumor drug and a derivative of bis(2,6-dioxopiperazine). The MST-16 Study Group. *Cancer* 1993; 71:2217–2221.
198. Seifert CF, Nesser ME, Thompson DF. Dexrazoxane in the prevention of doxorubicin-induced cardiotoxicity [published erratum appears in Ann Pharmacother 1994 Dec;28(12):1413]. *Ann Pharmacother* 1994; 28:1063–1072.
199. Buss JL, Hasinoff BB. Ferrous ion strongly promotes the ring opening of the hydrolysis intermediates of the antioxidant cardioprotective agent dexrazoxane (ICRF-187). *Arch Biochem Biophys* 1995; 317:121–127.
200. Buss JL, Hasinoff BB. The one-ring open hydrolysis product intermediates of the cardioprotective agent ICRF-187 (dexrazoxane) displace iron from iron-anthracycline complexes. *Agents Actions* 1993; 40:86–95.
201. Cooney DA, Covey JM, Kang GJ, Dalal M, McMahon JB, Johns DG. Initial mechanistic studies with merbarone (NSC 336628). *Biochem Pharmacol* 1985; 34:3395–3398.
202. Chen M, Beck WT. Differences in inhibition of chromosome separation and G2 arrest by DNA topoisomerase II inhibitors merbarone and VM-26. *Cancer Res* 1995; 55:1509–1515.
203. Chen M, Beck WT. Teniposide-resistant CEM cells, which express mutant DNA topoisomerase IIα, when treated with the non-complex-stabilizing inhibitors of the enzyme, display no cross-resistance and reveal aberrant functions of the mutant enzyme. *Cancer Res* 1993; 53: 5946–5953.
204. Flanigan RC, Saiers JH, Wolf M, Kraut EH, Smith AY, Blumenstein B, Crawford ED. Phase II evaluation of merbarone in renal cell carcinoma. *Invest New Drugs* 1994; 12:147–149.
205. Poplin EA, Tangen CM, Harvey WH, Macdonald JS. Hepatoma/merbarone. A Southwest Oncology Group study. *Invest New Drugs* 1994; 12:337–340.
206. Jones DV, Jr., Ajani JA, Winn RJ, Daugherty KR, Levin B, Krakoff IH. A phase II study of merbarone in patients with adenocarcinoma of the pancreas. *Cancer Invest* 1993; 11:667–669.
207. Kraut EH, Bendetti J, Balcerzak SP, Doroshow JH. Phase II trial of merbarone in soft tissue sarcoma. A Southwest Oncology Group study. *Invest New Drugs* 1992; 10:347–249.
208. Ajani JA, Winn R, Baez L, Pollock T, Maher T, Hallinan-Fueger B, Newman J. Phase II study of merbarone (NSC 336628) in patients with advanced gastric carcinoma. *Cancer Invest* 1994; 12:488–490.
209. Chang AY, Kim K, Glick J, Anderson T, Karp D, Johnson D. Phase II study of taxol, merbarone, and piroxantrone in stage IV non-small-cell lung cancer: The Eastern Cooperative Oncology Group Results (*see* comments). *J Natl Cancer Inst* 1993; 85:388–394.
210. Jackson RC, Fry DW, Boritzki TJ, Roberts BJ, Hook KE, Leopold WR. The biochemical pharmacology of CI-920, a structurally novel antibiotic with antileukemic activity. *Adv Enzyme Regul* 1985; 23:193–215.
211. Scheithauer W, Von Hoff DD, Clark GM, Shillis JL, Elslager EF. In vitro activity of the novel antitumor antibiotic fostriecin (CI-920) in a human tumor cloning assay. *Eur J Cancer Clin Oncol* 1986; 22:921–926.
212. Baguley BC, Calveley SB, Crowe KK, Fray LM, O'Rourke SA, Smith GP. Comparison of the effects of flavone acetic acid, fostriecin, homoharringtonine and tumour necrosis factor alpha on colon 38 tumours in mice. *Eur J Cancer Clin Oncol* 1989; 25:263–269.
213. Roberge M, Tudan C, Hung SM, Harder KW, Jirik FR, Anderson H. Antitumor drug fostriecin inhibits the mitotic entry checkpoint and protein phosphatases 1 and 2A. *Cancer Res* 1994; 54:6115–6121.
214. Guo XW, Th'ng JP, Swank RA, Anderson HJ, Tudan C, Bradbury EM, Roberge M. Chromosome condensation induced by fostriecin does not require p34cdc2 kinase activity and histone H1 hyperphosphorylation, but is associated with enhanced histone H2A and H3 phosphorylation. *EMBO J* 1995; 14:976–985.

8 The Taxoids

Marie-Christine Bissery, PhD and François Lavelle, PhD

CONTENTS

1. INTRODUCTION

Paclitaxel and docetaxel belong to the taxoid family, a new class of antineoplastic drugs. The name taxoids refers to compounds, natural or modified, having a taxane skeleton.

Paclitaxel (Taxol®, NSC 125973) was extracted in the late 1960s from the bark of the Pacific Yew, *Taxus brevifolia*. Because of the scarcity of the drug and the difficulties of formulation, the development was initially slow. Once these problems were solved, development accelerated. Docetaxel (Taxotere®, PR 56976) was obtained by hemisynthesis, using the starting material, 10-deacetyl baccatin III extracted from the needles of the European Yew tree, *Taxus baccata* (Fig. 1). This drug was more readily available because of the renewability of the source, and somewhat more soluble, and thus its development was rapid. Paclitaxel consists of an eight-member taxane ring with a four-member oxetane ring and a side chain at the C-13 position (Fig. 1). Docetaxel differs from paclitaxel in the 10-position on the baccatin ring and in the 3′-position of the lateral chain (Fig. 1).

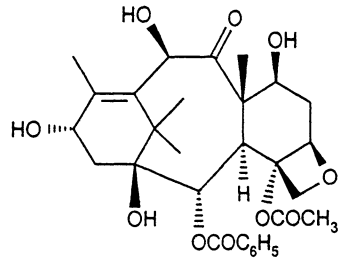
This chapter will summarize the key steps in the development process of these two new exciting antitumor agents.

2. DISCOVERY

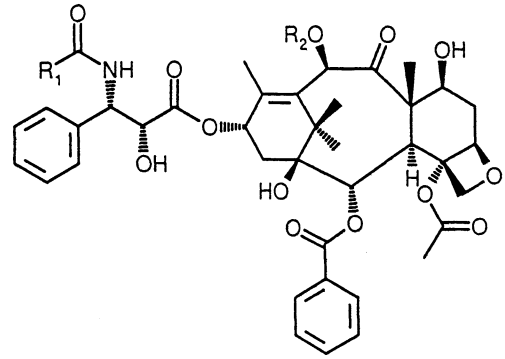
2.1. Paclitaxel

In 1960, a vast screening program for antitumor agents derived from plants was initiated by the Cancer Chemotherapy National Service Center under J. L. Hartwell (*1–3*). In 1962, a US Department of Agriculture botanist, A. S. Barclay, collected 650

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ



10-Deacetyl Baccatin III



$R_1 = -C_6H_5$	$R_2 = -COCH_3$	paclitaxel
$R_1 = -OC(CH_3)_3$	$R_2 = -H$	docetaxel

Fig. 1. Structures of paclitaxel (Taxol®), docetaxel (Taxotere®) and 10-deacetyl-baccatin III.

samples in the West Coast States of the US, including samples of *T. brevifolia*, the Pacific Yew tree. Initial screening of crude extracts showed cytotoxicity toward 9KB cells derived from a nasopharyngeal tumor. M. Wall at Research Triangle Institute was particularly interested in plants with 9KB activity because of his previous experience with *Camptotheca* extracts. This is why he received among other plants, *T. brevifolia*. The isolation procedure of the drug substance was laborious and involved numerous steps (ethanol extraction; partition of the ethanolic residue between water and chloroform; followed by Craig countercurrent distribution), each of them being monitored by an in vivo assay using the rat Walker 256 carcinosarcoma (*2–4*) or/and the P1534 leukemia (*1*). Approximately 0.5 g of paclitaxel could be isolated starting with 12 kg of dried stem and bark (yield 0.004%). The isolation of the pure compound was achieved in 1966 (*1*). In 1971 the structure of paclitaxel was published, and antitumor efficacy was reported in L1210, P388, and P1534 leukemias, and in Walker carcinosarcoma 256 (*5*). However, the activity levels seen against the L1210 and P388 leukemia models were very modest compared to that of other compounds. The only tumor system showing good efficacy, the P1534 leukemia, was not thought to be of predictive value (*1*). Finally, the compound was poorly soluble (i.e., the in vivo evalu-

ation was performed with drug suspension), and supplies were difficult to obtain. Because of this, paclitaxel was not selected for further preclinical development (*1*). Luckily in 1974–1975, extensive in vivo testing was conducted by the National Cancer Institute (NCI), and paclitaxel showed high activity against the murine B16 melanoma model, newly introduced to the NCI screen (*1*). This prompted the NCI to evaluate the compound further. Additional efficacy was noted in colon 26 carcinoma and in MX-1 human breast carcinoma implanted under the renal capsule in nude mice (*6*). However, no efficacy was observed at that time in most of the other models tested, colon 38 adenocarcinoma, Lewis lung carcinoma, CD8F1 mammary, and human xenografts implanted subcutaneously (*6*). Preliminary studies of its mechanism of action indicated that it was a spindle poison that inhibited the cell proliferation at the G2-M phase in the cell cycle and that it blocked mitosis (*7*). The turning point occurred with the demonstration that paclitaxel had a unique mechanism of action by Horwitz. It was established that paclitaxel stabilized microtubules and inhibited depolymerization back to tubulin. This differed from the mechanism of action of other spindle poisons, such as vinca-alkaloids, that bind to tubulin and inhibit its polymerization (*8*). On the basis of the in vivo B16 melanoma efficacy and the uniqueness of mechanism of action, the NCI initiated a very large effort to collect barks and wood to obtain enough material to initiate clinical trials (*1–3*).

Formulation was also an issue, and in 1980, it was reported that toxicology studies would proceed if a satisfactory formulation was developed (*6*). These toxicology and clinical formulation development studies were completed by 1983. Clinical Phase I trials started in 1983. Progress of these trials was hampered by hypersensitivity reactions, which led to the premature closure of some Phase I studies (*9,10*). Since these reactions were observed more commonly with infusions of shorter durations, a decision was made to pursue clinical trials using a 24-h continuous infusion, with premedication to lessen the reactions. The dose-limiting toxicity was neutropenia in seven out of the nine Phase I trial initiated (*1,9,10*).

The final major step in paclitaxel's development was the recognition of its activity against ovarian cancer with responses in approx 30% of patients, many of them having cisplatin or carboplatin refractory disease. These clinical results were reported in 1989 (*11*) (i.e., 6 yr after first clinical entry). Even as evidence of paclitaxel's activity increased, with the report of efficacy in breast and nonsmall-cell lung carcinoma (*12,13*), the clinical development was still prevented by the supply shortage. To address this issue, the NCI sought the assistance of the pharmaceutical industry. A cooperative research and development agreement (CRADA) was awarded to Bristol Myers Squibb in 1991 (*3,9*). In 1992, the company filed a New Drug Application. The Food and Drug Administration approved paclitaxel that same year for the treatment of patients whose ovarian carcinoma had progressed with other chemotherapy (*3,9*) and, in 1994, for metastatic breast cancer.

2.2. Docetaxel

The Institut de Chimie des Substances Naturelles (ICSN) of the Centre National de la Recherche Scientifique and Rhône-Poulenc were interested by the ongoing work on paclitaxel in the US and, in particular, of the newly described mechanism of action of paclitaxel (*8*). The ICSN had expertise in the chemistry and the biochemistry of antimitotic compounds, especially vinca-alkaloids, and it was using a test to measure the

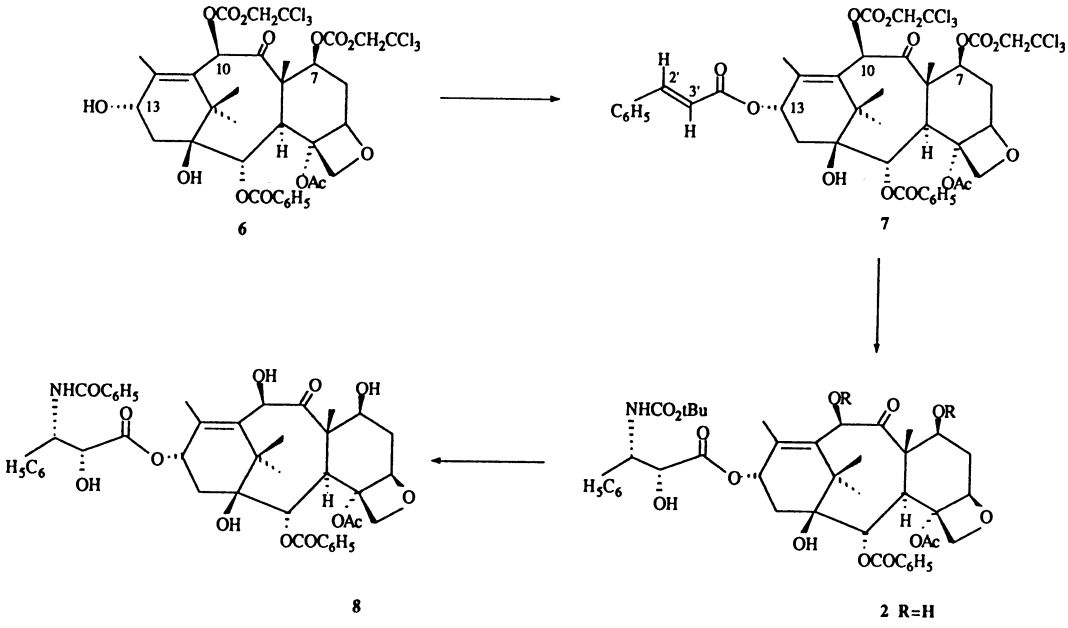


Fig. 2. First hemi-synthesis of docetaxel *2* and 10-deacetyl-paclitaxel *8*.

inhibition of polymerization or depolymerization of tubulin, based on the Shelanski's method (*14*). Finally, the ICSN had a large supply of *T. baccata*, the European species of yew tree that is widely dispersed in France and Europe. Therefore, in 1979, the ICSN decided to undertake research in this area. In 1980, Rhône-Poulenc decided to stop a 20-year period of research on anthracyclines and signed a research agreement concerning taxoids with the ICSN with three main objectives: to explore the chemistry of taxoids, bearing in mind the issue of supply, to build structure–activity relationships, and to select new and patentable antitumor compounds in these series.

From the beginning, P. Potier and colleagues were convinced that semisynthesis was the only realistic approach for preparing paclitaxel and analogs in sufficient quantities for pharmaceutical research and clinical trials. They decided to explore systematically and extensively the different chemical components present in the European yew tree *T. baccata*, in particular in the needles, a renewable source of biological material. The purification of the components present in the needles was monitored by measuring their interactions with tubulin purified from calf brain (*14*). This purification led to the isolation of an abundant precursor of paclitaxel, 10-deacetyl-baccatin III, with a yield of 1 g/kg of fresh needles (*15*) (Fig. 1). This yield was important especially in light of the 150 mg of paclitaxel that could be extracted from 1 kg of dried bark (*16*). It was considered an interesting precursor for hemisynthesis of paclitaxel and other taxoids (*17*). It was 50- to 100-fold less active than paclitaxel in inhibiting microtubules depolymerization (*18*). The closer precursor, baccatin III, was also detected but at much lower concentrations.

2.2.1. First Access to Paclitaxel: Discovery of Docetaxel (Fig. 2)

10-Deacetylbaccatin III, protected at both the C-7 and C-10 positions (compound 6), was converted into the cinnamoyl ester at the C-13 position (compound 7) with a 90% yield. The cinnamic double bond was then oxyminated (*19*), leading to docetaxel (compound 2) and to 10-deacetylpaclitaxel (compound 8) after cleavage of the Boc

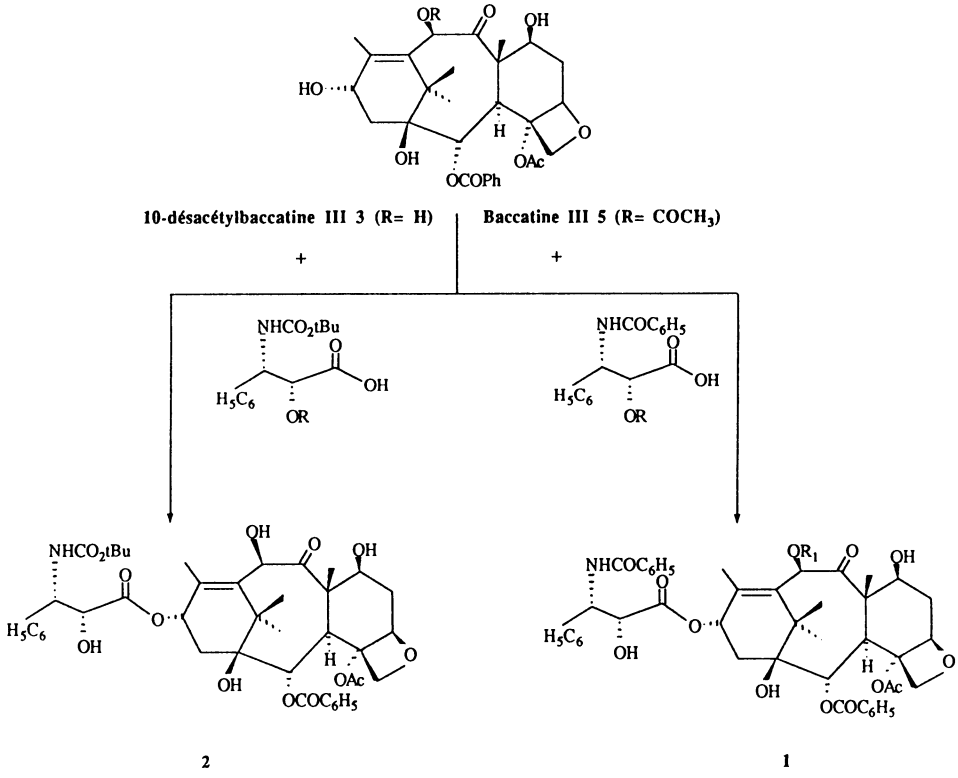


Fig. 3. Convergent synthesis of docetaxel *2* and paclitaxel *1*.

and reacylation (*20*). Paclitaxel was obtained using the same pathway, starting from baccatin III. Similar to all new derivatives of paclitaxel, docetaxel was tested for its interaction with tubulin/microtubules and, surprisingly, was found to be twice more potent than paclitaxel in inhibiting the cold-induced reaction of microtubule depolymerization. At the end of 1985, a small batch of docetaxel was available, and the first demonstration of its in vitro cytotoxicity and in vivo antitumor properties was obtained in the Oncology Department of Rhône-Poulenc, using P388 leukemia. Further in vivo evaluation revealed efficacy against L1210, Lewis lung carcinoma and B16 melanoma (*16,21*).

However, although very successful, this first semisynthetic approach was not applicable at an industrial scale owing to the use of toxic and very expensive reagents, such as osmium tetraoxide.

2.2.2. Convergent Synthesis of Taxoids (Fig. 3)

The convergent synthesis of paclitaxel and docetaxel was performed by direct esterification of 10-deacetyl-baccatin III or baccatin III, with the acids corresponding to the lateral chains present in paclitaxel and docetaxel, respectively. The first asymetric synthesis of the C-13 phenylisoserine chain of paclitaxel was done by Denis and collaborators (*22*). The lateral chain of docetaxel was prepared using benzaldehyde and terbutyl chloroacetate (*23*). Finally, esterification of the acids by baccatin III and 10-deacetyl-baccatin III yielded paclitaxel (*24*) and docetaxel (*23*), respectively. Since these first experiments, the yield of the esterification methods and of the synthesis of the lateral chain have been improved by different teams (*25–28*).

In 1989, the compound was obtained in sufficient amount to initiate extensive pharmacological and toxicological studies and a suitable formulation for iv evaluation was developed. Using the sc B16 melanoma murine model, it was found that iv docetaxel was more active than paclitaxel at an equitoxic dose (*29*).

The compound was also found to be highly active against a large number of murine tumor models, most importantly when treated at an advanced stage (i.e., measurable disease), and schedule studies revealed that the compound was schedule-independent (*29*). Finally, a pharmacokinetic/distribution study in tumor-bearing mice showed that at optimal dosage, the area under the plasma and tumor concentration vs time curves (AUC) were much higher than the AUC required to kill human cancer cell lines in vitro. Toxicology studies were performed according to the NCI guidelines.

Phase I clinical trials were initiated in 1990 in Europe and in the US (*30*). Five different schedules were investigated up front in record time. At the end of the Phase I trials, it was shown that neutropenia was the major dose-limiting toxicity, and responses were reported in different tumor types. Based on considerations, such as dose intensity, toxicity profile, and preclinical data, suggesting absence of schedule dependency, the recommended dose and schedule for Phase II studies were 100 mg/m^2 adminisetred as a 1-h infusion every 3 wk, without prophylactic measures. Broad Phase II testing was initiated in 1992 throughout Europe, North America, and Japan, and a CRADA was signed by the NCI and Rhône-Poulenc Rorer. A broad spectrum of efficacy was reported, including breast, nonsmall-cell lung, and ovarian cancers. A New Drug Application was filed in 1994, and docetaxel has now been approved in more than 30 countries.

3. MECHANISM OF ACTION OF TAXOIDS

3.1. The Cellular Target of Taxoids

Together with actin microfilaments and intermediate filaments, microtubules form the cytoskeleton of eukaryotic cells. The microtubules are involved in a variety of cell functions, including chromosome movement and the regulation of cell shape and motility (*31*). These activities are modulated through associations with several biochemical components, such as guanosine triphosphate (GTP), and a wide range of proteins, the microtubule-associated proteins (MAPs). When a cell begins to divide, interphasic microtubules totally vanish and the mitotic spindle assembles. The depolymerization of mitotic spindle microtubules is essential for specific mitotic events, such as the movement of the chromosomes to the metaphase plate and their correct segregation during anaphase (*32*). Microtubules are long, hollow cylinders assembled from a heterodimeric (α/β) globular protein called tubulin. They consist of 13 aligned protofilaments within which the tubulin subunits interact through longitudinal and lateral bonds (*33*). Not all the tubulin pool assembles into microtubules: a steady state is maintained between assembled tubulin and a concentration of free tubulin called the critical concentration.

3.2. Taxoids Stabilize Microtubules

The polymerization of tubulin purified from mammalian brain usually enhances the turbidity of the solution; thus, the degree of polymerization can be monitored simply by measuring turbidity (*34*). Figure 4 depicts, for example, the effects of doce-

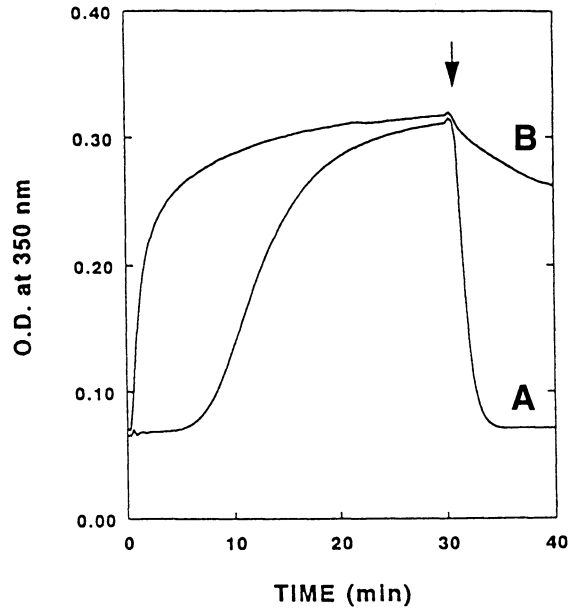


Fig. 4. Effect of docetaxel on polymerization of tubulin and depolymerization of microtubules. Tubulin was polymerized by heating from 3 to 37 °C. Depolymerization of microtubules was obtained by cooling from 37 to 3 °C (arrow). Polymerization or depolymerization was monitored by following the turbidimetry at 350 nm. (**A**) 10 μM porcine brain tubuline. (**B**) 10 μM tubulin and 3 μM docetaxel.

taxel on the kinetics of tubulin assembly and disassembly. The lag time corresponding to the activation and nucleation of tubulin is notably reduced, and the rate of polymerization is increased (*35*). Finally, microtubules, stabilized by taxoids, do not depolymerize on cold treatment. In fact, paclitaxel and docetaxel analogs are usually evaluated on the basis of the drug concentration that inhibits half of the cold-induced depolymerization. Docetaxel is about twice as efficient as paclitaxel in this respect (*36,37*). The thermodynamic parameters of tubulin assembly are also modified by the taxoids, and the critical concentration is significantly reduced in the presence of paclitaxel (*8*). Docetaxel is twice as efficient as paclitaxel in decreasing the critical concentration of tubulin assembly (*38*).

It should be pointed out that the mechanism of action of the taxoids is unique, since all other known mitotic spindle poisons, in particular, the vinca-alkaloids, shift the tubulin-microtubule equilibrium toward tubulin (*39*) (Fig. 5).

3.3. Characterization of the Interaction Site

Tritiated paclitaxel cosediments with microtubules and dissociates rapidly on the addition of paclitaxel. Thus, a rapid and reversible equilibrium exists between paclitaxel and the microtubules. There is only one high-affinity binding site of paclitaxel per α/β tubulin subunit, indicating that the interaction between microtubules and paclitaxel is specific. The equilibrium dissociation constant was originally found to be 870 nM (*40*). Docetaxel competes with paclitaxel for binding to the microtubules, but its equilibrium dissociation constant is two times less, i.e., it has better affinity (*38*). This difference could account for the higher efficiency of docetaxel to promote tubulin polymerization, to stabilize microtubules against cold-induced disassembly, and to

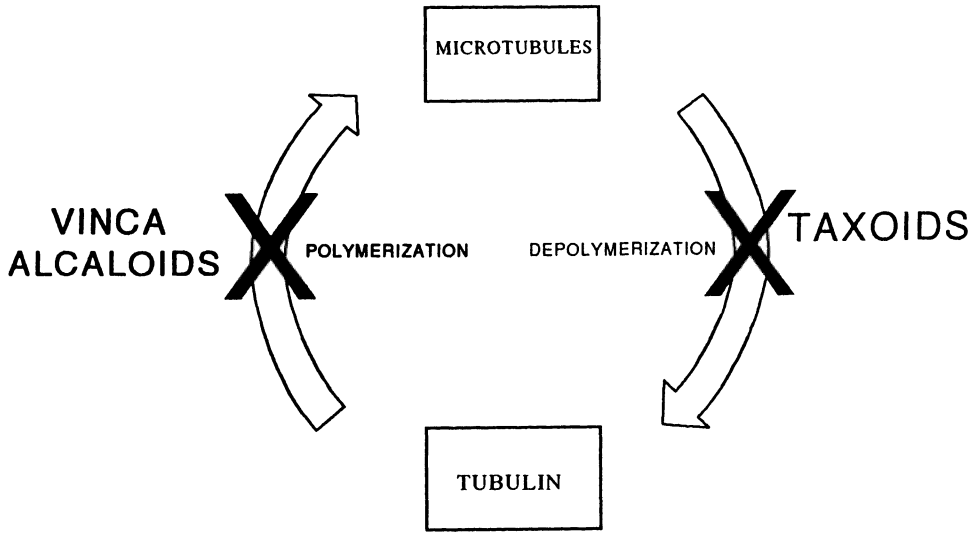


Fig. 5. Mechanism of action of vinca alkaloids and taxoids.

decrease the critical concentration of tubulin assembly. To acquire further insight into the taxoid–microtubule interaction at the molecular level, it is essential to locate the taxoid binding site. So far, it is known that the binding of taxoids is linked to the polymerization process. Furthermore, no binding of taxoids to dimeric tubulin has been detected, indicating that the site is located on assembled tubulin (*38*). This site does not overlap those of other known ligands, such as colchicine, podophyllotoxin, vinblastine, or GTP (*41,42*).

3.4. Models of the Mechanism of Action

Paclitaxel-bound microtubules, the structure of which has been resolved at 3 nm employing X-ray scattering, appear to be constituted of 12 protofilaments instead of the 13 protofilaments usually observed (*43,44*). The solution structure of microtubules induced by docetaxel has been also characterized using the same technique (*45*). The substructures of the microtubule walls are identical in paclitaxel- and docetaxel-induced microtubules; however, the population of docetaxel microtubules has an average of 13 protofilaments like the control microtubules. It is proposed that the chemical substitutions present in docetaxel side chain in its binding site increases slightly the contact angle between adjacent protofilaments. The simple working hypothesis is that taxoids bind between adjacent tubulin molecules, and such a hypothesis is fully compatible with the observed thermodynamic behavior of the taxoid-induced microtubule assembly system (*43*).

4. ANTITUMOR PROPERTIES

4.1. In Vitro Activities

4.1.1. Cellular Cytotoxicity

Both taxoids have been found extremely potent against a wide variety of murine and human cancer cell lines. Using the COMPARE computer program, it was con-

cluded that docetaxel response profile on 50 human tumor cell lines in the new NCI screening panel, correlated with the data pattern of test agents acting on the tubulin/microtubule system, the closest compound being paclitaxel (NCI, unpublished results). Several in vitro studies have been done comparing their activities under various experimental conditions (liquid medium, semisolid medium, short- and long-term exposures). The cytotoxicity of paclitaxel and docetaxel at submicromolar concentrations was compared in several murine, P388, SVras, and human tumor cell lines, breast Calc18, colon HCT 116, bladder T24, and nasopharyngeal KB (*46*). Docetaxel was found to be 1.3- to 12-fold more potent than paclitaxel. The cytotoxic properties of paclitaxel and docetaxel were also compared against nine cell lines established from human ovarian tumors and having intrinsic or acquired resistance to cisplatin. These cell lines were not crossresistant to the taxoids, and docetaxel was found to be active at a twofold lower concentrations than paclitaxel (*47*).

In addition, the activities of docetaxel and paclitaxel, in a human tumor cloning stem cell assay (starting from fresh human tumor biopsies), were compared at concentrations of taxoids similar to the plasma levels obtained after treatment of patients (*48,49*). Melanoma, breast, lung, ovarian, and colon tumors cells were significantly inhibited, regardless of the schedule of incubation (1-h exposure or continuous exposure for 14–28 d). Interestingly, 29 samples were found to be more sensitive to docetaxel, whereas 13 were more sensitive to paclitaxel, suggesting partial crossresistance between these two drugs (*49*). Finally, the in vitro cellular effects of docetaxel and paclitaxel have been recently assessed against a wide range of human normal and tumor samples, including tumor cell lines, primary cultures from tumor biopsies and normal bone marrow samples (*50*). IC_{50} (50% inhibitory concentrations) values of the two taxoids were in the nanomolar range and docetaxel appeared to be two- to fourfold more cytotoxic than paclitaxel (*50*).

4.1.2. Mechanism of Cytotoxicity and Cellular Effects

Uptake and efflux studies were performed on P388 leukemia cells in vitro with radiolabeled docetaxel and paclitaxel. Uptake experiments revealed that a threefold higher intracellular concentration of docetaxel was obtained as compared to paclitaxel, for the same initial extracellular concentration (0.1 μM) (*51*). Efflux studies revealed that the half-time of efflux of docetaxel from P388 cells was at least three times slower than that of paclitaxel (150 vs 45 min, respectively).

Thus, the higher potency of docetaxel observed in vitro may be explained by the combination of its higher affinity for microtubules, its higher achievable intracellular concentration, and the slower cellular efflux.

Cell-cycle studies revealed that paclitaxel was mainly cytotoxic during mitosis (M phase), as demonstrated by experiments on CHO and A 2780 ovarian tumor cell lines (*52*). Inhibition of cytokinesis has been observed, but some cells can progress through new cell cycles, leading to the formation of polyploid cells (*53,54*). Using synchronized HCT116 cells, it was demonstrated that paclitaxel inhibits formation of mitotic spindles in cells without affecting function of preformed spindles and without arresting cells in mitosis (*54*). Docetaxel has been found to be more active on proliferating than on nonproliferating KB cells (*46*) and to inhibit mitosis in several cell lines, such as J82 and KB (*55*). Surprisingly, using synchronized HeLa cells, it has been shown that docetaxel exerts cell killing specifically during the S phase of the cell cycle; no

Table 1
In Vivo Antitumor Activity of Paclitaxel Against Human Tumor Xenografts

sc Human tumor	*Highest nontoxic iv dosage mg/kg/dose*	*Schedule days*	*Activity[a] rating*
A 2780 ovarian	18	7, 9, 11, 13, 15	+ + +
LX-1 lung	24	5, 7, 9, 11, 13	+ + + +
H 2981 lung	24	5, 7, 9, 11, 13	+ +
L 2987 lung	36	14, 16, 18, 20, 22	+ + +
RCA colon	36	4, 6, 8, 10, 12	> + +
HCT-116 colon	36	3, 5, 7, 9, 11	> + + +
A431 vulva	36	3, 5, 7, 9, 11	+ + +

[a]Activity rating: + + + + = highly active (log cell kill > 2.8), + + + = highly active (log cell kill = 2.0 to 2.8), + + = active (log cell kill = 1.3–1.9; T/C ≥ 150% for L1210, + = active (log cell kill = 0.7–1.2 for s.c. tumors, T/C = 125–174% for P388), − = inactive.

cytotoxicity was observed during mitosis, a different situation from what is observed with paclitaxel (*56*).

Finally, it was found that paclitaxel greatly increases the pool of polymerized tubulin in cells, and new short microtubules free in the cytoplasm were observed (*57*). In addition, at high concentration, it induced the formation of microtubules bundles (*58*). Using J82 human bladder and KB 3-1 human carcinoma cells, it was shown that paclitaxel and docetaxel lead to the formation of bundles and asters in a dose- and time-dependent manner (*55*). Asters were observed in mitotic cells, and bundles were seen in interphase cells. The effects of docetaxel as compared to paclitaxel appeared at a twofold lower concentration (*55*).

4.2. In Vivo Activity

Paclitaxel and even more docetaxel have been studied in many murine tumor models and human tumor xenografts.

4.2.1. Paclitaxel

The development of an adequate formulation for paclitaxel led to a re-evaluation of its in vivo antitumor efficacy, using better experimental conditions, i.e., avoiding the previous ip/ip evaluation and administering the drug iv at a site different from the tumor site. The formulation used was 10% Cremophor®, 10% ethanol, 80% NaCl 0.9%. These studies have been recently reviewed, and most of them were performed after initial clinical trials (*59,60*). Indeed, these studies demonstrated that paclitaxel delivered iv was active against several tumors implanted in distal sites and treated at an early stage: sc Madison 109 murine lung carcinoma and A 431 vulva, A 2780 ovarian, H 2981 and LX-1 lung, and RCA and HCT-116 colon human tumor xenografts implanted under the renal capsule of nude mice (Table 1) (*60*). When administered sc five times weekly for three consecutive weeks, paclitaxel caused the complete regression of a human breast tumor xenograft, and significantly delayed the growth of endometrial, ovarian, brain, tongue, and lung human tumor xenografts (*61*). Paclitaxel was also evaluated against ovarian carcinoma xenografts HOC8, HOC18, and HOC22, and was found to have similar efficacy to docetaxel (*62*).

Table 2
In Vivo Antitumor Activity of Docetaxel Against Human Tumor Xenografts

sc Human tumor	*Highest nontoxic iv dosage mg/kg/dose*	*Schedule days*	*Activity[a] rating*
Calc 18 mammary	32.2	11, 15, 19	+ + +
MX-1 mammary	22	11, 15, 19	+ + + +
LX-1 lung	22	9, 13, 17	+ +
SKMEL-2 melanoma	33	27, 31, 35	+ + + +
CX-1 ovarian	15	12,16, 20	+ + +
KM20L2 ovarian	33	14,18,22	+ +
OVCAR-3	33	3, 7, 11	+ + + +

[a]For activity rating, *see* Table 1.

Further schedule-dependency studies were performed and showed that daily injection for 7 d was the best schedule as opposed to longer spaced administration (*60*).

4.2.2. Docetaxel

The experimental antitumor activity of docetaxel has been evaluated against a panel of 30 tumors of mice and human tumors xenografted in nude mice, representing a variety of tissue types and chemosensitivity patterns. The tumors were grafted in distal sites, and several tumors were treated at advanced and metastatic stages. Dose response was evaluated in all trials to determine accurately the maximum tolerated dose. The formulation used was a 1:1 ethanol polysorbate 80 solution, administered after a 1:10 dilution in glucose 5% in water (*29*). Docetaxel had a broad spectrum of antitumor activity, since 28/30 models responded to this agent (*29,63–65*) (Tables 2 and 3). The experimental antitumor activities of paclitaxel and docetaxel were compared by testing these drugs against B16 melanoma, a tumor sensitive to taxoids using an intermittent schedule, every 2 d × 3. Antitumor activity was expressed by the tumor growth delay (T-C) and by the log cell kill (LCK) of tumor cells obtained at the maximal tolerated dose (MTD) of each drug. According to these criteria, docetaxel was approximately two times more active and potent than paclitaxel (docetaxel: T-C = 12.2 d, LCK = 2.9, MTD = 11.3 mg/kg/d; paclitaxel: T-C = 4.7 d, LCK = 1.1, MTD = 21.7 mg/kg/d) (*29*) (Fig. 6). Among the murine models tested, good activity was observed, with in some cases cures of early stage tumors. However, the most meaningful data were those obtained against advanced stage tumors (i.e., tumors at least 200 mg at start of therapy) where complete tumor regressions could be observed. This occurred with the murine mammary adenocarcinomas 16/C and 13/C, pancreatic ductal carinoma 03, colon 38 adenocarcinoma, and the human xenografts MX-1 mammary and SK-MEL-2 melanoma (*63,64*). Prolonged tumor growth delays were also observed with Calc-18 breast, LX-1 lung, CX-1 colon, head and neck HNX-14C, and HNX-22B xenografts (*64,66*). Since the clinical activity of paclitaxel against ovarian tumors was impressive, five human ovarian xenografts having different sensitivities to the reference drug cisplatin were included in this study. Docetaxel was active against the three tumors sensitive to cisplatin; interestingly, it was also active against OV-Pe, which is resistant to cisplatin (*65*).

Table 3
In Vivo Antitumor Activity of Docetaxel Against Murine Tumors

Tumor	*Highest nontoxic iv dosage mg/kg/dose*	*Schedule days*	*Activity[a] rating*
Solid tumors sc			
Melanoma B16 early	24	3, 5, 7, 9	+ + + +
Pancreas			
PO2	32.2	3, 5, 7	±
PO3 early	20.5	3, 5, 7, 9	+ + + +
PO3 advanced	18.3	22, 24, 26, 28	5/6 CR
Mammary			
MA16/C early	15	3, 5, 7	+ + +
MA16/C advanced	10.8	7, 9, 11	5/5 CR
MA13/C early	14.2	3, 5, 7	+ + + +
MA13/C advanced	15	24, 27, 30	3/5 CR[b]
MA44 early	22	3, 5, 7	±
Colon			
C26 early	5	1–4	+
C38 early	23.5	3, 5, 7	+ + + +
C38 advanced	26.8	14, 16, 18	5/5 CR
C51 early	12.7	3, 5, 7	+ + +
C51 advanced	15.2	10, 12, 14	+ +
Lewis lung early	23.2	3–7	+
Osteosarcoma GOS early	18.6	3–7	+
Hystiocytosarcoma M5076 early	8.6	3–7	–
Leukemias ip			
P388 10^6 cells	23.2	1–4	+
L1210 10^5 cells	21.7	1–4	+ +

[a]For activity rating, *see* Table 1.
[b]CR = complete regressions.

Scheduling studies were performed against advanced colon 38 adenocarcinoma. Docetaxel was tested using three different schedules comparing the effect of 2, 3, and 10 administrations over the same duration of treatment. Overall, the administration schedule did not influence markedly the total dosage that can be administered and, thus, the compound was considered schedule-independent for the MTD (*29*).

4.3. Combination Chemotherapy

Since taxoids have clinical activity in ovarian, breast, and lung tumors, most of the experimental studies have been done with drugs active in these diseases: doxorubicin, 5-fluorouracil, cyclophosphamide, cisplatin, and etoposide.

4.3.1. Paclitaxel

Both in vitro and in vivo studies were performed. The efficacy of combination therapy consisting of paclitaxel plus a topoisomerase II inhibitor, doxorubicin or

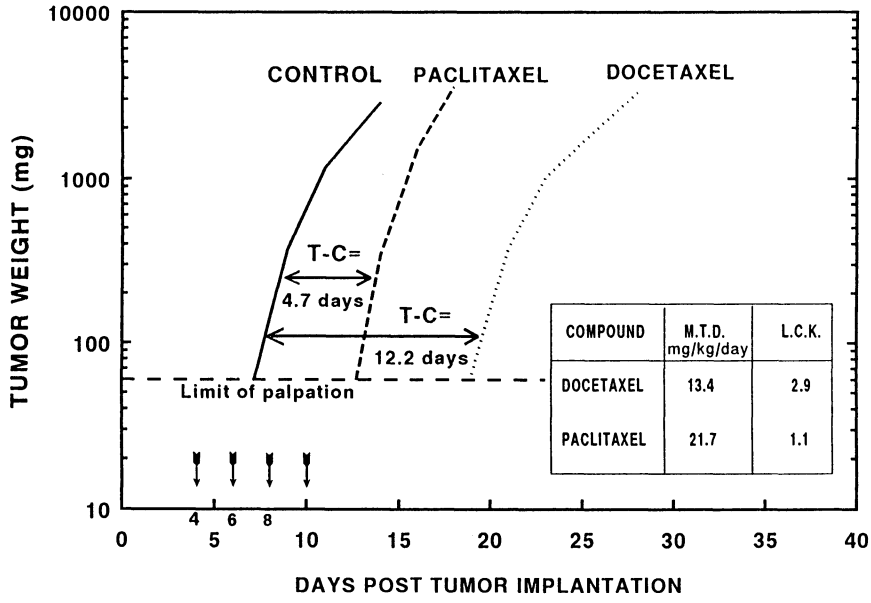


Fig. 6. Comparative in vivo activities of paclitaxel and docetaxel against B16 melanoma. Detailed experimental conditions were described in ref. (*29*). Briefly, B6D2F$_1$ mice (7 mice/group) were grafted sc on day 0 with 30 mg B16 tumors fragments. Drugs were injected iv on days 4, 6, 8, and 10 at the MTD (21.7 mg/kg/d for paclitaxel; 13.4 mg/kg/d for docetaxel). Tumor growths were measured biweekly. Activity is expressed by the T-C (where T and C are the median time in days necessary for the tumors of the treated group T and the control group C to reach a size of 1 g). Activity is also expressed by the LCK, which quantifies the number of tumor cells killed by the chemotherapy.

etoposide, against various cell lines has been studied in vitro; better results were obtained when cells were first incubated with paclitaxel (*67*). Cisplatin–paclitaxel combination was evaluated using L1210 leukemic cells: maximal effects were observed when cells were incubated for 24 h with paclitaxel, and then treated for 30 min with cisplatin (*68*). Combinations of taxoids and tubulin-interactive agents are of interest because of their complementary mechanism of action. Paclitaxel–estramustine was found to give supra-additive cytotoxic effects on several lines of human prostatic adenocarcinoma. No additive properties were noted when taxoids were combined with vinblastine (*69*).

In vivo combination chemotherapy studies have also been performed with paclitaxel using the M109 tumor model (*60*). The combined agents included cisplatin, etoposide, doxorubicin, cyclophosphamide, methotrexate, pentamethylmelamine, and bleomycin. Taxol-cisplatin and Taxol-bleomycin were the two combinations reported as showing hints of therapeutic synergy (*60*).

In mice grafted with MA16/C mammary adenocarcinoma, the association of doxorubicin-paclitaxel has a synergistic effect, which is observed only when compounds are administered sequentially every 4 d; such a protocol of administration makes this regimen, which is otherwise toxic, acceptable (*70*).

4.3.2. Docetaxel

Much of the work has been performed in vivo. Ten two-drug combinations were evaluated in mice bearing sc transplantable tumors (*63, 71,72*). The effects of the

optimal docetaxel-based combination were found to be greater than the effect of the best single agent in the case of docetaxel-vinorelbine (against MA16/C), docetaxel-etoposide (against B16 melanoma), docetaxel-cyclophosphamide (against MA13/C), docetaxel-5-fluorouracil (against colon 38), and docetaxel-methotrexate (against P388 leukemia). A similar level of efficacy was obtained in the case of docetaxel-vincristine (against P388) and docetaxel-mitomycin C (against MA13/C), compared to the activity of the best single agent. Good activity was obtained with the docetaxel-vinblastine and docetaxel-doxorubine combinations, both tested against MA13/C. However, their activity was lower than that of docetaxel alone.

In terms of toxicity, the combination toxicity index (CTI, i.e., sum of the fractions of the LD_{50} of each agent used in the combination) ranged from 0.75 for the most toxic combination (docetaxel/cisplatin), indicating complete overlap in dose-limiting toxic effects, to 2 for the least toxic combination docetaxel/vinca alkaloids, indicating that the maximum tolerated dose of each agent could be administered without additional toxicity. All the other combinations had a CTI around 1.2, indicating that approximately 60% of the full dose of each agent can be used in combination without an increase in the overall toxicity (*63, 71,72*).

Although most of the combinations were evaluated using simultaneous administration, a few studies were performed using different schedules. This factor was found to be important in the case of the docetaxel vincristine combination, where administering the two drugs 24 h apart led to a greater level of host toxicity (*71*).

5. ANIMAL PHARMACOLOGY

Pharmacokinetic data on paclitaxel in animals are scarce because of the lack of a sensitive assay when the drug was being investigated only in the laboratory. None of the bioassays that were developed were suitable for detailed studies. With an assay with a detection limit of 0.1 μM, however, it has been shown that in animals, paclitaxel was almost totally bound to proteins, and had distribution and elimination half-lives of 2.7 and 42 min, respectively, in rabbits (*73*).

In contrast, a sensitive and selective high-performance liquid chromatographic (HPLC) assay for docetaxel was available early in its development (*74*) and was used for studies in animals (*75,76*). In tumor-bearing mice, $t1/2\alpha$ and $t1/2\beta$ were 7 min and 1.1 h, respectively. The pharmacokinetics were linear. The plasma clearance was 2.2 L/h/kg, and the apparent volume of distribution at steady state 2.2 L/kg. The AUC at doses of 13–62 mg/kg ranged from 4.5 to 29.6 μg/mL/h. Interestingly, the elimination half-life from tumor tissue was more than 20 h, compared to a 2- to 4- h elimination half-life in all other tissues. It was of interest that, at all doses, tumor levels were considerably higher than the IC_{50} values of cytotoxicity in tumor cell cultures, up to 24 h after administration. This long exposure of tumor tissue may be an essential factor to docetaxel activity in human patients, where plasma exposure at therapeutic doses were found later to be in the same range as in the mouse (*77*).

Administration of radiolabeled docetaxel led to a rapid diffusion into all tissues except those of the CNS, with the highest levels seen in liver, bile, intestines, and gastric contents. The plasma protein binding in mice was 76–89%, and the elimination was almost complete at 96 h after administration. In the dog and mouse, the primary route of elimination of radiolabeled docetaxel was hepatic extraction and biliary excretion, whereas urinary excretion was < 10% (*78*). Docetaxel metabolism was found

to be similar across species in vivo and in vitro models (*79*). The facts that docetaxel was generally the main circulating compound and that the major metabolites were much less active than docetaxel indicated that parent drug analysis was an appropriate parameter for pharmacokinetic/pharmacodynamic studies of this drug.

6. ANIMAL TOXICOLOGY

Toxicology studies with paclitaxel in rodents were completed by 1982. They were performed with ip drug administration because of the solubility limitation and because of volume constraints. In dogs, the drug was given iv (*1*). The single-dose LD_{10} ip in Sprague-Dawley rats was 138 mg/m^2, and the LD_{50} was 206 mg/m^2. For the daily-times-five administration schedule, these doses were 36 and 51 mg/m^2/d, respectively, in rats, and 67 and 82 mg/m^2/d in CD2F1 mice. The single-dose toxic low (TDL) iv in beagle dogs was 45 mg/m^2. The major toxic effect in rats and dogs was reversible myelosuppression. In rodents, oligospermia was noted. Gastrointestinal toxicity was most pronounced in the daily-times-five schedule in dogs, and consisted of diarrhea, mucosal ulcerations, and emesis. In dogs, hypotension was also observed, which was believed to be related to histamine released by Cremophor®.

For docetaxel the single-dose LD_{10} in mice was 345 mg/m^2, and the LD_{50} was 414 mg/m^2. In dogs, the TDL was 15 mg/m^2 (*75*). The main toxic effects of docetaxel were reversible myelosuppression and epithelial necrosis in the digestive tract. In mice, cumulative and reversible neurotoxicity was observed. Dogs experienced hypotension, which was thought to be related to the vehicle polysorbate 80. Although there are minor differences, animal toxicology in general appears to be similar for paclitaxel and docetaxel, with similar target organs and more pronounced toxicity with repeated-administration schedules.

The results of the acute toxicity study in dogs served as a basis for the entry dose in humans. Following recommendations of the NCI, one-third of the TDL in dogs was selected as the initial dose level for Phase I clinical trials: 15 mg/m^2 for paclitaxel and 5 mg/m^2 for docetaxel.

7. CONCLUSION

New chemical structure, new mechanism of action, coupled with solid preclinical antitumor activity are considered to be key features for the selection of antitumor agents. The taxoids do fit these criteria. Although the discovery process has been very long and tedious, numerous challenges have been overcome. In the case of paclitaxel, a suitable formulation was developed, an adequate supply was ensured, and severe hypersensitivity reactions were diminished. This process took more than 30 years from bark collections to first approval. As a result of the supply difficulties, semisynthetic routes were unveiled, paving the way for the discovery of new compounds. Docetaxel was the first semisynthetic compound in clinical trial and has been developed in record time.

As predicted by their chemical analogies, paclitaxel and docetaxel bear similarities in terms of mechanism of action and experimental antitumor properties in cellular and animal models. These two compounds bind to the same site on microtubules, and consequently, share the same and unique mechanism of action: promotion of tubulin assembly into microtubules resistant to cold-induced depolymerization. However,

what seem to be minor chemical differences result in quantitative and qualitative differences. Docetaxel has a twofold increased affinity for binding to microtubules. The population of docetaxel microtubules has an average of 13 protofilaments like normal microtubules, whereas paclitaxel microtubules have an average of 12 protofilaments. Higher intracellular concentration can be achieved and the drug is retained longer owing to a slower efflux from the cells. In addition, whereas paclitaxel is cytotoxic during mitosis, docetaxel exerts its cytotoxic effect during S phase. Docetaxel has been found to be schedule-independent, whereas paclitaxel seems to be schedule-dependent. Finally, crossresistance has not been systematically observed between the two drugs in a tumor cloning assay.

Despite the extraordinary advances obtained during these last few years with the preparation of taxoids by semisynthesis and with the total synthesis of paclitaxel, the chemistry of taxoids is still in its infancy. The first structure-activity relationships for taxoids have been obtained and have revealed key positions both on the lateral chain and on the diterpenoid skeleton. Second-generation compounds could include soluble compounds that are easier to formulate without the use of detergents, such as Cremophor® or polysorbate 80, compounds that are not crossresistant with paclitaxel/docetaxel, and compounds that are better tolerated after acute and/or cumulative administrations.

REFERENCES

1. Suffness M. Taxol: from discovery to therapeutic use. In: Bristol JA ed. *Annual Reports in Medicinal Chemistry*. San Diego, CA: Academic 1993:305-314.
2. Wall ME. Camptothecin and taxol. In: Lednicer D, ed. *Chronicles of Drug Discovery*. Washington DC: American Chemical Society. 1993:327-348.
3. Wall ME, Wani MC. Campthothecin and taxol: discovery to clinic—Thirteenth Bruce F. Cain Memorial Award Lecture. *Cancer Res* 1995; 55:753-760.
4. Wall ME, Wani MC. Recent progress in plant anti-tumor agents. In: 153rd National Meeting of the American Chemical Society, Miami Beach FL, 1967; Paper M-006.
5. Wani MC, Taylor HL, Wall ME, Coggon P, Mc Phail AT. Plant antitumor agents VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J Am Chem Soc* 1971; 93:2325-2327.
6. Douros J, Suffness M. New natural products under development at the National Cancer Institute. Recent results. *Cancer Res* 1981; 76:153-175.
7. Fuchs DA, Johnson RK. Cytologic evidence that taxol, an antineoplastic agent from *Taxus brevifolia*, acts as a mitotic spindle poison. *Cancer Treat Rep* 1978; 62:1219-1222.
8. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly *in vitro* by Taxol. *Nature* 1979; 22:665-667.
9. Pazdur R, Kudelka AP, Kavanagh JJ, Cohen PR, Raber MN. The taxoids: paclitaxel (Taxol®) and docetaxel (Taxotere®). *Cancer Treatment Rev* 1993; 19:351-386.
10. Rowinsky EK, Cazenave LA, Donehower RC. Taxol: A novel investigational antimicrotubule agent. *J Natl Cancer Inst* 1990; 82 (15):1247-1259.
11. McGuire WP, Rowinsky EK, Rosenshein NB, Grumbine FC, Ettinger DS, Armstrong DK, Donehower RC. Taxol: a unique antineoplastic agent with significant activity in advanced ovarian epithelial neoplasms. *Ann Intern Med* 1989; 111:273-279.
12. Holmes FA, Walters RS, Theriault RL, Forman AD, Newton LK, Raber MN, Buzdar AU, Frye DK, Hortobagyi GN. Phase II trial of taxol, an active drug in the treatment of metastatic breast cancer. *J Natl Cancer Inst* 1991: 83:1797-1805.
13. Murphy WK, Fossella FV, Winn RJ, Shin DM, Hynes HE, Gross HM, Davilla E, Leimert JT, Dhingra HM, Raber MN, Krakoff IH, Hong WK. Phase II study of taxol in patients with untreated advanced non-small-cell lung cancer. *J Natl Cancer Inst* 1993; 85:384-388.

14. Shelanski ML, Gaskin F, Cantor CR. Microtubule assembly in the absence of added nucleotides. *Proc Natl Acad Sci USA* 1973; 70:765–769.
15. Senilh V, Blechert S, Colin M, Guénard D, Picot F, Potier P, Varenne P. Mise en évidence de nouveaux analogues du taxol, extraits de *Taxus baccata*. *J Natural Product* 1984; 47:131.
16. Lavelle F, Guéritte-Voegelein F, Guénard D, Le Taxotere: des aiguilles d'if a la clinique. *Bull Cancer* (Paris) 1993; 80:326–338.
17. Guéritte-Voegelein F, Senilh V, David B, Guénard D, Potier P. Chemical studies of 10-deacetylbaccatin III. Hemisynthesis of taxol derivatives. *Tetrahedron* 1986; 42:4451–4460.
18. Guénard D, Guéritte-Voegelein F, Potier P. Taxol and Taxotere: discovery, chemistry and structure-activity relationships. *Acc Chem Res* 1993; 26:160–167.
19. Herranz E, Biller SA, Sharpless KB. Osmium-catalyzed vicinal oxyamination of olefins by *N*-chloro-*N*-argentocarbamates. *J Am Chem Soc* 1978; 100:3596–3598.
20. Mangatal L, Adeline MT, Guénard D, Guéritte-Voegelein F, Potier P. Application of the vicinal oxyamination reaction with asymmetric induction to the hemisynthesis of taxol and analogues. *Tetrahedron* 1989; 45:4177–4190.
21. Lavelle F, Fizames C, Guéritte-Voegelein F, Guénard D, Potier P. Experimental properties of RP 56976, a Taxol derivative. *Proc Am Assoc Cancer Res* 1989; 30:2254.
22. Denis JN, Greene AE, Serra AA, Luche MJ. An efficient, enantioselective synthesis of the taxol side chain. *J Org Chem* 1986; 51:46–50.
23. Colin M, Guénard D, Guéritte-Voegelein F, Potier P. Preparation of baccatin III derivatives as antitumor agents. Eur. Pat. Appl EP 336841, 89/10/11, FR appl. 884513 (88/04/06).
24. Denis JN, Greene AE, Guénard D, Guéritte-Voegelein F, Mangatal L, Potier P. A highly efficient, practical approach to natural taxol. *J Am Chem Soc* 1988; 110:5917–5919.
25. Commercon A, Bézard D, Bernard F, Bourzat JD. Improved protection and esterification of a precursor of the Taxotere® and Taxol® side chain. *Tetrahedron Lett* 1992; 33:5185.
26. Holton RA. Eur. Pat. Appl. EP 400,971, 1990. Chem. Abstr. 1991: 114, 164568q.
27. Holton RA. Eur. Pat. Appl. EP 428,376, 1991. Chem. Abstr. 1991: 115, 114817v.
28. Ojima I, Habus I, Zhao M, Georg G, Jayasinghe LR. Efficient and practical asymmetric synthesis of the Taxol C-13 side chain, *N*-Benzoyl-(2R,3S)-3-phenylisoserine, and its analogues via chiral 3-hydroxy-4-aryl-β-lactams through chiral ester enolate-imine cyclocondensation. *J Org Chem* 1991; 56:1681.
29. Bissery MC, Guénard D, Guéritte-Voegelein F, Lavelle F. Experimental antitumor activity of Taxotere (RP 56976, NSC 628503), a taxol analogue. *Cancer Res* 1991; 51:4845–4852.
30. Verweij J, Clavel M, Chevalier B. Paclitaxel (Taxol™) and docetaxel (Taxotere™): Not simply two of a kind. *Ann Oncol* 1994; 5:495–505.
31. Hyams JS, Lloyd CW. in: Harford JB, ed. *Microtubules, Modern Cell Biology Series*, vol. 13. New York: Wiley Liss. 1993:460.
32. Wadsworth P. Mitosis: spindle assembly and chromosome motion. *Current Opinion Cell Biol* 1993; 5:123–128.
33. Beese L, Stubbs G, Cohen C. Microtubule structure at 18 Å resolution. *J Mol Biol* 1987; 194: 257–264.
34. Gaskin F, Cantor CR, Shelanski ML. Turbidimetric studies of the *in vitro* assembly and disassembly of porcine neurotubules. *J Mol Biol* 1974; 89:737–758.
35. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly *in vitro* by taxol. *Nature (Lond.)* 1979; 22:665–667.
36. Ringel I, Horwitz SB. Studies with RP 56976 (Taxotere): a semi-synthetic analog of taxol. *J Natl Cancer Inst* 1991; 83:288–291.
37. Guéritte-Voegelein F, Guénard D, Lavelle F, Le Goff M-T, Mangatal L, Potier P. Relationships between the structure of Taxol analogues and their antimitotic activity. *J Med Chem* 1991; 34:992–998.
38. Diaz JF, Andreu JM. Assembly of purified GDP-tubulin into microtubules induced by RP 56976 and paclitaxel: reversibility, ligand stoichiometry and competition. *Biochemistry* 1993; 32: 2747–2755.
39. Karsenti E. Vers une description du mécanisme d'assemblage du fuseau mitotique a l'échelle moléculaire. *Méd/Sci* 1993; 9:131–139.
40. Parness J., Horwitz SB. Taxol binds to polymerized tubulin in vitro. *J Cell Biol* 1981; 91: 479–487.

41. Schiff PB, Horwitz SB. Taxol assembles tubulin in the absence of exogenous guanosine-5'-triphosphate or microtubule associated proteins. *Biochemistry* 1981; 20:3247-3252.
42. Kumar N. Taxol induced polymerization of purified tubulin. *J Biol Chem* 1981; 256: 10,435-10,441.
43. Diaz JF, Menendez M, Andreu JM. Thermodynamics of ligand-induced assembly of tubulin. *Biochemistry* 1993; 32:10,067-10,077.
44. Andreu JM, Bordas J, Diaz JF, Garcia De Ancos J, Gil R, Medrano FJ, Nogales E, Pantos E, Towns-Andrews E. Low resolution structure of microtubules in solution. *J Mol Biol* 1992; 226:169-184.
45. Andreu JM, Diaz JF, Gil R, De Pereda JM, Garcia De Lacoba M, Peyrot V, Briand C, Towns-Andrews E, Bordas J. Solution structure of microtubules induced by the side chain taxol analogue Taxotere to 3 nm resolution. *J Biol Chem* 1994; 269 no 50:31,785-31,792.
46. Riou JF, Naudin A, Lavelle F. Effects of Taxotere on murine and human tumor cell lines. *Biochem Biophys Res Commun* 1992; 187:164-170.
47. Kelland LR, Abel G. Comparative *in vitro* cytotoxicity of taxol and Taxotere against cisplatin-sensitive and resistant human ovarian carcinoma cell lines. *Cancer Chemother Pharmacol* 1992; 30:444-450.
48. Vogel M, Hilsenbeck SG, Depenbrock H, Danhauser-Riedl S, Block T, Nekarda H, Fellbaum C, Aapro MS, Bissery MC, Rastetter J, Hanauske AR. Preclinical activity of Taxotere (RP 56976, NSC 628503) against freshly explanted clonogenic human tumour cells: comparison with taxol and conventional antineoplastic agents. *Eur J Cancer* 1993; 29A:2009-2014.
49. Hanauske AR, Degen D, Hilsensbeck SG, Bissery MC, Von Hoff DD. Effects of Taxotere and taxol on *in vitro* colony formation of freshly explanted human tumor cells. *Anti-Cancer Drugs* 1992; 3:121-124.
50. Braakhuis BJM, Hill BT, Dietel M, Kelland LR, Aapro MS, Zoli W, Lelieveld P. *In vitro* antiproliferative activity of docetaxel (Taxotere®), paclitaxel (Taxol®) and Cisplatin against human tumors and normal bone marrow cells. *Anticancer Res* 1994; 14:205-208.
51. Riou JF, Petitgenet O, Combeau C and Lavelle F. Cellular uptake and efflux of docetaxel (Taxotere®) and paclitaxel (Taxol®) in P388 cell line. *Proc Am Assoc Cancer Res* 1994; 35:385.
52. Lopes NM, Adams EG, Pitts TW, Bhuyan BK. Cell kill kinetics and cell cycle effects of taxol on human and hamster ovarian cell lines. *Cancer Chemother Pharmacol* 1993; 32:235-242.
53. Roberts JR, Allison DC, Donehower RC, Rowinsky EK. Development of polyploidization in Taxol-resistant human leukemia cells *in vitro*. *Cancer Res* 1990; 50:710-716.
54. Long BH, Fairchild CR. Paclitaxel inhibits progression of mitotic cells to G1 phase by interference with spindle formation without affecting other microtubule functions during anaphase and telophase. *Cancer Res* 1994; 54:4355-4361.
55. Garcia P, Braguer D, Carles G, El Khyari S, Barra Y, De Ines C, Barasoain I, Briand C. Comparative effects of taxol and Taxotere on two different human carcinoma cell lines. *Cancer Chemother Pharmacol* 1994; 34:335-343.
56. Hennequin N, Giocanti N, Favaudon V. S-phase specificity of cell killing by docetaxel (Taxotere) in synchronised HeLa cells. *Br J Cancer* 1995; 71:1194-1198.
57. De Brabander M, Geuens G, Nuydens R, Willebrords R, De Mey J. Taxol induces the assembly of free microtubules in living cells and blocks the organizing capacity of the centrosomes and kinetochores. *Proc Natl Acad Sci USA* 1981; 78:5608-5612.
58. Schiff PB, Horwitz SB. Taxol stabilizes microtubules in mouse fibroblasts cells. *Proc Natl Acad Sci* 1980; 77:1561-1565.
59. Rose WC. Taxol: a review of its preclinical *in vivo* antitumor activity. *Anti-Cancer Drugs* 1992; 3:311-321.
60. Rose WC. Taxol-based combination chemotherapy and other *in vivo* preclinical antitumor studies. *JNCI Monographs* 1993; 15:47-53.
61. Riondel J, Jacrot M, Picot F, Beriel H, Mouriquand C, Potier P. Therapeutic response to taxol of six human tumors xenografted into nude mice. *Cancer Chemother Pharmacol* 1986; 17:137-142.
62. Nicoletti MI, Lucchini V, D'Incalci M, Giavazzi R. Comparison of paclitaxel and docetaxel activity on human ovarian carcinoma xenografts. *Eur J Cancer* 1994; 30A:5:691-696.

63. Bissery MC, Vrignaud P, Lavelle F. Preclinical profile of Docetaxel (Taxotere): Efficacies as a single agent or in combination. *Seminars in Oncology* 1995; 22(Suppl):3-16.
64. Dykes DJ, Bissery MC, Harrison SD, Waud WR. Response of human tumor xenografts in athymic nude mice to docetaxel (RP 56976, Taxotere®). *Invest New Drugs* 1995; 13:1-11.
65. Boven E, Venema-Gaberscek E, Erkelens CAM, Bissery MC, Pinedo HM. Antitumor activity of taxotere (RP 56976, NSC 628503), a new taxol analog, in experimental ovarian cancer. *Ann Oncol* 1993; 4:321-324.
66. Braakhuis BJM, Kegel A, Welters MJP. The growth inhibiting effect of docetaxel (Taxotere®) in head and neck squamous cell carcinoma xenografts. *Cancer Lett* 1994; 81:151-154.
67. Hahn SM, Liebmann JE, Cook J, Fisher J, Goldspiel B, Venzon D, Mitchell JB, Kaufman D. Taxol in combination with doxorubicin or etoposide. *Cancer* 1993; 72:2705-2711.
68. Citardi MJ, Rowinsky EK, Schaefer KL, Donehower RC. Sequence dependent cytotoxicity between cisplatin (C) and the antimicrotubule agents taxol (T) and vincristine (V). *Proc Am Assoc. Cancer Res* 1990; 31:24-31.
69. Speicher LA, Barone L, Tew KD. Combined antimicrotubule activity of estramustine and taxol in human prostatic carcinoma cell lines. *Cancer Res* 1992; 52:4433-4440.
70. Lorusso PM, Demchik LL, Plowman J, Baker L, Corbett TH. Preclinical activity and toxicity of taxol combinations. *Proc Am Assoc Cancer Res* 1993; 34:1794.
71. Bissery MC, Vrignaud P, Bayssas M, Lavelle F. *In vivo* evaluation of Taxotere (RP 56976, NSC 628503) in combination with cisplatin, doxorubicin or vincristine. *Proc Am Assoc Cancer Res* 1992; 33:443.
72. Bissery MC, Vrignaud P, Bayssas M, Lavelle F. Taxotere synergistic combination with cyclophosphamide, etoposide and 5-fluorouracil in mouse tumor models. *Proc Am Assoc Cancer Res* 1993; 34:1782.
73. Hamel E, Lin CM, Johns DG. Tubulin dependent biochemical assay for the antineoplastic agent taxol and application of the drug in serum. *Cancer Treat Rep* 1982; 66:1381-1386.
74. Vergniol JC, Bruno R, Montay G, Frydman A. Determination of Taxotere in human plasma by a semiautomated high-performance liquid chromatographic method. *J Chromatog* 1992; 582: 273-278.
75. Bissery MC, Renard A, André S., et al. Preclinical pharmacology and toxicology of Taxotere (RP 56976, NSC 628503). *Ann Oncol* 1992; 3 (suppl 1):121.
76. Bissery MC, Renard A, Montay G, Bayssas M, Lavelle F. Taxotere:Antitumor activity and pharmacokinetics in mice. *Proc Am Assoc Cancer Res* 1991; 32:401.
77. Bruno R, Sanderink GJ. Pharmacokinetics and metabolism of Taxotere™. *Cancer Surveys* 1993; 17:305-313.
78. Marlard M, Gaillard C, Sanderink GJ, Roberts SA, Joannou PP, Facchini V, Chapelle P, Frydman A. Kinetics, distribution, metabolism and excretion of radiolabelled Taxotere® (^{14}C-RP 56976, docetaxel) in mice and dogs. *Proc Am Assoc Cancer Res* 1993; 34:393.
79. Sanderink GJ, Martinet M, Touzet A, Chapelle P, Frydman A. Docetaxel (Taxotere®), RP 56976) metabolizing enzymes and metabolic drug-drug interactions *in vitro*. *Proc ISSX* 1993; 3:35.

9 Sequence-Selective Groove Binders

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CONTENTS

1. INTRODUCTION

A large number of agents are known to bind to DNA, interfering with multiple DNA functions in living cells. Their ability to interact with DNA is associated with several biological effects, including antiviral, antibacterial, antiprotozoal, and antitumor activities. Their biological activities are probably related to different effects on cellular targets. From the pharmacological point of view, the most relevant DNA binding agents are antitumor drugs. They exert their cytotoxic effect principally as a consequence of the lack of selectivity by damaging cellular DNA. Cytotoxic and antiproliferative drugs have played and will likely continue to play a major role in cancer chemotherapy.

DNA-interacting cytotoxic agents belong to different chemical classes, and their mode of binding to DNA is quite different depending on the chemical structure and the presence of reactive groups. DNA binding ability is not *per se* a sufficient condition to achieve a significant therapeutic effect. In addition, as a consequence of the lack of selectivity, clinically useful cytotoxic agents often have a low therapeutic index. Indeed, they are expected to cause a large number of DNA lesions not only in tumor, but also in normal cells. However, a certain selectivity toward specific tumor types has been recognized for some agents, as documented by the efficacy of platinum compounds in the treatment of testicular and ovarian cancers. It is conceivable that the different chemosensitivity of tumor cells to cytotoxic agents is related to a different ability of the drug to damage critical genomic sites. However, the genes crucial for a selective effect remain unknown. Most of the known cytotoxic agents have a very limited ability to read sequence information compared to DNA binding proteins.

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

Molecular pharmacology studies have elucidated several aspects of drug–target interaction and provided a molecular basis for the design of more selective agents.

DNA-interactive drugs include chemically reactive compounds and agents that bind to DNA by noncovalent interactions. The major groups of noncovalently reacting agents are represented by intercalative and nonintercalative drugs. This classification is of course schematic, and there is a large overlap in these groupings. In particular, most known antitumor intercalators contain nonintercalating moieties (e.g., sugars, oligopeptides) able to interact in the helical grooves or along the surface of the phosphate backbone of the double helix. A large number of natural and synthetic DNA-interactive compounds have been described. These small organic ligands (in particular, nonintercalative groove binders) have been extensively used to elucidate aspects of DNA conformation and DNA sequence elements involved in the mechanisms of recognition by regulatory proteins and/or by DNA processing enzymes (*1,2*). Unfortunately, only a few compounds have proven to be effective antitumor agents. Indeed, DNA interaction can have cellular consequences distinct from cell death. Regardless of the mode of drug–DNA interaction, DNA binding ability is not a sufficient condition for a drug to be an effective antitumor agent. In general, a DNA-interacting agent is endowed with a significant cytotoxic effect when DNA damage (alteration of the nucleic acid structure or persistent inhibition of a critical cellular function) occurs as a consequence of DNA binding. If optimal repair is impossible, persistent lesions may be recognized as an apoptotic stimulus (*3*).

A number of reviews in the past decade have summarized the DNA binding properties of antitumor agents (*4,5*). In this chapter, we have focused on the development of antitumor agents for which groove binding ability is a determinant of sequence specificity in induction of DNA damage. Significant research efforts were directed toward elucidating the underlying mechanisms of cytotoxicity of groove binders. In an attempt to interpret the available information on their mode of action and to provide insight into current approaches for the rational development of new effective agents, this chapter will focus on the most representative classes of groove binding agents.

2. MAJOR GROOVE BINDERS

Although the major groove of DNA has greater recognition potential, as predicted on the basis of the number of hydrogen bonds (*4*), only a few DNA-interacting agents appear to be major-groove-specific. They include bifunctional alkylating agents, methylating agents, and cisplatin (*6,7*). Typically, such compounds covalently interact with the N-7 position of guanine located in the major groove. In spite of the fact that they apparently do not contain any sequence information, a preferential alkylation by nitrogen mustards occurs at guanines within guanine clusters. This selectivity has been related to the sequence dependence of the electrostatic potential of DNA (*8*). In fact, guanines surrounded by other guanines are associated with a more negative potential at the N-7 alkylation site than any isolated guanine. A contribution of specific binding interaction of the nonalkylating moieties in influencing selectivity has been proposed (*7*). Such a possibility may have relevant implications in the development of new agents with increased specificity of DNA binding, as suggested by bis (platinum) compounds, which exhibit a different profile of antitumor activity (*9*).

Although the sequence specificity of known alkylating (or platinating) agents is not enough to achieve tumor selectivity, the pattern of cell response to these cytotoxic agents is likely to be related to the localization of drug-induced DNA modification within chromatin (*10*) rather than to the variable expression of defense mechanisms. Experimental evidence in resistant cells supports this hypothesis. In tumor cell lines with variable degrees of resistance to cisplatin, no linear relationship has been found between the extent of DNA platination and cytotoxicity (*11*). In the presence of a comparable extent of total platinum-induced DNA-interstrand crosslinks, a marked decrease in formation of these lesions within the ribosomal RNA genes was found in resistant cells (*12*). The finding suggests that differences in the genomic sites of drug-induced lesions could contribute to the resistance phenotype. The molecular aspects of sequence selectivity exhibited by DNA-reactive drugs in DNA covalent modification have been extensively reviewed elsewhere (*5,6*).

3. INTERCALATING AGENTS

DNA intercalators have been extensively studied owing to their efficacy in cancer chemotherapy. The first class of intercalating antitumor agents proven to have clinical antitumor activity is represented by anthracyclines. One of them, doxorubicin, remains one of the most widely used antitumor drugs. The development of anthracyclines is the topic of another chapter of this book. Following identification of the clinical efficacy of anthracyclines, a large number of intercalating agents have been studied in an attempt to find more effective and selective antitumor agents. As already mentioned in Section 1., DNA binding and intercalation are necessary conditions, but not sufficient for optimal antitumor activity (*13*). Indeed, strong intercalators (e.g., ethidium bromide) may have marginal (if any) antitumor activity. It is likely that the mode and site of binding are more critical than the binding affinity. Stabilization of the intercalation complex involves several molecular interactions (*4*). In particular, side chains and bulky groups of the intercalating molecule placed either in the major or minor groove may have a critical role in determining the sequence specificity. Thus, the intercalation site depends not only on the planar drug chromophore, but also on a variety of intrinsic properties (steric and electronic factors) that involve external ligand moieties.

The mechanism of cytotoxic and antitumor activity of intercalating agents is ascribed to their ability to interfere with the function of topoisomerase II (*14*), a nuclear enzyme that regulates DNA topology during multiple metabolic DNA processes (*15*). The agents stabilize an intermediate of the enzyme reaction (the so-called cleavable complex), in which DNA strands are broken and enzyme subunits are covalently linked to DNA. Topoisomerase II inhibitors form a DNA–drug–enzyme ternary complex. A model of drug interaction in the ternary complex has been proposed in which the intercalator is placed at the interface between the topoisomerase II active site and the DNA cleavage site (*14*), thus preventing DNA religation by the enzyme. Studies of anthracycline analogs have not shown precise correlations between a drug's ability to induce DNA cleavage and DNA binding affinity (*13,16,17*). Although drug intercalation may have a role in the mechanism of enzyme inhibition, external interactions involving the sugar residue and the cyclohexene ring, therefore, may be more critical than the strength of intercalation. Relevant to this point is the observation that both these moieties are located in the minor groove (*18*).

Minor groove binders (*see* Section 4. for details on their biochemical and biological activities) have been reported to inhibit the induction of topoisomerase II-mediated DNA damage by *m*-AMSA and etoposide (*19,20*). Although minor groove binding is not a sufficient condition to stimulate enzyme-mediated cleavable complexes (*15*), several antitumor drugs known to be topoisomerase inhibitors have side chains that interact with the minor groove. For example, anthracyclines have a sugar moiety that is located in the minor groove. The presence of a bulky substituent at the 3′-position of doxorubicin (i.e, 3′-morpholinyl derivatives) totally abolishes the drug effect on topoisomerase II. Such derivatives were found to retain their ability to bind to DNA by intercalation (*21*). Available evidence suggests that the amino group at the 3′-position is not required for cytotoxic potency and topoisomerase II-trapping activity (*17*). Thus, the loss of activity of 3′-morpholinyl derivatives on topoisomerase II is not the result of a reduced stabilization of the intercalation complex consequent to loss of an electrostatic interaction of the free protonated amino group in the minor groove. These observations suggest that a bulky substituent at the 3′-position, but not at the 4′-position, is a steric hindrance for formation of the ternary complex and support the hypothesis that the minor groove binding moiety (i.e., amino sugar) is a critical determinant for drug poisoning of topoisomerase II (*17*). The importance of the 3′-substituent (and its orientation in the minor groove) in anthracycline's ability to inhibit topoisomerase II is emphasized by a recent study that indicated that epimerization of the 3′-amino group of the daunorubicin markedly influences the sequence specificity of topoisomerase II-mediated DNA cleavage (*22*). The observation is consistent with a critical role of the 3′-position for specific interaction of daunorubicin-related anthracyclines in the ternary complex. However, it is likely that multiple drug–enzyme interactions contribute to ternary complex stabilization, as suggested by reduced potency of 9-deoxy-doxorubicin in DNA-cleavage stimulation (*16*). Since the latter derivative has DNA binding parameters comparable to those of the parent drug, it is likely that the hydroxyl group at position 9 of the cyclohexene ring is involved in critical interactions with the enzyme. Again, the moiety is located in the minor groove (*18*). In addition to anthracyclines, another classical intercalating antitumor agent, actinomycin D, contains two pentapeptide rings located in the minor groove (*23*), and is a topoisomerase I and II inhibitor (*15,21*).

A peculiar feature of drug-stimulated topoisomerase II DNA cleavage is that the effects of antitumor drugs of different classes are sequence-specific (*24*). The sequence selectivity of different drugs has been rationalized in terms of distinct pharmacophores with similar interaction in the ternary complex. For example, amsacrine and bisantrene were shown to have similar sequence specificity, and also similar drug conformation and electronic properties. It is conceivable that specific interactions involving the drug domain(s) placed externally to the double helix critically influence the drug's ability to form the ternary complex and, hence, the site of DNA cleavage. This interpretation is supported by the effects of streptonigrin on topoisomerase II (*25*). This antitumor agent exhibits unique sequence specificity of topoisomerase II DNA cleavage, since it requires the dinucleotide 5′-TA-3′ from +2 to +3 positions at the DNA cleavage site. Streptonigrin does not intercalate into DNA, but has DNA binding properties resembling those of minor groove binders (*25*).

With few exceptions, known intercalators effective as antitumor agents (e.g., anthracyclines, anthracenediones, actinomycin D, and amsacrines) also have features of

external binders, since the intercalation of the planar chromophore is accompanied by groove binding of a part of the molecule. It should be emphasized that the external binding moiety of the drug molecule is a crucial determinant of antitumor efficacy. Other intercalating agents (saintopin, intoplicine, actinomycin D) are dual topoisomerase I and II inhibitors (*15,26–29*), although the structural requirements for topoisomerase I and topoisomerase II are different. Lack of a correlation between activity in stimulation of topoisomerase I DNA cleavage and DNA binding affinity of indolocarbazole derivatives suggests that the mode of binding rather than intercalation is critical for activity (*30*). An interesting feature of intercalator topoisomerase I inhibitors (e.g., 3′-morpholinyl-doxorubicin, actinomycin D) is their preferential inhibition of ribosomal gene transcription (*31*) compared to pure topoisomerase II inhibitors (i.e., doxorubicin). Such a finding suggests a relationship between topoisomerase I inhibition and ribosomal RNA synthesis, and is consistent with an increased cytotoxic potency and a low therapeutic index of these agents.

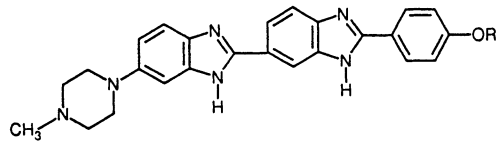
4. NONCOVALENT DNA MINOR GROOVE BINDERS

Among DNA-interactive drugs, nonintercalating minor groove binders have been most extensively studied for the sequence specificity in DNA binding (*1*). They include agents with broad-spectrum biological activity. Detailed aspects of their biochemical and pharmacological action have been extensively reviewed (*1*) and are not the subject of this chapter. The best studied of these agents are the antiviral antibiotics, distamycin and netropsin, which are known to bind noncovalently to the minor groove with AT preference and to cause widening of the minor groove (*4*). Although incorrectly referred to as antitumor agents, distamycin and noncovalent binders of this group have low cytotoxic potency and negligible antitumor activity. Despite their physical interaction with DNA that causes reversible inhibition of DNA functions, they do not induce a persistent DNA damage, and therefore, they do not have cytotoxic activity. Indeed, among noncovalent minor groove binders, only DNA cleaving agents and topoisomerase I inhibitors show a significant antitumor activity.

Enediyne antibiotics (neocarzinostatin, esperamicin A1, and calicheamicin γ1) are typical examples of cytotoxic drugs with high DNA cleaving ability in the minor groove (*32*). DNA-damaging properties of enediynes have been related to formation of diradical species (*32*). A very potent antitumor antibiotic, C-1027, containing a novel enediyne chromophore has been described (*33*). The compound exhibits high and specific DNA-cleaving ability even in the absence of thiols (*34*). An interesting observation is that in calicheamicin γ1, the sugar residue functions as a minor groove binding element (*35*); such an interaction may be the basis of the site-specific double-strand cleavage (*36*). A similar role has been described for the amino sugar residue of elsamicin A, an agent with a bimodal mechanism of binding (intercalation and minor groove binding) that induces selective DNA cleavage (*37*).

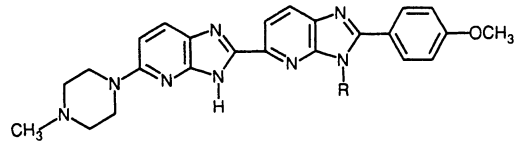
Bleomycin is a well-known glycopeptide antibiotic that exerts cytotoxic activity through oxidative DNA damage. Its binding to DNA involves a partial intercalation of the bithiazole moiety and binding in the minor groove (*38*).

An interesting group of minor groove binding agents is topoisomerase I inhibitors (*39*). In general, drugs that interact with DNA in the minor groove can inhibit the catalytic activity of DNA topoisomerases in a concentration-dependent manner and

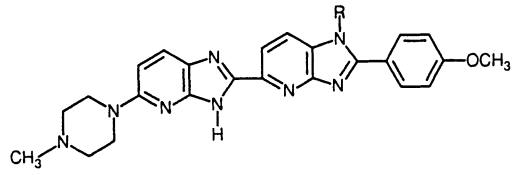


Hoechst 33342, R = OCH_2CH_3

Hoechst 33258, R = OH



1 R = $CH_2OCH_2CH_3$, $CH_2O(CH_2)_7CH_3$, $CH_2CH_2OCH_2CH_3$



2 R = CH_2OCH_3, $CH_2O(CH_2)_7CH_3$,

Fig. 1. Chemical structures of bis-benzimidazoles (Hoechst 33258 and 33342) and *N*1-alkoxyalkyl derivatives.

interfere with the stabilization of cleavable complexes by topoisomerase-targeted drugs (*40,41*). Thus, these agents act by impeding enzyme action without stabilizing DNA cleavable complexes. Since these compounds apparently inhibit DNA topoisomerase by competing with enzyme binding to DNA, Beerman et al. (*40*) proposed that the action of topoisomerases involves the minor groove. However, a number of minor groove binders (e.g., the bis-benzimidazoles, Hoechst 33342 and 33258 [Fig. 1]) were found to induce DNA cleavage in the presence of purified DNA topoisomerase I (*39*). A peculiar characteristic of single-strand DNA breaks induced by these minor groove binders is the high site specificity. The major cleavage sites have been found in AT rich regions (5′-TCATTTTT-3′ with cleavage occurring between T and C), thus suggesting that the T track is a potential binding site of these bis-benzimidazoles, which are indeed highly AT-selective. It has been proposed that this effect could be related to the drug's ability to alter DNA bending, thus preventing ligation of the transiently broken DNA ends (*15*). This interpretation is consistent with the ability of bulgarein to induce topoisomerase I-mediated DNA cleavage and to induce positive DNA supercoils (*42*). The DNA winding produced by low-mol-wt compounds is known only for AT-specific minor groove binders. Topoisomerase I has been involved in the regula-

tion of transcription, since the enzyme is associated with the general transcription factor, TATA box binding protein (TBP) (*43*). The factor has been documented to interact with DNA in the minor groove and share the same sequence preference of minor groove binders (*44*). In addition to a direct effect of minor groove binders as inhibitors of topoisomerase I, the agents are also effective inhibitors of the formation of the DNA/TBP complex (*45*). The structural aspects of minor groove drug–DNA complexes were recently examined by Neidle (*46*), who, on the basis of crystallographic studies, provided a detailed picture of the recognition processes involved. The structural studies emphasized, in addition to electrostatic interactions and hydrogen bonds, the hydrophobic interactions required to maintain the drug in an optimal position within the minor groove.

The pharmacological implications of the specific effects of minor groove binders on topoisomerase I remain to be established. The synthesis and preliminary biological evaluation of minor groove-selective *N*1-alkoxyalkyl-bis-benzimidazoles have been described (*47*) (Fig. 1). A correlation was found between DNA binding and cytotoxic potency. In particular, one of the derivatives with the inward-directed substituent exhibited a 15-fold increased cytotoxic potency compared to Hoechst 33258. Since the compounds may also have inhibitory effects on the catalytic activity of topoisomerases (*41*), the molecular basis of cytotoxicity remains to be defined. Only limited information is available on the cytotoxic and antitumor activity of the bis-benzimidazole derivatives. A selective cytotoxicity of Hoechst 33258 in human melanoma cells has been reported (*48*). The drug effect has been related to inhibition of transcription of specific genes. A plausible explanation for the cell type-specific effects of Hoecst 33258 is that tumor cell lines may differ in drug accessibility of certain AT-rich sequences. The low potency and high toxicity of Hoechst 33258 have precluded clinical evaluation. However, the more lipophylic Hoechst 33342 has been reported to be a potent cytotoxic agent (*49*). Detailed cytotoxicity studies were performed with Hoechst 33342, which exhibits an enhanced membrane permeability compared to the parent compound Hoechst 33258. The pattern of cell response to the former agent was different from that of camptothecin, since it was found less cytotoxic in cell lines expressing multidrug resistance, but only moderately crossresistant in camptothecin-resistant cells, which express a mutant form of topoisomerase I. The aforementioned studies support the potential interest of Hoechst 33342 in cancer chemotherapy.

Other DNA-interacting agents (including mithramycin, chromomycin A3, and nogalamycin) have been reported to stimulate topoisomerase I-mediated DNA cleavage (*15*). Again, such agents are known to bind to the minor groove or to have a moiety located in the minor groove, but with a different sequence specificity (i.e., GC-rich regions) (*50–54*). Although they are well-known cytotoxic agents, only a few have proven useful in clinical therapy. Thus, all DNA binding agents that are known to stimulate topoisomerase I-mediated DNA cleavage have a DNA minor groove binding mode (*15*). The drug interactions with DNA–enzyme in the ternary complex required for an effective stabilization of the cleavage complex have not yet been established. It is likely that minor groove binding is a critical determinant of sequence specificity of these "information-reading" molecules in inducing enzyme-mediated DNA damage.

The DNA sequence-recognizing properties of minor groove binders have suggested their use as carriers for delivering DNA-damaging moieties to specific sequences in DNA. In such approaches (aimed to functionalize minor groove binders), reactive

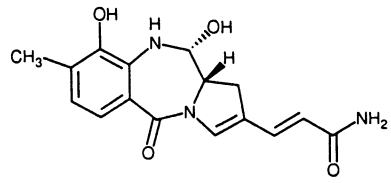
(alkylating/crosslinking) groups or intercalating chromophores have been used. Several minor groove binder–intercalator hybrid molecules (named combilexins) have been developed (*55–57*). An enhanced DNA binding affinity and selectivity, and an interference with DNA topoisomerases at specific DNA sites are the potential advantages of these hybrid molecules. The molecular interactions with DNA and biological activity of a netropsin–acridine hybrid have been described (*57*). The hybrid compound retains its ability to intercalate into DNA, and exhibits lower cytotoxic and antitumor activities than the reference compound *m*-AMSA.

Based on these preliminary observations, other hybrid molecules that combine minor groove ligands and more effective intercalating agents have been synthesized. The most interesting compounds of this category appear to be the distamycin analogs. The DNA binding properties of a series of distamycin–ellipticine hybrid molecules have been extensively studied (*55,56*). One of these agents, Distel (1+), was found to have a bimodal mechanism of interaction with DNA (i.e., intercalation of the ellepticin chromophore and binding of distamycin in the minor groove), without any apparent sequence specificity (*55*). Molecular modeling studies have suggested that the lack of sequence-selective interaction is related to the lack of a positively charged side chain on the distamycin moiety. Based on these theoretical predictions, a biscationic hybrid, Distel (2+), has been synthesized to study it interaction with DNA (*56*). The introduction of a second positive charge resulted in a highly sequence-specific, DNA-reading agent. Psoralen (or coumarin) conjugates of minor groove binding AT-selective pyrrole-, or GC-selective imidazole-containing analogs of netropsin have been described (*58*). The linking of psoralen to the AT-selective, pyrrole-containing analogs of netropsin produced a photoactive agent more potent than the corresponding imidazole compound and the 8-methoxy psoralen itself, thus documenting that the minor groove ligand had targeted the photosensitizing agent to cellular DNA. It is noteworthy that in the case of the distamycin–ellepticine hybrid molecule, no direct correlations between DNA binding and cytotoxicity could be found (*55*). The hybrid compound is less cytotoxic than the ellipticine derivative used for conjugation. Such biological effects were interpreted in terms of unfavorable cellular pharmacokinetics of the hybrid compound as a consequence of the hydrophilic nature of distamycin residue. The poor drug uptake may thus represent a relevant limitation in the use of oligopeptide analogs for the development of hybrid antitumor agents.

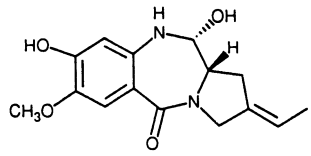
5. MINOR GROOVE ALKYLATING AGENTS

Mitomycin C is a well-known antitumor antibiotic currently used in clinical cancer chemotherapy. Its cytotoxic action is related to its ability to bind covalently to minor groove DNA, resulting in monofunctional and bifunctional adducts. These processes require reductive activation of the quinone system of the drug and involve exclusively N2 positions of guanines (*59*). A specific DNA sequence recognition by mitomycin C for crosslink formation has been described. A 4-bp sequence preference by the crosslink produced by mitomycin is the result of absolute specificity for CG.

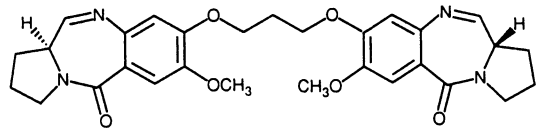
Another example of antitumor agents with minor groove GC sequence selectivity is anthramycin (*6*), which is a member of the pyrrole [1,4]-benzodiazepine group (Fig. 2). In spite of their low molecular weight, anthramycin and tomaymycin have shown a surprising degree of sequence selectivity with preference for adenine flanking the



Anthramycin



Tomaymycin



DSB - 120

Fig. 2. Pyrrolo[1,4]benzodiazepine antibiotics (anthramycin and tomaymycin) and dimer of anthramycin (DSB 120).

alkylated guanine (*60*). Crystal structure analysis of a covalent DNA-drug adduct provides insights to understand the molecular basis for sequence specificity in benzodiazepine antibiotics (*61*). This preference is related to a netropsin-like fitting of the acrylamide tail into the minor groove.

In an attempt to find more useful DNA-reading drugs, an interesting development of GC sequence-directed alkylating agents is the synthesis of a C8-linked dimer of anthramycin, with a flexible -0-$(CH_2)_3$-0- linker (DSB120) (*62*) (Fig. 2). The dimeric derivative, unlike anthramycin, forms interstrand crosslinks and exhibits a marked cytotoxicity in tumor cell lines. The compound binds to a 6-bp sequence (5 '-PuGATCPyr). DNA binding, interstrand crosslinking efficiency, and cytotoxic potency are dependent on the linker chain length, and compounds with even numbers of methylene groups show reduced effectiveness. The observation suggests that, in the design of effective DNA-groove binder alkylators, an optimal size and geometry of the linker are required to ensure that the reactive moiety is in optimal orientation with respect to the target DNA sequence (*63*).

Bifunctional alkylating agents are the first drugs proven to have clinical antitumor efficacy. They are still among the most widely used drugs, with a broad range of activity for the treatment of human tumors. The agents have a limited capacity to read sequence information (*see* Section 2.). In an attempt to increase sequence specificity and

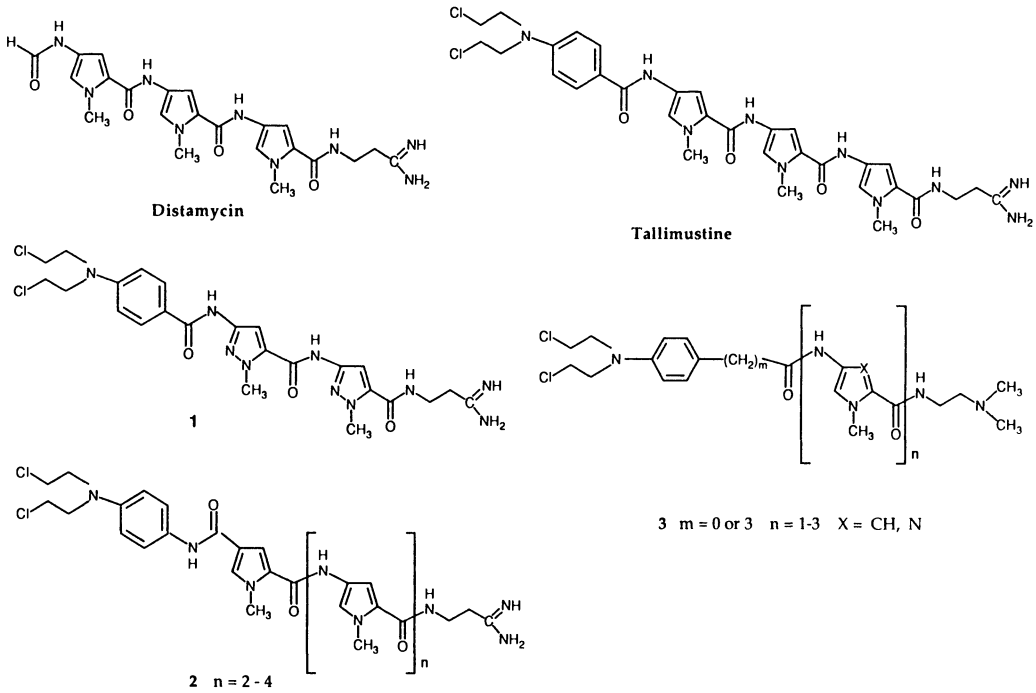


Fig. 3. Minor groove selective alkylating agents related to distamycin A.

to alter the covalent modification of DNA of alkylating/crosslinking agents, several minor groove-directed alkylators have been developed as potential antitumor agents (*64*). In such approaches, the naturally occurring pyrrolic oligopeptides, netropsin and distamycin, or their oligoimidazole analogs were used as a sequence-selective minor groove vectors. One of the most relevant observations in different series of DNA targeted alkylating agents is that the introduction of alkylating functions in minor groove binders results in a substantial increase in cytotoxic potency as compared to the carrier DNA binder itself (*65*). A similar finding was reported for 4-anilino-quinoline-based, minor groove-directed aniline mustards (*66*). Unfortunately, the latter compounds show a negligible in vivo antitumor activity, presumably related to their poor aqueous solubility.

Again, the most studied (and interesting) compounds appear to be the distamycin analogs (Fig. 3). Among recently developed alkylating derivatives of distamycin, a very interesting compound, tallimustine (FCE 24517), has been identified and is being tested in early clinical studies. The derivative was obtained by incorporation of a benzoyl mustard moiety at the N-terminus (*67*). As expected, the compound was found to react covalently with DNA. Whereas distamycin has a low cytotoxic activity and no significant antitumor efficacy, the alkylating derivative exhibits potent cytotoxic activity comparable or superior to that of melphalan, a structurally related alkylating agent (*68*). The compound was not crossresistant with melphalan in a subline of L1210 leukemia selected for resistance to melphalan, but was crossresistant with doxorubicin in a cell line (LoVo/DX) expressing a typical MDR phenotype. Tallimustine exhibits a broad spectrum of antitumor activity against a variety of murine and human solid tumor models, including human melanoma (M14) and small-cell lung carcinoma

(N592). An unexpected finding of in vivo evaluation of tallimustine was a marginal activity against conventional murine leukemia models (P388, L1210). The observation suggests that the pattern of tumor response is not related to the rate of tumor cell proliferation as observed for the conventional alkylating agents and supports a cell-type-specific cytotoxic effect of minor groove alkylators, presumably related to sequence-specific covalent modification of DNA and/or cell type-dependent drug accessibility to critical genes. This interpretation is supported by the toxicity profile observed in a clinical Phase I study of tallimustine (*69*). Drug treatment causes a highly selective and short-lasting neutropenia, but marginal thrombocytopenia. The pattern of hematological toxicity is peculiar, since treatment was conventional alkylating agents results in both neutropenia and thrombocytopenia.

Distamycin derivatives containing different pyrrole rings have been recently developed (*70*) (Fig. 3, *see* compounds 2). Among new compounds, preliminary preclinical evaluation suggests that MEN 10710, which is a synthetic derivative of distamycin possessing four pyrrole rings in which the bis-(2-chloroethyl)-aminophenyl moiety is linked to the oligopyrrole backbone by the flexible butanamido chain (*71*), is pharmacologically interesting. The molecular mechanism of DNA interaction, investigated by footprinting experiments and by in vitro DNA interstrand-induced cross-linking, indicated a remarkable difference from tallimustine. Cytotoxic potency was increased by 10- to 50-fold compared to melphalan or tallimustine in human tumor cell lines. In vivo experiments indicated a significant antitumor efficacy against human tumor xenografts, including an ovarian cisplatin-resistant (A2780/DDP) tumor line. In vitro studies also showed a reduced myelotoxicity compared to tallimustine, suggesting a potential degree of selectivity against tumor cells with respect to normal cells.

Since polypyrrole compounds (i.e., distamycin and netropsin) may be susceptible to oxidative breakdown, bispyrazole compounds were prepared as potentially more stable minor groove binding analogs (*72*) (Fig. 3, compound 1). Although the compounds bind less strongly to DNA and show lower specificity for AT-rich sequences than distamycin, a benzoic acid mustard derivative has a cytotoxic potency comparable to that of the alkylating distamycin analog. A negligible antileukemic activity of the bispyrazole analog was reported in the treatment of P388 murine leukemia. Unfortunately, no solid tumors were used in the preliminary evaluation. Since alkylating derivatives of distamycin exhibit a marginal effectiveness against leukemia systems (*68*), no definitive conclusions could be drawn on the pharmacological interest of the bispyrazole derivatives.

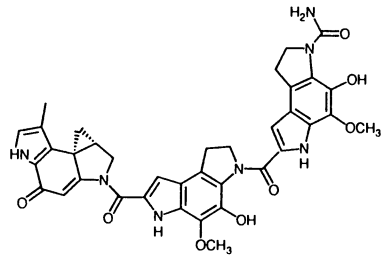
The increased cytotoxic potency of minor groove binders following incorporation of a reactive moiety is not related to the extent of DNA alkylation. For example, tallimustine is known to alkylate a very limited number of adenines at the N-3 position (*73*). In spite of the presence of a typical alkylating moiety, tallimustine does not produce detectable covalent links with N-7 guanine, which is the major site of alkylation for conventional bifunctional alkylating agents (*6*). It appears that the site of alkylation is dependent on the mode of DNA interaction with the polypyrrole chain. This interpretation is supported by a similar mode of action reported for C-1065, the first compound of the minor groove alkylators, that exhibits a somewhat similar sequence recognition (*6,64*). The sequence selectivity of adenine adduct formation by tallimustine has been recently identified as 5′-TTTTGA-3′ (*74*). A single base modification in the hexamer completely abolishes the adenine alkylation. Such a sequence

specificity is unexpected for a low-mol-wt agent and could account for the limited extent of alkylation found in cellular DNA.

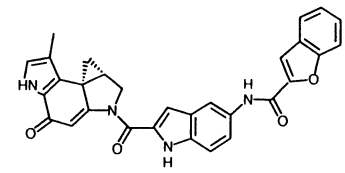
Several efforts have been made to modify DNA sequence selectivity of the distamycin/netropsin molecule. Structural modifications have been based on replacement of *N*-methylpyrrole by an *N*-methylimidazole to accommodate hydrogen bonding to the 2-amino group of guanine (*75*). Thus, a novel class of minor groove binding molecules (termed "lexitropsin" to identify information-reading oligopeptides) has been developed. A degree of GC recognition has been found in this series of compounds, but this is associated with a reduction of DNA binding affinity, presumably related to a marked sequence dependence of minor groove width (*46*). Recently, a GC sequence-specific recognition has been reported for an *N*-formamido C-terminus-modified lexitropin (*76*), which represents a new carrier molecule for the development of minor groove alkylators with a different sequence specificity. Alkylating lexitropsins have been used to document groove- and sequence-selective adduct formation by small-mol-wt alkylating agents, thereby providing an insight into the sequence and orientation preferences of the groove binder (*77*). Imidazole-containing analogs of distamycin were used as vectors for delivery of a variety of alkylating agents to GC-rich sequences (*78,79*). The most representative compounds of this series are shown in Fig. 3 (scheme 3). In an attempt to identify compounds with GC sequence selectivity and increased efficacy, oligoimidazolecarboxamido analogs of distamycin, wherein the N-terminus contains either a benzyl-mustard or a chloroambucil moiety, were prepared (*79*). The pattern of DNA alkylation was markedly different from that of other minor groove binder benzoyl mustards, since the former compounds were found to alkylate guanine-N7 in the major groove and to form crosslinks (*79*). In contrast, benzoyl mustards do not alkylate guanine N-7, but produce highly cytotoxic monoalkylation. Lack of a correlation between the mode of drug binding to DNA (hence the extent of lesions) and cytotoxic potency suggests that comparable cytotoxic potency could be achieved through different mechanisms.

An interesting series of sequence-selective minor groove binders are cyclopropylpirroloindole antitumor antibiotics with unique features (*64*) (Fig. 4). The parent compound of the series is CC-1065, a fermentation product of *Streptomyces zelensis* containing two identical benzopyrrole units and an indolequinone system bearing a reactive cyclopropane ring. CC-1065 binds covalently and noncovalently in the minor groove of AT-rich regions of double-stranded DNA. The covalently bound species results from an alkylation reaction between the cyclopropyl group and N-3 of adenine. A sequence-selective alkylation was observed, with a reactive adenine located inside a 5-bp consensus, i.e., 5′AAAAA and 5′PyNTTA (*N* = any nucleotide) (*80–82*). Preclinical studies indicated that CC-1065 is one of the most potent cytotoxic agents ever found, with activity at nanomolar levels (*83*). However, it was not developed as a clinical candidate because of delayed and irreversible toxicity in preclinical studies (*84*).

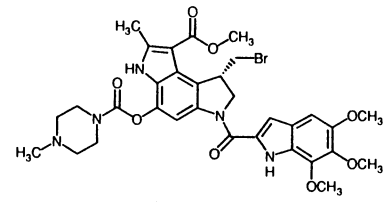
The unique molecular and pharmacological features of CC-1065 led to a marked effort in the chemical modification of parent compound. Among a number of analogs, the first identified as a candidate for clinical development was adozelesin (U-73975) (*85*). The analog exhibits a similar mechanism of action, with a sequence-selective interaction overlapping at least in part with the parent compound (*80*). Adozelesin is characterized by a high potency and a promising profile of preclinical activity against murine and human tumor models, but devoided of delayed or irreversible toxicity of the parent compound (*64*). Phase I studies of adozelesin have been presented using



(+) - CC-1065



Adozelesin (U - 73975)



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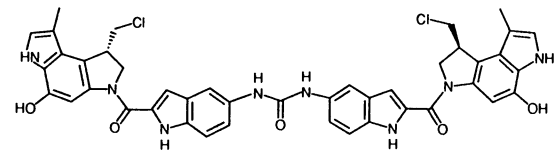


Bizelesin (U-77779)

Fig. 4. Chemical structures of CC-1065 and cyclopropyl pyrroleindole analogs.

different iv infusion schedules (*85,86*). The maximum tolerated dose in a 3-wk schedule of brief iv infusion was 188 $\mu g/m^2$, thus documenting the potency expected on the basis of preclinical studies (*85*). The dose-limiting toxicity was myelosuppression with thrombocytopenia and leukopenia. The toxicity profile was similar, but not identical to that of other minor groove alkylators (*69*).

A possible drawback of adozelesin is its pharmacokinetic behavior characterized by a short half-life. In an attempt to modulate the pharmacologic properties, synthetic efforts were directed to the preparation of prodrugs in this series. A very promising compound is carzelesin (U-80244). Although less potent than adozelesin, it possesses a superior efficacy in preclinical evaluation and a more favorable pharmacological behavior (*87*). As found in other minor groove reactive agents (*79*), CC-1065 and adozelesin are monoalkylating agents. Based on the observation that clinically useful alkylators are crosslinking agents, cyclopropylpyrrolondole dimers were designed in an attempt to cause DNA damage in a bifunctional manner. Bizelesin (U-77779) emerged from this effort. Bizelesin produces stable interstrand crosslinks with an increased sequence selectivity (*81,88*). The dimeric bisalkylator derivative inhibits growth of human tumor cells at picomolar levels (*89*), thus supporting the view that crosslinks are more cytotoxic lesions than monoadducts. On the basis of its potency and good therapeutic index, bizelesin has been chosen for preclinical development (*90*). A comparative study of DNA binding properties and cytotoxic activity of simplified or extended nonalkylating oligomers of the dihydropyrroloindole ring structure of CC-1065 defined the structural features that contribute to noncovalent DNA minor groove binding, thus identifying the optimal binding unit (*91*). An interesting observation of the study was a correlation between cytotoxic activity of these oligomers and their molecular size. Although less potent than the alkylating parent compounds, these oligomers were found to be markedly more cytotoxic than other AT-selective, noncovalent minor groove binders (distamycin and netropsin). The finding

is consistent with a critical contribution of the noncovalent complex in determining the pharmacological activity of the cyclopropylpyrroloindole drugs.

Duacarmycins are a novel antitumor antibiotic structurally related to CC-1065 (*92*). Duacarmycin A contains a reactive cyclopropane ring responsible for adenine alkylation at the 3′-end of sequences of three or more consecutive A or T. KW-2189, a synthetic analog of duacarmycin B2, has been selected for development on the basis of its promising pharmacological profile (*93*). The derivative is considered a prodrug; indeed, although its cytotoxic potency is substantially reduced, it is more effective than the parent compound against experimental tumor models, including drug-resistant human tumors.

On the basis of the mode of interaction with DNA, minor groove binders with alkylating functions should be considered as a novel class of alkylating agents, with a promising preclinical activity. Their clinical efficacy remans to be documented. In contrast to bifunctional alkylating agents (known to interact with N7 or O6 of guanines and cause crosslinks as typlical cytotoxic lesions), minor groove alkylators interact with the N3 of adenines with formation of a mono-adduct. It is likely that this mode of DNA interaction is related to the location of the drug in the minor groove before alkylation (*74*). In contrast to conventional alkylating agents (which cause selective inhibition of DNA synthesis), no correlation has been found between inhibition of DNA synthesis and cytotoxicity of adozelesin (*94*) or tallimustine (*73*). The inhibition of DNA synthesis is a delayed effect that reflects an arrest in G2 phase (*73*). Although the alkylation of DNA is considered as the primary lesion, the detailed mechanism of the cytotoxic action thus remains to be elucidated. A stimulating hypothesis is that the drug-induced DNA damage (caused by covalent binding) in consensus sequences for the binding of transcription factors may result in persistent impairment of the regulatory mechanisms of transcription of specific genes (*7,46*). A number of observations support this possibility. For example, distamycin A and its alkylating derivative inhibit the binding of transcription factors that recognize AT-rich consensus sequences in vitro (*95*). A similar observation has been reported for some synthetic *N*-methylpiperazine derivatives (Hoechst 33258 and 33342) (*48*). Minor groove binding drugs are effective inhibitors of formation of the TBP/DNA complex (*45*). CC-1065, which binds covalently to the DNA, is more effective than the noncovalent binder distamycin A. It is conceivable that the irreversible interaction of the alkylating derivatives may produce a persistent and more effective inhibition of transcription. TFIID, a member of a group of general transcription factors, plays a key role in the initiation step of transcription by RNA polymerase II (*44*). If the primary mechansim of cell toxicity of minor groove alkylators is related to inhibition of transcription through interference with these regulatory proteins, the mode of actions could not be regarded as tumor cell-specific. Indeed, the cytotoxic potency of these agents is also reflected by their in vivo potency. Thus, the therapeutic index is expected to be a critical pharmacological determinant of the clinical usefulness of the minor groove alkylators.

6. CONCLUSIONS

Most of currently used antitumor agents proven to be clinically effective were discovered through empirical approaches, including random screening of natural and synthetic compounds or systematic chemical maniputations of active agents (i.e., analog development). The identification of their preclinical efficacy led to therapeutic

application long before the mechanistic basis of the antitumor effects was understood. Progress in molecular pharmacology of DNA-damaging agents and in molecular biology of tumor cells (oncogene activation, gene regulation, signal transduction) has provided a more rational basis for new drug discovery by exploiting specific macromolecular targets for pharmacological intervention (*96*).

Groove binding drugs exhibit a striking sequence specificity in DNA interaction and DNA damage (*7,46*). However, it is evident that the recognition potential of available DNA-interacting agents is not sufficient to provide selective cytotoxicity against tumor cells. Although it appears that DNA lesions produced by DNA-reactive agents are not randomly distributed in the entire genome (*10*), many of these lesions are probably not required for therapeutic activity. Indeed, cell-specific effects may not be related to the bulk DNA damage (*97*). Studies performed with tallimustine at the molecular level indicate that a low level of DNA damage sustained at relevant sites of the genome may mediate drug action. Unfortunately, although malignant transformation is known to involve a stepwise accumulation of specific alterations in genes normally regulating growth stimulatory or inhibitory pathways, the critical genes and therefore the DNA sequences most appropriate as useful targets for tumor-specific effects remain to be identified (*98*). It is possible that efforts directed to the identification of gene-specific sites of DNA damage may provide insight for a better understanding of the molecular/cellular basis of the relative tumor selectivity of available effective antitumor agents. A critical aspect of this molecular pharmacology approach concerns the cellular implications of sequence specificity of drug-induced DNA interaction (and DNA damage) observed in vitro with isolated DNA or oligonucleotide models. Drug interaction is dependent not only on specific sequence, but also on target accessibility and DNA conformation in chromatin. For example, in the presence of the minor groove binder, distamycin A, duocarmycin A induces alkylation of guanine residues that are usually not alkylated by duocarmycin A alone (*99*). DNA alkylation sites induced by adozelesin and bizelesin in human colon carcinoma cells were mapped in individual genes at the single-nucleotide level (*100*). The pattern of alkylation induced by these drugs is similar, but not identical to that observed in isolated cell-free DNA. These findings indicated that drug reaction with DNA may be critically influenced by DNA conformation in chromatin.

Among known DNA-interacting compounds, only DNA-damaging drugs have a significant antitumor activity. These agents cause different types of DNA lesions depending on the mode of DNA interactions. It is conceivable that their antitumor efficacy is related to the type of drug-induced lesions and to the persistence of DNA damage. A critical question for the identification of more specific and effective DNA-damaging agents concerns the role played by the sequence specificity and, therefore, the genomic localization of DNA damage. It is conceivable that the cytotoxic and antitumor potency is dependent on the site of drug-induced DNA lesions and the tumor-specific effects of known DNA-kamaging agents are the result of selective DNA damage to critical genes presumably relevant for growth and/or survival (*10*). Of course, the introduction of a DNA-damaging moiety in more specific DNA-recognizing elements (e.g., oligonucleotides as major groove binders or oligopeptides as minor groove binders) (*101*) is expected to cause DNA damage at selected genomic sites. However, the exploitation of these highly selective molecules for drug design is still hindered by several chemical and pharmacological problems, including stability of the carriers, poor cellular penetration, and difficulties of synthesis. The available

sequence-selective agents can be viewed as structural models for further development of drugs designed to interfere with DNA sites recognized by specific regulatory proteins. The continued development of molecular structural principles in the design process will also provide an increased understanding of the mechanism of tumor inhibition, thus leading to the synthesis of more effective antitumor agents (*46*).

ACKNOWLEDGMENTS

We thank B. Gatto for her critical reading of the manuscript and Laura Zanesi for careful preparation of the manuscript. We gratefully acknowledge grant supports from Associazione Italiana per la Ricerca sul Cancro (Milan), from Progetto Finalizzato ACRO, Consiglio Nazionale delle Ricerche (Rome) and Ministro della Sanita', Italy.

REFERENCES

1. Zimmer C, Wähnert U. Nonintercalating DNA-binding ligands: specificity of the interaction and their use as tools in biophysical, biochemical and biological investigations of the genetic material. *Prog Biophys Mol Biol* 1986; 47:31-112.
2. Hurley LH, Boyd FL. Approaches towards the design of sequence specific drugs for DNA. *Annu Rep Med Chem* 1987; 22:259-268.
3. Smets LA. Programmed cell death (apoptosis) and response to anti-cancer drugs. *Anticancer Drugs* 1994; 5:3-9.
4. Neidle S, Pearl LH, Skelly JV. DNA structure and perturbation by drug binding. *Biochem J* 1987; 243:1-13.
5. Hurley LH. DNA and associated targets for drug design. *J Med Chem* 1989; 32:2027-2033.
6. Warpehoski MA, Hurley LH. Sequence selectivity of DNA covalent modification. *Chem Res Toxicol* 1988; 1:315-333.
7. D'Incalci M, Broggini M, Hartley JA. Sequence and gene-specific drugs. In: Workman P, ed. *New Approaches in Cancer Pharmacology: Drug Design and Development.* Berlin: Springer Verlag, 1992:5-11.
8. Pullman A, Pullman B. Molecular electrostatic potential of the nucleic acids. *Q Rev Biophys* 1981; 14:289-380.
9. Farrell N, Qu Y, Feng L, Van Houten B. Comparison of the chemical reactivity, cytotoxicity, interstrand cross-linking and DNA sequence specificity of bis(platinum) complexes containing monodentate or bidentate coordination spheres with their monomeric analogues. *Biochemistry* 1990; 29:9522-9531.
10. Broggini M, D'Incalci M. Modulation of transcription factor-DNA interactions by anticancer drugs. *Anticancer Drug Design* 1994; 9:373-387.
11. Hamilton TC, Johnson SW, Godwin AK, Bookman MA, O'Dwyer PJ, Hamaguchi K, Jackson K, Ozols RF. Drug resistance in ovarian cancer and potential for its reversal. In: Sharp F, Mason P, Blackett T, Berek J, eds. *Ovarian Cancer 3.* Chapman & Hall Medical, 1994: 203-213.
12. Zhen WP, Link CJ, O'Connor PM. Increased gene-specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines. *Mol Cell Biol* 1992; 12: 3689-3698.
13. Zunino F, Capranico G. DNA topoisomerase II as the primary target of antitumor anthracyclines. *Anticancer Drug Design* 1990; 5:307-317.
14. Capranico G, Zunino F. DNA topoisomerase-trapping antitumour drugs. *Eur J Cancer* 1992; 28A:2055-2060.
15. Chen AY, Liu LF. DNA topoisomerases: essential enzymes and lethal targets. *Annu Rev Pharmacol Toxicol* 1994; 34:191-218.
16. Capranico G, De Isabella P, Penco S, Tinelli S, Zunino F. Role of DNA breakage in cytotoxicity of doxorubicin, 9-deoxydoxorubicin, and 4-demethyl-6-deoxydoxorubicin in murine leukemia P388 cells. *Cancer Res* 1989; 49:2022-2027.

17. Capranico G, Supino R, Binaschi M, Capolongo L, Grandi M, Suarato A, Zunino F. Influence of structural modifications at the 3′ and 4′ positions of doxorubicin on the drug ability to trap topoisomerase II and to overcome multidrug resistance. *Mol Pharmacol* 1994; 45: 908–915.
18. Moore MH, Hunter WN, d'Estaintot BL, Kennard O. DNA-drug interactions. The crystal structure of d(CGATCG) complexed with daunomycin. *J Mol Biol* 1989; 206:693–705.
19. Woynarowski JM, Sigmund RD, Beerman TA. DNA minor groove binding agents interfere with topoisomerase II mediated lesions induced by epipodophyllotoxin derivative VM-26 and acridine derivative m-AMSA in nuclei from L1210 cells. *Biochemistry* 1989; 28:3850–3855.
20. Fesen M, Pommier Y. Mammalian topoisomerase II activity is modulated by the DNA minor groove binder distamycin in simian virus 40 DNA. *J Biol Chem* 1989; 264:11,354–11,359.
21. Wassermann K, Markovits J, Jaxel C, Capranico G, Kohn KW, Pommier Y. Effects of morpholinyl doxorubicins, doxorubicin, and actinomycin D on mammalian DNA topoisomerase I and II. *Mol Pharmacol* 1990; 38:38–45.
22. Capranico G, Butelli E, Zunino F. Change of the sequence specificity of daunorubicin-stimulated topoisomerase II DNA cleavage by epimerization of the amino group of the sugar moiety. *Cancer Res* 1995; 55:312–317.
23. Kamitori S, Takusagawa F. Crystal structure of the 2:1 complex between d(GAAGCTTC) and the anticancer drug actinomycin D. *J Mol Biol* 1992; 225:445–456.
24. Capranico G, Palumbo M, Tinelli S, Mabilia M, Pozzan A, Zunino F. Conformational drug determinants of the sequence specificity of drug-stimulated topoisomerase II DNA cleavage. *J Mol Biol* 1994; 235:1218–1230.
25. Caprianico G, Palumbo M, Tinelli S, Zunino F. Unique sequence specificity of topoisomerase II DNA cleavage stimulation and DNA binding mode of streptonigrin. *J Biol Chem* 1994; 269:25,004–25,009.
26. Riou J-F, Fossé P, Nguyen CH, Larsen AK, Bissery M-C, Grondard L, Saucier J-M, Bisagni E, Lavelle F. Intoplicine (RP 60475) and its derivatives, a new class of antitumor agents inhibiting both topoisomerase I and II activities. *Cancer Res* 1993; 53:5987–5993.
27. Trask DK, Muller MT. Stabilization of type I topoisomerase–DNA covalent complexes by actinomycin D. *Proc Natl Acad Sci USA* 1988; 85:1417–1421.
28. Yamashita Y, Kawada SZ, Fujii N, Nakano H. Induction of mammalian DNA topoisomerase I and II mediated DNA cleavage by saintopin, a new antitumor agent from fungus. *Biochemistry* 1991; 30:5838–5845.
29. Riou JF, Helissey P, Grondard L, Giorgi-Renault S. Inhibition of eukaryotic DNA topoisomerase I and II activities by indoloquinolinedione derivatives. *Mol Pharmacol* 1991; 40: 699–706.
30. Yamashita Y, Fujii N, Murakata C, Ashizawa T, Okabe M, Nakano H. Induction of mammalian DNA topoisomerase I mediated DNA cleavage by antitumor indolocarbazole derivatives. *Biochemistry* 1992; 31:12,069–12,075.
31. Wassermann K, Newman RA, Davis FM, Mullins TD, Rose KM. Selective inhibition of human ribosomal gene transcription by the morpholinyl anthracyclines cyanomorpholinyl- and morpholinyldoxorubicin. *Cancer Res* 1988; 48:4101–4106.
32. Nicolaou KC, Smith AL, Yue EW. Chemistry and biology of natural and designed enediynes. *Proc Natl Acad Sci USA* 1993; 90:5881–5888.
33. Sugiura Y, Matsumoto T. Some characteristics of DNA strand scission by macromolecular antitumor antibiotic C-1027 containing a novel enediyne chromophore. *Biochemistry* 1993; 32:5548–5553.
34. Xu YJ, Zhen YS, Goldberg IH. C1027 chromophore, a potent new enediyne antitumor antibiotic, induces sequence-specific double-strand DNA cleavage. *Biochemistry* 1994; 33:5947–5954.
35. Hawley RC, Kiessling LL, Schreiber SL. Model of the interactions of calicheamicin gamma 1 with a DNA fragment from pBR322. *Proc Natl Acad Sci USA* 1989; 86:1105–1109.
36. Zein N, Sinha AM, McGahren WJ, Ellestad GA. Calicheamicin gamma 1I: an antitumor antibiotic that cleaves double-stranded DNA site specifically. *Science* 1988; 240:1198–1201.
37. Uesugi M, Sekida T, Matsuki S, Sugiura Y. Selective DNA cleavage by elsamicin A and switch function of its amino sugar group. *Biochemistry* 1991; 30:6711–6715.

38. Kenani A, Lohez M, Houssin R, Helbecque N, Lemay P, Hénichart JP. Chelating, DNA-binding and DNA-cleaving properties of a bleomycin synthetic model. *Anticancer Drug Design* 1987; 2:47-59.
39. Chen AY, Yu C, Gatto B, Liu LF. DNA minor groove-binding ligands: a different class of mammalian DNA topoisomerase I inhibitors. *Proc Natl Acad Sci USA* 1993; 90:8131-8135.
40. Beerman TA, Woynarowski JM, Sigmund RD, Gawron LS, Rao KE, Lown JW. Netropsin and bis-netropsin analogs as inhibitors of the catalytic activity of mammalian DNA topoisomerase II and topoisomerase cleavable complexes. *Biochim Biophys Acta* 1991; 1090:52-60.
41. Beerman TA, McHugh MM, Sigmund R, Lown JW, Rao KE, Bathini Y. Effects of analogs of the DNA minor groove binder Hoechst 33258 on topoisomerase II and I mediated activities. *Biochim. Biophys Acta* 1992; 1131:53-61.
42. Fujii N, Yamashita Y, Saitoh Y, Nakano H. Induction of mammalian DNA topoisomerase I-mediated DNA cleavage and DNA winding by bulgarein. *J Biol Chem* 1993; 268:13,160-13,165.
43. Merino A, Madden KR, Lane WS, Champoux JJ, Reinberg D. DNA topoisomerase I is involved in both repression and activation of transcription. *Nature* 1993; 365:227-232.
44. Lee DK, Horikoshi M, Roeder RG. Interaction of TFIID in the minor groove of the TATA element. *Cell* 1991; 67:1241-1250.
45. Chiang S-Y, Welch J, Rauscher FJ, Beerman TA. Effects of minor groove binding drugs on the interaction of TATA box binding protein and TFIIA with DNA. *Biochemistry* 1994; 33: 7033-7040.
46. Neidle S. Principles in the design of DNA-interactive molecules. In: Workman P, ed. *New Approaches in Cancer Pharmacology: Drug Design and Development,* vol. II. Heidelberg: Springer Verlag, 1994:9-22.
47. Wang H, Gupta R, Lown JW. Synthesis, DNA binding, sequence preference and biological evaluation of minor groove-selective N1-alkoxyalkyl-bis-benzimidazoles. *Anticancer Drug Design* 1994; 9:153-180.
48. Wong SSC, Sturm RA, Michel J, Zhang X-M, Danoy PAC, McGregor K, Jacobs JJ, Kaushal A, Dong Y, Dunn IS, Parsons PG. Transcriptional regulation of differentiation, selective toxicity and ATGCAAAT binding of bisbenzimidazole derivatives in human melanoma cells. *Biochem Pharmacol* 1994; 47:827-837.
49. Chen AY, Y C, Bodley A, Peng LF, Liu LF. A new mammalian DNA topoisomerase I poison Hoechst 33342: cytotoxicity and drug resistance in human cell cultures. *Cancer Res* 1993; 53: 1332-1337.
50. Kam M, Shafer RH, Berman E. Solution conformation of the antitumor antibiotic chromomycin A3 determined by two-dimensional NMR spectroscopy. *Biochemistry* 1988; 27:3581-3588.
51. Fox KR, Cons BM. Interaction of mithramycin with DNA fragments complexed with nucleosome core particles: comparison with distamycin and echinomycin. *Biochemistry* 1993; 32: 7162-7171.
52. Silva DJ, Goodnow R Jr, Kahne D. The sugars in chromomycin A3 stabilize the Mg(2+)-dimer complex. *Biochemistry* 1993; 32:463-471.
53. Gao XL, Mirau P, Patel DJ. Structure refinement of the chromomycin dimer-DNA oligomer complex in solution. *J Mol Biol* 1992; 223:259-279.
54. van Houte LP, van Garderen CJ, Patel DJ. The antitumor drug nogalamycin forms two different intercalation complexes with d(GCGT).d(ACGC). *Biochemistry* 1993; 32:1667-1674.
55. Bailly C, Leclere V, Pommery N, Colson P, Houssier C, Rivalle C, Bisagni E, Hénichart J-P. Binding to DNA, cellular uptake and biological activity of a distamycin-ellipticine hybrid molecule. *Anticancer Drug Design* 1993; 8:145-164.
56. Bailly C, Michaux C, Colson P, Houssier C, Sun J-S, Garestier T, Hélène C, Hénichart J-P, Rivalle C, Bisagni E, Waring MJ. Reaction of a biscationic distamycin-ellipticine hybrid ligand with DNA. Mode and sequence specificity of binding. *Biochemistry* 1994; 33:15,348-15,364.
57. Bailly C, Collyn-D'Hooghe M, Lantoine D, Fournier C, Hecquet B, Fosse P, Saucier J-M, Colson P, Houssier C, Hénichart J-P. Biological activity and molecular interaction of a netropsin-acridine hybrid ligand with chromatin and topoisomerase II. *Biochem Pharmacol* 1992; 43:457-466.
58. Hartley JA, McAdam SR, Das S, Roldan MC, Haskell MK, Lee M. Molecular and cellular pharmacology of novel photoactive psoralen and coumarin conjugates of pyrrole- and imidazole-containing analogues of netropsin. *Anticancer Drug Design* 1994; 9:181-197.

59. Borowy-Borowski H, Lipman R, Tomasz M. Recognition between mitomycin C and specific DNA sequences for cross-link formation. *Biochemistry* 1990; 29:2999-3006.
60. Kizu R, Draves PH, Hurley LH. Correlation of DNA sequence specificity of anthramycin and tomaymycin with reaction kinetics and bending of DNA. *Biochemistry* 1993; 32:8712-8722.
61. Kopka ML, Goodsell DS, Baikalov I, Grzeskowiak K, Cascio D, Dickerson RE. Crystal structure of a covalent DNA-drug adduct: anthramycin bound to C-C-A-A-C-G-T-T-G-G and a molecular explanation of specificity. *Biochemistry* 1994; 33:13,593-13,610.
62. Subhas Bose D, Thompson AS, Ching J, Hartley JA, Berardini MD, Jenkins TC, Neidle S, Hurley LH, Thurston DE. Rational design of a highly efficient non-reversible DNA interstrand cross-linking agent based on the pyrrolobenzodiazepine ring system. *J Am Chem Soc* 1992; 114:4939-4941.
63. Wang JJ, Hill GC, Hurley LH. Template-directed design of a DNA-DNA cross-linker based upon a bis-tomaymycin-duplex adduct. *J Med Chem* 1992; 35:2995-3002.
64. Wierenga W. Sequence-selective DNA-interactive antitumor agents. *Drugs Future* 1991; 16: 741-750.
65. Krowicki K, Balzarini J, De Clercq E, Newman RA, Lown JW. Novel DNA groove binding alkylators: design, synthesis, and biological evaluation. *J Med Chem* 1988; 31:341-345.
66. Gravatt GL, Baguley BC, Wilson WR, Denny WA. DNA-directed alkylating agents. 4. 4-anilinoquinoline-based minor groove directed aniline mustards. *J Med Chem* 1991; 34:1552-1560.
67. Arcamone FM, Animati F, Barbieri B, Configliacchi E, D'Alessio R, Geroni C, Giuliani FC, Lazzari E, Menozzi M, Mongelli N, Penco S, Verini MA. Synthesis, DNA-binding properties, and antitumor activity of novel distamycin derivatives. *J Med Chem* 1989; 32:774-778.
68. Pezzoni G, Grandi M, Biasoli G, Capolongo L, Ballinari D, Giuliani FC, Barbieri B, Pastori A, Pesenti E, Mongelli N, Spreafico F. Biological profile of FCE 24517, a novel benzoyl mustard analogue of distamycin A. *Br J Cancer* 1991; 64:1047-1050.
69. Sessa C, Pagain O, Zurlo MG, de Jong J, Hofmann C, Lassus M, Marrari P, Benedetti MS, Cavalli F. Phase I study of the novel distamycin derivative tallimustine (FCE 24517). *Ann Oncol* 1994; 5:901-907.
70. Animati F, Arcamone F, Bigioni M. DNA sequence selectivity of novel distamycin analogues exhibiting remarkable cytotoxic activity. 8th NCI-EORTC Symposium on New Drugs in Cancer Therapy. Amsterdam, March 15-18, 1994.
71. Bigioni M, Ciucci A, Palma C. Biologicl profile of MEN 10710, a distamycin A derivative possessing antitumor activity and peculiar mode of DNA interaction. 86th Annual Meeting of the American Association for Cancer Research. Toronto, Ontario, Canada, March 18-22, 1995.
72. Lee HH, Boyd M, Gravatt GL, Denny WA. Pyrazole analogues of the bispyrrolocarboxamide anti-tumour antibiotics: synthesis, DNA binding and antitumour properties. *Anticancer Drug Design* 1991; 6:501-517.
73. Broggini M, Erba E, Ponti M, Ballinari D, Geroni C, Spreafico F, D'Incalci M. Selective DNA interaction of the novel distamycin derivative FCE 24517. *Cancer Res* 1991; 51:199-204.
74. Broggini M, Coley H, Mongelli N, Presenti E, Wyatt MD, Hartley JA, D'Incalci M. DNA sequence-specific adenine alkylation by the novel antitumor drug tallimustine (FCE 24517), a benzoyl nitrogen mustard derivative of distamycin. *Nucleic Acids Res.* 1995; 23:81-87.
75. Lown JW. Lexitropsins: rational design of DNA sequence reading agents as novel anti-cancer agents and potential cellular probes. *Anticancer Drug Design* 1988; 3:25-40.
76. Lee M, Preti CS, Vinson E, Wyatt MD, Hartley JA. GC sequence specific recognition by an *N*-formamido, C-terminus-modified and imidazole-containing analogue of netropsin. *J Med Chem* 1994; 37:4073-4075.
77. Zhang Y, Chen F-X, Mehta P, Gold B. Groove- and sequence-selective alkylation of DNA by sulfonate esters tethered to lexitropsins. *Biochemistry* 1993; 32:7954-7965.
78. Lee M, Rhodes AL, Wyatt MD, D'Incalci M, Forrow S, Hartley JA. In vitro cytotoxicity of GC sequence directed alkylating agents related to distamycin. *J Med Chem* 1993; 36:863-870.
79. Lee M, Rhodes AL, Wyatt MD, Forrow S, Harley JA. Design, synthesis, and biological evaluation of DNA sequence and minor groove selective alkylating agents. *Anticancer Drug Design* 1993; 8:173-192.
80. Hurley LH, Warpehoski MA, Lee C-S, McGovren JP, Scahill TA, Kelly RC, Mitchell MA, Wicnienski MA, Gebhard I, Johnson PS, Bradford VS. Sequence specificity of DNA alkylation by the unnatural enantiomer of CC-1065 and its synthetic analogues. *J Am Chem Soc*

1990; 112:4633–4649.
81. Ding Z-M, Hurley LH. DNA interstrand cross-linking, DNA sequence specificity, and induced conformational changes produced by a dimeric analog of (+)-CC-1065. *Anticancer Drug Design* 1991; 6:427–452.
82. Krueger WC, Hatzenbuhler NT, Prairie MD, Shea MH. DNA sequence recognition by the antitumor antibiotic CC-1065 and analogs of CC-1065. *Chem-Biol Interact* 1991; 79:265–286.
83. Wierenga W, Bhuyan BK, Kelly RC, Krueger WC, LI LH, McGovren JP, Swenson DH, Warpehoski MA. Antitumor activity and biochemistry of novel analogs of the antibiotic, CC-1065. *Adv Enzyme Regul* 1986; 25:141–155.
84. McGovren JP, Clarke GL, Pratt EA, DeKoning TF. Preliminary toxicity studies with the DNA-binding antibiotic, CC-1065. *J. Antibiot.* 1984; 37:63–70.
85. Shamdas GJ, Alberts DS, Modiano M, Wiggins C, Power J, Kasunic DA, Elfring GL, Earhart RH. Phase I study of adozelesin (U-73,975) in patients with solid tumors. *Anticancer Drugs* 1994; 5:10–14.
86. Fleming GF, Ratain MJ, O'Brien SM, Schilsky RL, Hoffman PC, Richards JM, Vogelzang NJ, Kasunic DA, Earhart RH. Phase I study of adozelesin administered by 24-hour continuous intravenous infusion. *J Natl Cancer Inst* 1994; 86:368–372.
87. Li LH, DeKoning TF, Kelly RC, Krueger WC, McGovren JP, Padbury GE, Petzold GL, Wallace TL, Ouding RJ, Prairie MD, Gebhard I. Cytotoxicity and antitumor activity of carzelesin, a prodrug cyclopropylpyrroloindole analogue. *Cancer Res* 1992; 52:4904–4913.
88. Lee C-S, Gibson NW. DNA damage and differential cytotoxicity produced in human carcinoma cells by CC-1065 analogues, U-73,975 and U-77,779. *Cancer Res* 1991; 51:6586–6591.
89. Walker DL, Reid JM, Ames MM. Preclinical pharmacology of bizelesin, a potent bifunctional analog of the DNA-binding antibiotic CC-1065. *Cancer Chemother Pharmacol* 1994; 34: 317–322.
90. Weiss GR, Burris III HA, Eckardt JR, Fields S, O'Rourke T, Rodriguez GI, Rothenberg ML. New anticancer agents. In: Pinedo HM, Longo DL, Chabner BA, eds. *Cancer Chemotherapy and Biological Response Modifiers Annual 15.* Elsevier Science. 1994:130–151.
91. Boger DL, Invergo BJ, Coleman RS, Zarrinmayeh H, Kitos PA, Collins Thompson S, Leong T, McLaughhlin LW. A demonstration of the intrinsic importance of stabilizing hydrophobic binding and non-covalent van der Waals contacts dominant in the non-covalent CC-1065/B-DNA binding. *Chem-Biol Interact* 1990; 73:29–52.
92. Sugiyama H, Hosoda M, Saito I. Covalent alkylation of DNA with duocarmycin A. Identification of a basic site structure. *Tetrahedron Lett* 1990; 31:7197–7200.
93. Hoshi A, Castaner J. KW-2189. *Drugs Future* 1993; 18:1112,1113.
94. Bhuyan BK, Smith KS, Adams EG, Wallace TL, Vonhoff DD, Li LH. Adozelesin, a potent new alkylating agent. Cell-killing kinetics and cell-cycle effects. *Cancer Chemother Pharmacol* 1992; 30:348.
95. Broggini M, Ponti M, Ottolenghi S, D'Incalci M, Mongelli N, Mantovani R. Distamycins inhibit the binding of OTF-1 and NFE-1 transfactors to their conserved DNA elements. *Nucleic Acids Res* 1989; 17:1051–1059.
96. Zunino F, Capranico G, Pratesi G, Spinelli S. Current approaches to new drug development in cancer chemotherapy. *Farmaco* 1992; 47:1115–1132.
97. Gewirtz DA. Does bulk damage to DNA explain the cytostatic and cytotoxic effects of topoisomerase II inhibitors? *Biochem Pharmacol* 1991; 42:2253–2258.
98. Schwab G, Guroux I, Chavany C, Hélène C, Saison-Behmoaras E. An approach for new anticancer drugs: oncogene-targeted antisense DNA. *Ann Oncol* 1994; 5:S55–S58.
99. Yamamoto K, Sugiyama H, Kawanishi S. Concerted DNA recognition and novel site-specific alkylation by duocarmycin A with distamycin A. *Biochemistry* 1993; 32:1059–1066.
100. Lee CS, Pfeifer GP, Gibson NW. Mapping of DNA alkylation sites induced by adozelesin and bizelesin in human cells by ligation-mediated polymerase chain reaction. *Biochemistry* 1994; 33:6024–6030.
101. Hélène C. Rational design of sequence-specific oncogene inhibitors based on antisense and antigene oligonucleotides. *Eur J Cancer* 1991; 27:1466–1471.

10 Bis-Naphthalimides

Synthesis and Preclinical Evaluation

Cynthia A. Romerdahl, PhD and Miguel F. Braña, PhD

CONTENTS

1. INTRODUCTION

DNA intercalating agents are among the most common anticancer drugs used in the clinical therapy of human tumors. Although some drugs, such as doxorubicin and daunomycin, were originally isolated from natural sources, other compounds are synthetic organic molecules specifically designed as antineoplastic agents. Arcamone has classified these drugs on the basis of DNA interaction into:

1. Compounds that are able to bind covalently to DNA;
2. Agents that cause cleavage of the DNA; and
3. Drugs that bind reversibly to double-helical DNA (*1*).

Our synthetic efforts have focused on this last group, in the hopes that the lack of covalent binding to DNA or DNA nicking would lead to compounds that were less toxic to nontumor tissues. Naphthalimides were synthesized as a new series of potential antitumor compounds in the late 1970s (*2–4*). Since bis-intercalating agents have an even higher binding affinity for DNA (*5*), we later synthesized and evaluated a number of bis-intercalating naphthalimides (designated as bis-naphthalimides) (*6*). In this chapter, we review the origins of this new class of cytotoxic intercalators and summarize the data on the efficacy of these compounds in preclinical models.

2. MONOMERIC NAPHTHALIMIDES

2.1. Synthesis

The naphthalimide series was originally derived by trying to combine structural components of several antitumor compounds into a single molecule (Fig. 1). After

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

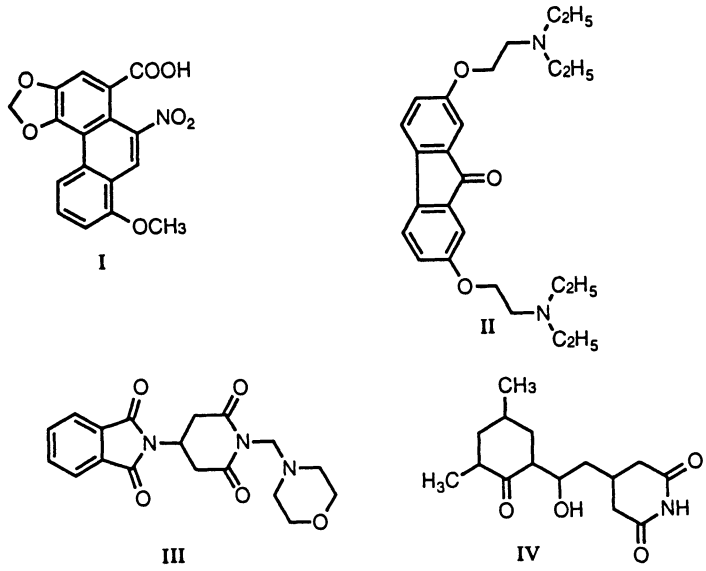


Fig. 1. Cytotoxic compounds used in the design of the naphthalimides. Pictured are aristolochic acid I, tilorone II, CG-603 III, and cycloheximide IV.

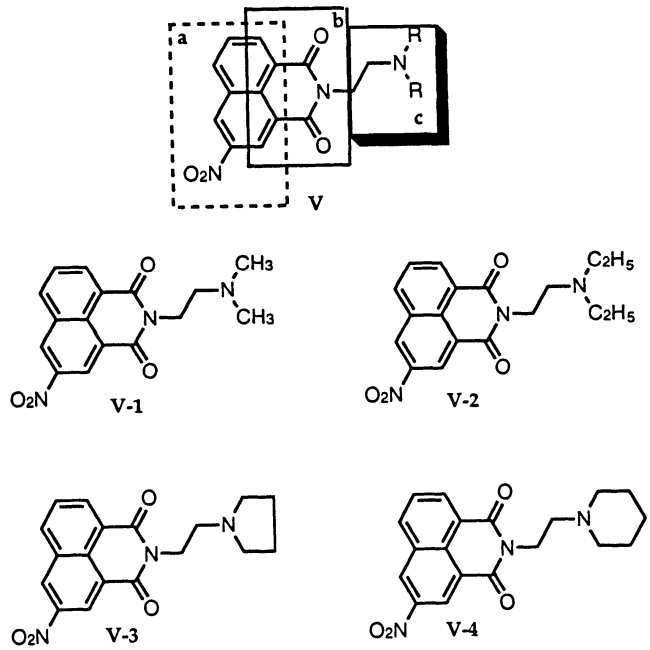


Fig. 2. Initial naphthalimides synthesized for possible antitumor activity.

studying many structures, we selected the following: aristolochic acid 1, a natural product with the intercalating phenanthrene system isolated from different strains of *Aristolochiaceae*; tilorone II, another intercalator that is also an interferon inducer; CG-603 III, a antiproliferative compound; and the protein synthesis inhibitor cycloheximide IV. Different moieties were combined into the naphthalimide V system shown in Fig. 2, retaining the β-nitro-naphthalene a of the aristolochic acid, the gluta-

Table 1
Cytotoxic Activity and Acute Toxicity of Selected Naphthalimides

Comp.	IC_{50} μg/mL HeLa Cells	IC_{50} μg/mL KB Cells	LD_{50} mg/kg mouse ip	LD_{50} mg/kg rat ip
V - 1	0.15	0.20	10	6.5
V - 2	2.50	3.50	13	32.5
V - 3	0.30	0.35	12.6	4.5
V - 4	2.0	2.0	40	9.1

rimide rings b that appear in GC-603 and cycloheximide, and the basic chains c present in tilorone and CG-603. The compounds designated as V-1–4 were synthesized and tested for activity using the HeLa and KB cell lines in culture. The data are summarized in Table 1 (*3*). Two of these derivatives were more active than 6-mercaptopurine, which was used as a reference compound for the cytotoxicity assays. This excellent antitumor activity suggested this was a promising strategy, and a large series of these naphthalimides were then synthesized.

2.2. Biological Activity

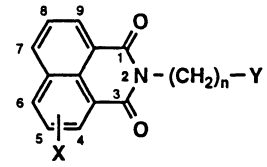
The structures and antitumor activities of the naphthalimide derivatives are presented in Table 2. The compounds were compared based on the concentration of drug required to inhibit 50% of the growth of HeLa cells. Growth inhibition was maximal when the side chain was formed by two methylene groups (position *Y*) and the presence of a basic terminal N group was required at position *X* (*4*). The positioning of the substituents in the naphthalic ring seemed optimal at position 5, instead of 4 or 6. These results were subsequently confirmed by other groups (*7,8*).

Samples from this series were sent to the National Cancer Institute (NCI) for screen- and evaluation (*9,10*). The activity of these compounds was confirmed by the NCI. One of the series, designated as Amonafide by the WHO (V-10 in Table 2, NSC 308847), was active against the P388 and L1210 leukemia models being used as the primary screen. When Amonafide is given ip 17.5 mg/kg (q 1 d × 5), the increase in life-span is ~50% over the untreated mice. Amonafide was selected for exploratory clinical development by the NCI. Another active agent from this series, one of the first synthesized (V-1) was designated as Mitonafide (NSC 300288) by the WHO. Mitonafide has in vitro activity against the KB and HeLa cells, as well as in vivo activity against murine and human tumor cell lines (*3*). The structures of these two compounds are shown in Fig. 3.

Amonafide and Mitonafide bind to double-stranded DNA by intercalation. These drugs stabilize DNA against thermal denaturation, inhibit RNA and DNA synthesis, and initiate DNA cleavage by topoisomerase II, resulting in single-strand breaks (*11–14*). DNA intercalating agents are known to trap topoisomerase II (*15*). These compounds appear to hinder the religation step of topoisomerase II action by stabilizing the cleavable complex (*16*). Mitonafide and Amonafide specifically induce DNA cleavage near nucleotide No. 1830 on pBR322 DNA. It remains unclear, however, if the antitumor effects are due to the alterations of topoisomerase II activity.

In addition to their cytotoxity against tumor cells Amonafide and Mitonafide have other biological activities. Both compounds were active against herpes simplex and

Table 2
Cytotoxic Activity of Naphthalimides V



Comp. V	*X*	*Y*	*n*	*IC_{50} μM*
1	5-NO_2	$N(CH_3)_2$	2	0.47
2	5-NO_2	$N(C_2H_5)_2$	2	7.00
3	5-NO_2	$N(CH_2)_4$	2	0.80
4	5-NO_2	$N(CH_2)_5$	2	5.80
5	5-NO_2	$N(CH_2)_4$	0	> 100
6	5-NO_2	$N(CH_2)_4$	1	100
7	5-NO_2	$N(CH_3)_2$	3	3.00
8	5-NO_2	$N(C_2H_5)_2$	3	14.00
9	5-NH_2	$N(C_2H_5)_2$	3	76.00
10	5-NH_2	$N(CH_3)_2$	2	8.80
11	5-NO_2	$N(CH_2CH_2)_2O$	2	56.00
12	5-NH_2	$N(CH_2CH_2)_2O$	2	30.00
13	5-NO_2	$(CH_2)_5NC_2H_5$	0	16.00
14	5-NH_2	$N(C_2H_5)_2$	2	9.60
15	5-NH_2	$N(CH_2)_4$	2	4.80
16	5-NO_2	$N(CH_2CH_2)_2NCH_3$	3	5.20
17	5-NH_2	$N(CH_2CH_2)_2NCH_3$	3	28.00
18	5-NH_2	$N(CH_2)_4$	2	24.00
19	5-Cl	$N(CH_2CH_2)_2O$	2	> 100
20	5-Cl	$N(CH_3)_2$	3	6.00
21	5-Cl	$N(CH_2)_4$	2	4.50
22	5-Cl	$N(CH_2)_5$	2	10.00
23	5-Cl	$N(CH_3)_2$	2	2.60
24	5-OH	$N(CH_3)_2$	2	10.00
25	5-OH	$N(CH_2CH_2)_2O$	2	> 100
26	5-OH	$N(CH_2)_4$	2	11.00
27	5-NO_2	$N(CH_3)_2$	0	> 100
28	5-NO_2	$N(CH_3)_2$	0	> 100
29	5-OCH_3	$N(CH_3)_2$	2	5.00
30	5-$NHCO_2C_2H_5$	$N(CH_3)_2$	2	28.00
31	5-$NHCO_2C_2H_5$	$N(CH_2)_4$	2	26.00
32	5-OCH_3	$N(CH_2)_4$	2	2.00
33	5-$NHCOCH_3$	$N(CH_3)_2$	2	12.00
34	5-$NHCOCH_3$	$N(CH_2)_4$	2	14.00
35	5-NO_2	$CH(CH_3)_2$	2	> 100
36	5-NO_2	SH	2	> 100
37	H	$N(CH_2)_4$	2	11.00
38	H	$N(CH_3)_2$	2	3.70
39	5-t-C_4H_9	$N(CH_3)_2$	2	43.00
40	5-NO_2	OH	2	> 100

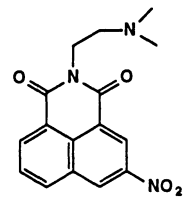
Table 2 (Continued)

Comp. V	X	Y	n	IC_{50} μM
41	5-NO_2	OCH_3	2	> 100
42	5-NO_2	NH_2	2	2.40
43	5-NO_2	$NHCH_3$	2	13.00
44	5-NO_2	$NHCOCH_3$	2	> 100
45	5-$NHCO_2C_2H_5$	$N(C_2H_5)_2$	2	26.00
46	5-$NHCO_2C_2H_5$	$N(C_2H_5)_2$	3	20.00
47	5-NH_2	$N(CH_3)_2$	3	16.00
48	5-OH	$N(C_2H_5)_2$	2	16.00
49	5-$NHCOCH_3$	$N(C_2H_5)_2$	2	16.00
50	5-$NHCOCH_3$	$N(C_2H_5)_2$	3	16.00
51	5-NO_2	$+N(CH_3)_3,I^-$	2	100
52	6-NO_2	$N(CH_3)_2$	2	6.30
53	6-NO_2	$N(CH_2)_4$	2	8.80
54	6-OCH_3	$N(CH_2)_4$	2	2.30
55	6-OCH_3	$N(CH_3)_2$	2	5.00
56	6-OCH_3	$N(C_2H_5)_2$	2	12.00
57	6-OCH_3	$N(CH_2)_5$	2	13.00
58	6-NH_2	$N(CH_3)_2$	2	3.50
59	6-NH_2	$N(CH_2)_4$	2	6.00
60	6-NO_2	$N(C_2H_5)_2$	2	16.00
61	6-NO_2	$N(CH_2)_5$	2	14.00
62	6-Br	$N(CH_3)_2$	2	23.00
63	6-Br	$N(CH_2)_4$	2	16.00
64	6-Cl	$N(CH_3)_2$	2	16.00
65	6-Cl	$N(CH_2)_4$	2	21.00
66	6-NH-Bu^n	$N(CH_3)_2$	2	11.00
67	6-NH-Bu^n	$N(CH_2)_4$	2	10.00
68	6-OH	$N-(CH_3)_2$	2	> 100
69	6-OH	$N(CH_2)_4$	2	> 100
70	4-OCH_3	$N(CH_3)_2$	2	10.00

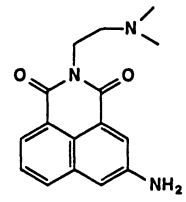
vaccinia viruses in in vitro assays (*17*). This antiviral activity appears to be specific for DNA viruses, with no activity observed against influenza viruses. Amonafide and Mitonafide were also tested by the NCI in its HIV screen, where the results were negative (data not shown). Mitonafide is an antibiotic with maximum activity against *Bacillus subtilis, Salmonella typi*, and *Shigella dysenteriae (18)*. Amonafide and Mitonafide have also been tested for the ability to kill *Trypanosoma cruzii*, which causes Chagas' disease (*19*). Both compounds were able to kill the parasite in vitro, at concentrations of $10-^7M$. However, in vivo studies with infected mice showed toxicity, so the compounds were not pursued for therapy of Chagas' disease.

2.3. Clinical Evaluation

Both Mitonafide and Amonafide have been tested in clinical trials. Mitonafide was studied in Phase I and II trials using a short administration schedule (1-h infusion every day × 3–5 d), and it had activity against solid tumors (*20*). However, this dosing



MITONAFIDE
NSC 300288



AMONAFIDE
NSC 308847

Fig. 3. Chemical structures of Amonafide and Mitonafide.

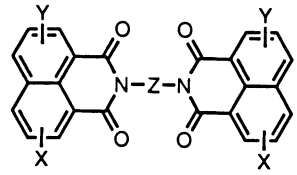
schedule was associated with central neurotoxicity. When the administration schedule was changed to a 5-d continuous infusion, Mitonafide could safely be administered. Unfortunately, the slow adminstration schedule lacked efficacy in the solid tumors that were tested (non-small cell lung, colorectal, and head and neck cancers) (*21*). Therefore, the clinical development was stopped.

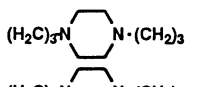
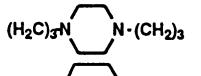
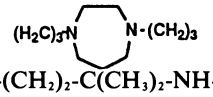
Amonafide has completed several Phase I and II trials. The dose-limiting toxicity appears to be myelosuppression (*22,23*). When given alone, it has limited activity in some solid tumors (*24–26*). When given in combination with cytosine arabinoside, it induced complete remissions in some poor-risk acute leukemias (*27*). Amonafide at a dose of 400 mg/m^2 was eliminated from plasma with a terminal half-life of 3.5 h (*28*). The peak plasma concentrations were variable and ranged from 2.6 to 9.1 mg/mL. Amonafide undergoes extensive metabolism in vivo, including *N*-acetylation. The rate of acetylation varies owing to genetic differences among patients (*29*). Fast acetylator phenotyping may allow the Amonafide dose to be adjusted for this variability and could be used for future clinical studies.

3. SYNTHESIS OF THE BIS-NAPHTHALIMIDES

Several antibiotics isolated from microorganisms, such as Echinomycin or Luzopeptin, have the ability to intercalate doubly into DNA (*30*). Several groups have attempted to design such bis-intercalators, looking for an improved therapeutic profile. Some new synthetic products have been described, for example, diacridines or ditercalinium (*30*). In the early 1980s, we were interested in improving the activity of naphthalimides by increasing the binding capacity to DNA. Therefore, a new series of bis-intercalating agents was designed using structural features of the lead compounds, Mitonafide and Amonafide. Two of the chromophore units were linked together by bridges containing at least one amine group, because a preliminarily synthesized compound with polymethylenic bridge, V-35, did not showed in vitro activity (*31*). This suggested that the presence of at least an amino group is essential for significant cytotoxic activity, as in the mononaphthalimide series (*4*). We therefore prepared a series of compounds with different substituents in the aromatic system, as well as bridges differing in length and the number of amine groups. The synthesis of these compounds has been described in detail elsewhere (*31,32*). The structures of selected bis-naphthalimides are shown in Table 3. The aromatic substituents were predominantly amino and nitro, since these were the most active in the mononaphthalimide series. In addition, we added the acetylamino substituent, since the main metabolite of Amonafide is the acetyl derivative V-33.

Table 3
Selected Bis-Naphthalimides



Comp.	X	Y	Z	IC_{50} μM
1	3-NO_2	H	$(CH_2)_2$-NH-$(CH_2)_2$	0.51
2	3-NH_2	H	$(CH_2)_2$-NH-$(CH_2)_2$	30.20
3	3-NH_2	6-NO_2	$(CH_2)_2$-NH-$(CH_2)_2$	0.27
4	3-NH_2	H	$(CH_2)_2$-NCH_3-$(CH_2)_2$	2.45
5	3-NH_2	H	$(CH_2)_2$-NH-$(CH_2)_3$	0.21
6	3-NH_2	H	$(CH_2)_2$-NH-$(CH_2)_3$	15.50
7	3-NH_2	H	$(CH_2)_3$-NCH_3-$(CH_2)_3$	0.07
8	3-NH_2	H	$(CH_2)_3$-NH-$(CH_2)_3$	0.16
9	3-NH_2	H	$(CH_2)_3$-NH-$(CH_2)_3$	4.96
10	3-NH_2	6-NO_2	$(CH_2)_3$-NH-$(CH_2)_3$	2.31
11	3-NH_2	6-NH_2	$(CH_2)_3$-NH-$(CH_2)_3$	> 100
12	4-NO_2	H	$(CH_2)_3$-NH-$(CH_2)_3$	2.78
13	3-NO_2	H	$(CH_2)_3$-NCH_3-$(CH_2)_3$	0.24
14	3-NH_2	H	$(CH_2)_2$-NCH_3-$(CH_2)_3$	1.87
15	3-$NHCOCH_3$	H	$(CH_2)_3$-NCH_3-$(CH_2)_3$	7.38
16	3-NO_2	6-NO_2	$(CH_2)_2$-NCH_3-$(CH_2)_3$	0.77
17	3-NO_2	6-NH_2	$(CH_2)_3$-NCH_3-$(CH_2)_3$	1.70
18	3-NH_2	6-NH_2	$(CH_2)_2$-NCH_3-$(CH_2)_3$	> 100
19	3-NO_2	H	$(CH_2)_3$-NH-$C(CH_3)_2$-$(CH_2)_2$	0.29
20	3-NO_2	H	$(CH_2)_3$-NH-$(CH_2)_4$	0.19
21	3-$NHCOCH_3$	H	$(CH_2)_3$-NH-$(CH_2)_4$	> 100
22	3-NO_2	H	$(CH_2)_2$-$C(CH_3)_2$-NH-$(CH_2)_4$	0.97
23	3-NH_2	H	$(CH_2)_3$-NH-$(CH_2)_2$-NH-$(CH_2)_3$	3.49
24	H	H	$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$	2.70
25	3-NH_2	H	$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$	39.00
26	3-$NHCOCH_3$	H	$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$	> 100
27	3-NO_2	H	$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$	0.72
28	3-NO_2	6-NO_2	$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$	4.00
29	3-NO_2	6-NH_2	$[(CH_2)_2$-C-$(CH_3)_2$-NH-$(CH_2)_2]_2$	4.50
30	3-NO_2	H		0.23
31	3-NH_2	H		> 100
32	3-NH_2	H		2.30
33	3-NO_2	6-NH_2	$(CH_2)_3$-NH-$(CH_2)_2$-$C(CH_3)_2$-NH-CH_2-CH_2 $CH_2)_3$-NH-$(CH_2)_2$-$C(CH_3)_2$-NH-CH_2-CH_2	> 100
34	3-NO_2	6-NH_2	$(CH_2)_2$-$C(CH_3)_2$-NH-$(CH_2)_3$-NH-CH_2-CH_2 $(CH_2)_2$-$C(CH_3)_2$-NH-$(CH_2)_3$-NH-CH_2-CH_2	1.77
35	3-NH_2	H	$(CH_2)_7$	> 100

(continued)

Table 3 (Continued)

Comp.	X	Y	Z	IC_{50} μM
36	H	H	$(CH_2)_2$-NH-$(CH_2)_2$-NH-$(CH_2)_2$	0.03
37	H	H	$(CH_2)_2$-NH-$(CH_2)_3$-NH-$(CH_2)_2$	0.004
38	H	H	$(CH_2)_2$-NH-$(CH_2)_4$-NH-$(CH_2)_2$	0.005
39	H	H		0.002
40	H	H	$CH(CH_3)$-CH_2-NH-$(CH_2)_2$-NH-CH_2-$CH(CH_3)$	0.7
41	H	H	$(CH_2)_2$-NH-$(CH_2)_2$-NH-$(CH_2)_2$-NH-$(CH_2)_2$	0.4
42	H	H	$[(CH_2)_2$-NH-$(CH_2)_2$-NH-$CH_2]_2$	0.6
43	3-NO_2	H	$(CH_2)_2$-NH-$(CH_2)_2$-NH-$(CH_2)_2$	0.002
44	3-NO_2	H	$(CH_2)_2$-NH-$(CH_2)_3$-NH-$(CH_2)_2$	0.0005
45	3-NO_2	H	$(CH_2)_2$-NCH_3-$(CH_2)_3$-NCH_3-$(CH_2)_2$	0.0006
46	3-NO_2	H	$(CH_2)_2$-NCH_3-$(CH_2)_2$-NCH_3-$(CH_2)_2$	0.04
47	4-NO_2	H	$(CH_2)_2$-NH-$(CH_2)_3$-NH-$(CH_2)_2$	0.1
48	3-NH_2	H	$(CH_2)_2$-NH-$(CH_2)_2$-NH-$(CH_2)_2$	0.03
49	3-NH_2	H	$(CH_2)_2$-NH-$(CH_2)_3$-NH-$(CH_2)_2$	0.009
50	3-NH_2	H	$CH(CH_3)$-CH_2-NH-$(CH_2)_2$-NH-(CH_2)-CH-(CH_3)	0.1
51	3-$NHCOCH_3$	H	$(CH_2)_2$-NH-$(CH_2)_2$-NH-$(CH_2)_2$	10
52	3-$NHCOCH_3$	H	$(CH_2)_2$-NH-$(CH_2)_3$-NH-$(CH_2)_2$	3
53	3-$NHCOCH_3$	H	$CH(CH_3)$-CH_2NH-$(CH_2)_2$-$NHCH_2$-$CH(CH_3)$	1
54	3-Br	H	$(CH_2)_2$-NH-$(CH_2)_3$-NH-$(CH_2)_2$	0.008
55	2-OH	H	$(CH_2)_2$-NH-$(CH_2)_3$-NH-$(CH)_2$	0.004
56	4-OH	H	$(CH_2)_2$-NH-$(CH_2)_3$-NH-$(CH_2)_2$	8
57	3-OH	H	$(CH_2)_2$-NH-$(CH_2)_3$-NH-$(CH_2)_2$	0.2

4. ANTITUMOR ACTIVITY

All the compounds were initially evaluated for cytotoxic activity in a standard monolayer cell culture of the HT-29 human colon carcinoma cell line. The IC_{50} values of symmetric and asymmetric bis-naphthalimides are shown in Table 3. In general, bis-naphthalimides have higher cytotoxic activities than the parental compounds, Amonafide and Mitonafide, with some exceptions. Certain aromatic substituents tend to increase cytotoxicity, in the order $CH_3CONH < NH_2 < H < NO_2$ for compounds with the same bridge. These results are consistent with those found for the mononaphthalimides. The length and nature of the bridge also alter the cytotoxicity. Nevertheless, any atempt to obtain accurate quantitative structure–activity relationship studies has failed (*32*). This suggests the concurrence of different mechanisms of intercalation, which is in agreement with the bis-acridine DNA polyintercalation studies done by Le Pecq and colleagues (*33*).

Selected bis-naphthalimides were tested for efficacy in animal tumor models. Surprisingly, the most active compound was one that lacked any aromatic substituent (number 37 in Table 3). This compound, designated LU 79553, shows excellent antitumor activity in a number of preclinical models (*34*). Our initial screen for activity in vivo was to test the compounds at the maximum tolerated dose in the MX-1 model. The MX-1 is a human mammary carcinoma that is xenografted sc into athymic nude mice (*35*). When LU 79553 is administered to the mice as two cycles of five daily injections, the compound is curative at doses of 20, 25, or 30 mg/kg/d (Fig. 4). This

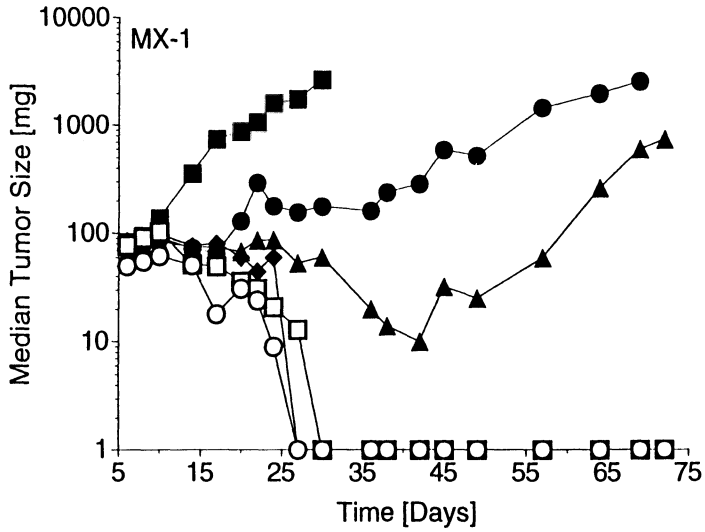


Fig. 4. MX-1 human mammary carcinoma was serially passaged as tumor pieces (3 mm in diameter) implanted sc on day 0. Treatment was initiated on d 6 and consisted of five daily iv injections of LU 79553 (dissolved in sterile water) at 0 (■), 5(●), 10 (▲), 20 (◆), 25 (□), or 30 (○) mg/kg/d. A second cycle was initiated on day 20. Tumors were measured two to three times a week with calipers, and a volume was calculated using the formula $V = (W^2 \times L)/2$. Median tumor size for each treatment group is plotted ($n = 5$).

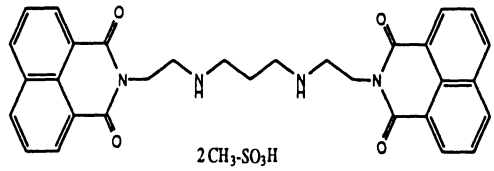
treatment schedule is also effective when the xenografts are allowed to grow tumor sizes of 1–2 g (*34*). LU 79553 was then tested in a number of different tumor models. The data are summarized in Table 4. Whereas the mononaphthalimide Amonafide has little activity against solid tumor xenografts, LU 79553 treatment has resulted in not only tumor growth inhibition, but tumor regression and tumor-free survivors in several of these models. Based on this dramatic antitumor activity, LU 79553 was selected for clinical development. Phase I clinical trials are currently in progress in Europe and the USA. The absence of a basic terminal nitrogen in the chromophore of LU 79553 may be advantageous, since the nitro substitution on the monomeric compounds appears to be responsible for the CNS toxicity observed in the Mitonafide clinical trials (*20*).

The mechanism of LU 79553's antitumor activity is unknown. The compound intercalates into DNA (M. Waring, in press), as do the monomeric derivatives (*11*). LU 79553 alters the catalytic activity of topoisomerase II in a decatenation assay, but is a much more potent inhibitor than Amonafide (*34*). Although the compound appears to exert its antitumor activity via DNA damage, the critical biochemical target of the bis-naphthalimides remains to be identified.

5. OTHER BIS-NAPHTHALIMIDES

Another series of bis-naphthalimides has been synthesized by investigators at Du Pont Merck (Wilmington, DE) (*36*). The compounds showed a similar spectrum of activity in vitro and in vivo to the bis-naphthalimides described above (*36–39*). The compounds bind to DNA with a high affinity, are active at killing many tumor cell

Table 4
Summary of In Vivo Activity of LU 79553



LU 79553

Tumor designation	*Tissue type*	*Activity level*[a]
MX-1 early stage	Breast	Curative
MX-1 late stage	Breast	Curative
LX-1	Lung	PR, CR TGD = 42–46 d
LOX	Melanoma	Curative
CX-1	Colon	Some PR TGD = 32–33 d
OVCAR-3	Ovarian	T/C = 198%

[a]PR = partial regressions, CR = complete regressions, TGD = tumor growth delay.

lines in vitro, and some derivatives have impressive activity in preclinical tumor models. One compound, DMP 840, was selected for clinical development. DMP 840 has been evaluated in Phase I studies at several sites (*40–44*). The dose-limiting toxicities are myelosuppression and stomatitis. DMP-840 is currently being studied in Phase II trials.

ACKNOWLEDGMENTS

We thank our many colleagues who have been involved in this research, including T. Barlozzari, P. Bousquet, J. M. Castellano, C. Cocchiaro, D. Conlon, M. D'Autilia, F. Emling, J. George, K. Fitzgerald, R. Kamen, G. Keilhauer, M. Kluge, R. Miller, M. Moran, M.J. Perez de Vega, D. Perron, X. -D. Qian, S. Robinson, E. Schlick, and M. Spigelman.

REFERENCES

1. Arcamone FM, Animati F, Brunella B, Configliacchi E, D'Alessio R, Geroni C, Giuliani FC, Lazzari E, Menozzi M, Mongelli N, Penco S, Verini MA. Synthesis, DNA-binding properties, and antitumor activity of novel dystamycin derivatives. *J Med Chem* 1989; 32:774–778.
2. Braña MF, Castellano JM, Jimenez A, Llombart A, Rabadán FP, Roldán CM, Roldán C, Santos A, Vazquez D. Synthesis, cytostatic activity and mode of action of a new series of imide derivatives of 3-nitro-1,8-naphthalic acid. *Curr Chemother* 1978; 2:1216,1217.
3. Braña MF, Castellano JM, Roldán CM, Santos A, Vazquez D, Jimenez A. Synthesis and mode(s) of action of a new series of imide derivatives of 3-nitro-1,8-naphthalic acid. *Cancer Chemother Pharmacol* 1980; 4:61–66.

4. Braña MF, Sanz AM, Castellano JM, Roldán CM, Roldan C. Synthesis and cytostatic activity of benz[de]isoqinolin-1,3-diones. Structure-activity relationships. *Eur J Med Chem* 1981; 16:207-212.
5. Walkelin LPG, Waring MJ. DNA Intercalating Agents. In: Sammes PG, ed. *Comprehensive Medicinal Chemistry*. Oxford: Pergamon. 1990:702-720.
6. Braña MF, Castellano JM, Morán M, Pérez de Vega MJ, Romerdahl CA, Qian X-D, Bousquet P, Emling F, Schlick E, Keilhauer G. Bis-naphthalimides: a new class of antitumor agents. *Anti-Cancer Drug Design* 1993; 8:257-268.
7. Stevenson KA, Yen S-F, Yang N-C, Bookin DW, Wilson WD. A substituent constant analysis of the interaction of substituted naphthalene monoimides with DNA. *J Med Chem* 1984; 27: 1677-1682.
8. Zee-Cheng RKY, Cheng CC. *N*-(aminoalkyl)imide antineoplastic agents. Synthesis and biological activity. *J Med Chem* 1985; 28:1216-1222.
9. Paull KD, Nasr M, Narayanan VL. Computer assisted structure-activity correlations. *Arzneim-Forsch/Drug Res* 1984; 34:1243-1246.
10. National Cancer Institute. Clinical Brochure, Nafidimide, NSC 308847, November 1984; 6-22.
11. Waring MJ, González A, Jimenez A, Vazquez D. Intercalative binding to DNA of antitumor drugs derived from 3-nitro-1,8-naphthalic acid. *Nucleic Acid Res* 1979; 7:217-230.
12. Andersson BS, Berian M, Bakic M, Silberman LE, Newman RA, Zwelling LA. In vitro toxicity and DNA cleaving capacity of benzisoquinolinedione (nafidimide; NSC-308847) in human leukemia. *Cancer Res* 1986; 47:1040-1044.
13. Nishio A, Uyeki, EM. Induction of DNA strand breaks and chromosome abnormalities by an imide derivative of 3-nitro-1,8-naphthalic acid (mitonafide) in Chinese hamster ovary cells. *J Natl Cancer Inst* 1983; 70:1097-1102.
14. De Isabella P, Zunino F, Capranico G. Base sequence determinants of amonafide stimulation of topoisomerase II DNA cleavage. *Nucleic Acids Res* 1995; 23:223-229.
15. Capranico G, Zunino F. DNA topoisomerase-trapping antitumor drugs. *Eur J Cancer* 1992; 28A:2055-2060.
16. Hsiang Y-H, Jiang JB, Liu LF. Topoisomerase II-mediated DNA cleavage by Amonafide and its structural analogs. *Mol Pharmacol* 1989; 36:371-376.
17. Gancedo APG, Gil C, Vilas P, Pérez S, Paez E, Rodriguez F, Braña MF, Roldán CM. Imide derivatives of 3-nitro-1,8-naphthalic acid: their inhibitory activity against DNA viruses. *Arch Virol* 1982; 74:157-165.
18. Roldán CM, Cubero SJ, Rubio F. Actividad antibiótica del nuevo producto 2-(2-dimetilamino-etil)-5-nitrobenzo[d,e]isoquinolein-1,3-diona (Made-4212). *Diag. Biol* 1979; XXVIII:443-446.
19. Osuna A, Castanys C, Mascaró C, Adroher FJ, Braña MF, Roldán CM. In vitro action of three benzo[de]isoquinoline-1,3-dione derivatives against Trypanosoma cruzi. *Rev Inst Med Trop Sao Paulo* 1983; 25:254-258.
20. Llombart M, Poveda A, Forner E, Martos CF, Gaspar C, Muñoz M, Olmos T, Ruiz A, Soriano V, Benavides A, Martín M, Schlick E, Guillem V. Phase I study of Mitonafide in solid tumors. *Invest New Drugs* 1992; 10:177-181.
21. Rosell R, Carles J, Abad A, Ribelles N, Barnadas A, Benavides A, Martín M. Phase I study of Mitonafide in 120 hour continuous infusion in non-small cell lung cancer. *Invest New Drugs* 1992; 10:171-175.
22. Legha SS, Ring S, Raber M, Felder TB, Newman RA, Krakoff IH, Phase I clinical investigation of benzisoquinolinedione. *Cancer Treat Rep* 1987; 71:1165-1169.
23. Saez R,, Craig JB, Kuhn JG, Weiss GR, Koeller J, Phillips J, Havlin K, Harman G, Hardy J, Melink TJ, Sarosy G, VonHoff DD. Phase I investigation of Amondafide. *J Clin Oncol* 1989; 7:1351-1358.
24. Scheithauer W, Kornek G, Haider K, Depisch D. Amonafide in metastatic colorectal carcinoma. *Eur J Cancer* 1990; 26:923-924.
25. Kornek G, Raderer M, Depisch D, Haider K, Fazeny B, Dittrich C, Scheithauer W. Amonafide as first-line chemotherapy for metastatic breast cancer. *Eur J Cancer* 1994; 30A:398-400.
26. Craig J, Crawford E. Phase II trial of amonafide in advanced prostate cancer. *Proc Am Soc Clin Oncol* 1989; 8:147.
27. Allen SL, Budman DR, Fusco D, Kolitz J, Kreis W, Schulman P, Schuster M, DeMarco L,

Marsh J, Haptas K, Fischkoff S. Phase I trial of Amonafide + cytosine arabinoside for poor risk acute leukemias. *Proc Am Assoc Cancer Res* 1994; 35:225.

28. Felder TB, McLean MA, Vestal ML, Lu K, Farquhar D, Legha SS, Shaw R, Newman RA. Pharmacokinetics and metabolism of the antitumor drug Amonafide (NSC-308847) in humans. *Drug Metab Dispos* 1987; 15:773–778.
29. Ratain MJ, Mick R, Berezin F, Janisch L, Schilsky RL, Vogelzang NJ, Lane LB. Phase I study of Amonafide dosing based on acetylator phenotype. *Cancer Res* 1993; 53:2304–2308.
30. Wakelin LPG, Waring MJ. DNA intercalating agents. In: Hansch C, Sammes PG, Taylor JB, eds. *Comprehensive Medicinal Chemistry,* vol. 2. Oxford: Pergamon, 1990:718–721.
31. Braña MF, Castellano JM, Morán M, Pérez de Vega MJ, Romerdahl CR, Qian X-D, Bousquet P, Emling F, Schlick E, Keilhauer G. Bis-naphthalimides: a new class of antitumor agents. *Anti-Cancer Drug Design* 1993; 8:257–268.
32. Braña MF, Castellano JM, Morán M, Perez de Vega MJ, Qian X-D, Romerdahl CA, Keilhauer G. Bis-naphthalimides. 2. Synthesis and biological activity of 5,6-acenaphthalimidoalkyl-1,8-naphthalimidoalkyl amines. *Eur J Med Chem* 1995; 30:235–239.
33. Le Pecq JB, Le Bret M, Barbet J, Roques B. DNA polyintercalating drugs: DNA binding of diacridine derivatives. *Proc Natl Acad Sci USA* 1975; 72:2915–2919.
34. Bousquet PF, Braña MF, Conlon D, Fitzgerald KM, Perron D, Cocchiaro C, Miller R, Moran M, George J, Qian X-D, Keilhauer G, Romerdahl CA. Preclinical evaluation of LU 79553: A novel Bis-naphthalimide with potent antitumor activity. *Cancer Res* 1995; 55:1176–1180.
35. Goldin A, Venditti JM, MacDonald JS, Muggia FM, Henney JE, DeVita JT Jr. Current results of the screening program at the division of cancer treatment, NCI. *Eur J Cancer* 1981; 17: 129–142.
36. Chen SF, Behrens DL, Behrens CH, Czerniak PM, Dexter DL, Dusak BL, Fredericks JR, Gale KC, Gross JL, Jiang JB, Kirshenbaum MR, McRipley RJ, Papp LM, Patten AD, Perrella FW, Seitz SP, Stafford MP, Sun JH, Sun T, Wuonola MA, Von Hoff DD. XB596, a promising bis-naphthalimide anti-cancer agent. *Anticancer Drugs* 1993; 4:447–457.
37. McRipley RJ, Burns-Horwitz PE, Czerniak PM, Diamond RJ, Miller LD, Page RJ, Dexter DL, Chen S-F, Sun J-H, Behrens CH, Seitz SP, Gross JL. Efficacy of DMP-840: a novel bis-naphthalimide cytotoxic agent with human solid tumor xenograft selectivity. *Cancer Res* 1994; 54:159–164.
38. Kirshenbaum MR, Chen S-F, Behrens CH, Papp LM, Stafford MM, Sun J-H, Behrens DL, Fredericks JR, Polkus ST, Sipple P, Patten AD, Dexter D, Seitz SP, Gross JL. (R,R)-2,2′-[1,2-Ethanediylbis [imino(1-methyl-2,1-ethandiyl)]]-bis[5-nitro-1H-benz[de]isoquinoline 1,3-(2H)-dione] dimethanesulfonate (DMP 840), a novel bis-naphthalimide with potent non-selective tumoricidal activity in vitro. *Cancer Res* 1994; 54:2199–2206.
39. Houghton PJ, Cheshire PJ, Hallman JC, Gross JL, McRipley RM, Sun J-H, Behrens CH, Dexter DL, Houghton JA. Evaluation of a novel bis-naphthalimide anticancer agent, DMP 840, against human xenografts derived from adult, juvenile, and pediatric cancers. *Cancer Chemother Pharmacol* 1994:33:265–272.
40. Slichenmyer W, Finizio M, Sartorius S, Rowinsky E, Lai C-M, Grochow L, Pieniaszek H, O'Reilly S, Bunitsky K, Brogdon B, Mabring D, Shifflet C, Donehower R. Phase I and pharmacologic study of DMP 840 as a single infusion every three weeks. *Proc Am Soc Clin Oncol* 1994; 13:142.
41. Cobb P, Burris H, Finizio M, Lai C, Eckardt J, Fields S, Kuhn J, Nelson J, Bunitsky K, Pieniaszek H, Brogdon B, Von Hoff D. Phase I trial and pharmacokinetic study of a new bis-naphthalimide, DMP 840, given daily × 5. *Proc Am Soc Oncol* 1994; 13:159.
42. Maroun J, Stewart D, Goel R, Goss G, Verma S, Yau J, Finizio M, Lai C-M, Bunitsky K, Pieniaszek H, Brodgon B. Phase I Pharmacokinetic study of DMP 840 given in a 3 weekly dosage in a 5 weeks schedule. *Proc Am Soc Clin Oncol* 1994; 13:151.
43. O'Reilly S, Rowinsky EK, Grochow L, Adjei A, Bowling K, Slichenmyer W, Sartorius S, Finizio M, Gray JR, Pieniaszek HJ, Peterman VC, Mabring D, Donehower KC. Phase I and pharmacologic study of DMP 840 as a 24 hour infusion in patients with solid tumors. *Proc Am Soc Clin Oncol* 1995; 14:472.
44. Cobb P. Burris H, Drengler R, Fields S, Smith L, White I, Pieniaszek H, Gray J, Peterman V, Finizio M, Von Hoff D. Phase I trial of DMP 840 given as a 120-hour continuous infusion every 28 days. *Proc Am Soc Clin Oncol* 1995; 14:484.

II NEWER STRATEGIES AND TARGETS

11 The Enediynes

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CONTENTS

1. INTRODUCTION

In 1965, Ishida et al. (1) reported the identification of an antimicrobial compound isolated from *Streptomyces carzinostaticus*. This compound was given the designation "neocarzinostatin" and was thereafter determined to be an antimitotic agent (*2*). What followed was a long series of structural and chemical studies of neocarzinostatin, largely spearheaded by Goldberg and his colleagues (reviewed in *3*), and culminating in clinical trials in Japan and the US (*4–7*). The Japanese experience demonstrated difficulty with drug delivery to solid tumors and rapid clearance of the drug from the bloodstream (*8*). The identification of these limitations led to a series of derivitizations of neocarzinostatin, in an effort to reproduce the demonstrated in vitro efficacy of this drug in an in vivo setting. In addition to lack of efficacy in solid tumors, the American experience was marked by problems with drug toxicity, both of dose-related and idiosyncratic types (*4,7*), and led to curtailment of preclinical and clinical studies of this drug in this country, and efforts to synthesize and isolate novel enediyne compounds that might not engender these difficulties. This chapter deals with the enediynes as a class. Because of its rich structural and mechanistic history, and because of the reliance of all subsequent developments in this area on prior studies involving this prototypic compound, neocarzinostatin is discussed in detail in each section, followed by a discussion of the compounds, both natural and synthesized, which followed in its wake.

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

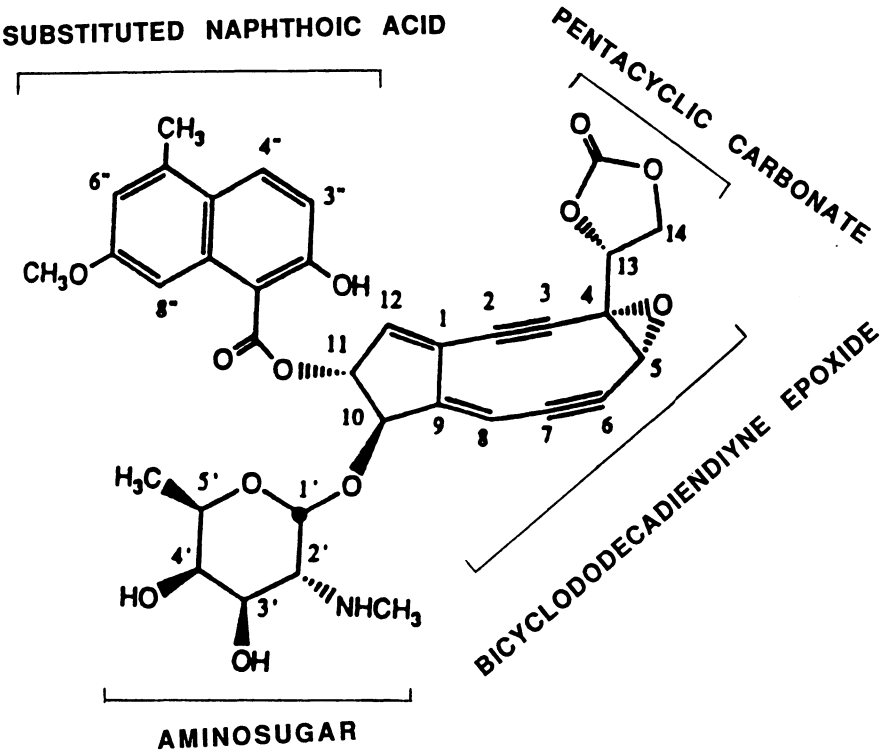

Fig. 1. Structural components of neocarzinostatin chromophore. The molecule consists of four functional components to each of which has been ascribed a role in the cellular activity of the drug.

2. CHEMISTRY AND MECHANISM OF ACTION

Neocarzinostatin consists of a 113-residue protein (M_r = 11,000) noncovalently associated with a chromophore of M_r 540 (*9–11*). The biological activity of the molecule lies primarily in its chromophore (*12,13*), and it is after this component of neocarzinostatin and other natural enediynes that all of the newer synthesized enediynes have been modeled (*14,15*). The chromophore consists of four structural subunits: a substituted naphthoic acid, an amino sugar, a pentacyclic carbonate, and a bicyclododecadiendiyne epoxide (*16*; *see* Fig. 1). Although the epoxide is the "business end" or "warhead" (*17*) of the molecule, required for DNA strand cleavage and adduct formation (*see this section, below*), the other components play important roles in the cytotoxicity of neocarzinostatin. The naphthalene moiety is believed to be important for minor groove binding of the drug; the amino sugar facilitates drug binding to the phosphate backbone of DNA; and the cyclic carbonate appears to mediate traversing of the drug across the cell membrane (*16*).

Despite the primary importance of the chromophore for the activity of neocarzinostatin, recent studies suggest that the protein does more than just "protect" the chromophore from extracellular inactivation. Modification of the protein moiety can alter cellular uptake of neocarzinostatin chromophore (*18*), which is believed in large measure to dissociate from the protein en route across the cell membrane (*19,20*).

The principal mode of action of neocarzinostatin is by the induction of DNA breaks, most of them single-stranded, preferentially at thymidine and adenine residues in AT-rich regions (*11*). The initial interaction of the chromophore with DNA is a two-step process (*21*). First, an external complex is formed between the amino sugar of the drug and the phosphate backbone of the DNA. Next, there is a slow "internalization" of the drug into the minor groove.

Single-strand breaks so predominate in the action of neocarzinostatin that it was originally thought that the occasional double-stranded breaks that arose were the result of close apposition of two randomly placed single-stranded breaks on opposite strands (*12,22*). It has recently become clear, however, that these double-stranded breaks are the result of direct, bistranded cleavage at AGC·GCT sequences (*23–25*).

The reaction of neocarzinostatin with DNA can result in several outcomes: DNA strand breakage, the release of free thymidylic aldehyde, or the formation of adducts between the chromophore and DNA. Both the cleavage of DNA and the liberation of free base aldehydes by neocarzinostatin require oxygen and sulfhydryl reagents. In contrast, the formation of DNA-neocarzinostatin adducts occurs largely under anaerobic conditions (*16*). Furthermore, the requirement of the former reactions for oxygen is independent of that for sulfhydryl groups; that is, in the presence of oxygen, one can observe the formation of spectrophotometrically different products depending on the presence or absence of sulfhydryl reagents (*26*). The kinetics of these reactions suggest that there is sequential reaction with neocarzinostatin of the sulfhydryl reagent followed by the oxygen molecule. The reaction of one sulfhydryl group with the neocarzinostatin chromophore is enough to change its fluorescence spectrum. However, the reaction of the second sulfhydryl reagent molecule with the chromophore requires the prior oxidation of the chromophore molecule (*26*).

Neocarzinostatin has also been found to inhibit *de novo* DNA synthesis (*12*) and to induce mutagenesis, especially at G·C pairs (*27*). However, the roles of these activities in the antineoplastic efficacy of neocarzinostatin are not known.

Since the time of discovery of neocarzinostatin, several other enediynes have been both isolated from natural sources or synthesized *de novo*. All of these compounds have some structural and mechanistic similarity to neocarzinostatin. The first of these to be described was calicheamicin γ^{1}_{1} (*28*). Calicheamicin was isolated from fermentation broths of *Micromonospora echinospora* ssp. calichensis, and, like neocarzinostatin, it binds in the minor groove of DNA and requires oxygen, but not peroxide or superoxide, for its action. Unlike neocarzinostatin, however, calicheamicin consists of a chromophore unassociated with a protein (*28*), and makes sequence-specific double-stranded cuts in DNA (*28,29*). Its particular sequence specificity is apparently partially determined by the carbohydrate domain of this molecule (*30*), although other structural components of the molecule also contribute to this property (*31,32*). Esperamicin A_1 is another enediyne chromophore that makes single-stranded cuts in DNA that differ from those made by neocarzinostatin in their particular base and sequence selectivity. Esperamicin requires thiol, heat, or UV-light activation analogous to that required by neocarzinostatin (*33,34*). Dynemicin A, a hybrid compound containing anthraquinone and enediyne cores, also requires thiol or UV-light activation (*35,36*), but has nicking sequence selectivity that differs from those of esperamicin and calicheamicin. This enediyne is also a natural product, isolated from the fermentation broth of *Micromonospora chersin (37*). It induces double-stranded cleavage to

the 3′-side of a purine base and staggered by 3 bases on opposite strands. Molecular modeling studies indicate that this cleavage pattern can be predicted from the intercalation position and capacity for enediyne activation of the anthraquinone subunit (*38,39*). Recently, new natural products with a protein component analogous to aponeocarzinostatin have been described (*40–42*). One of these, C1027, cleaves duplex DNA to produce single-strand breaks, double-strand breaks, and abasic sites in the absence of thiols (*43*).

By modeling compounds after dynemicin. A, Nicolaou and colleagues (*14*) have synthesized a series of "self-triggering" enediynes that do not require sulfydryl compounds for activation. They have made the observation that "pretriggered" enediynes (called stable enediynes) in their series interfere in a competitive fashion with the biological activity of unstable or reactive enediynes, raising the possibility that there is a non-DNA intracellular receptor for the enediynes (*15*).

3. CELLULAR EFFECTS

There are four principal effects of the enediynes on cells:

1. Mutagenicity;
2. Antimitotic activity associated with cell-cycle arrest;
3. Apoptosis induction; and
4. Differentiation induction.

These effects are not mutually exclusive, and some of them are undoubtedly the result of triggering of endogenous cell-determined responses to antimitosis or to particular mutations (*44*).

The mutagenicity of enediynes has been shown to be sulfhydryl-dependent and to vary with concentration in parallel with the cytotoxicity of these agents. That is, as the surviving fraction of cells diminishes, so increases the percentage of the remaining cells exhibiting a mutant phenotype. This has been interpreted to mean that the same mechanism, namely DNA cleavage, is responsible for both of these outcomes, and that the quantity of irreparable DNA damage determines whether cell death or mutation is seen in a particular cell or population. In any case, neocarzinostatin is mutagenic in bacteria, yeast, fungi, and mammalian cells (*45*). Although it had originally been said that cytolysis by neocarzinostatin is dependent only on the concentration of the drug present and not on the drug exposure time, these studies failed to take into account degradation of the chromophore over time at 37 °C, and subsequent studies have demonstrated independent exposure time- and concentration-dependent effects (whether cytolysis, mitotic arrest, or differentiation) of this agent (*46,47*). Hypertonicity of the bathing medium of the cell increases the DNA damage rate and decreases the DNA repair rate in response to neocarzinostatin treatment, but not to DNA crosslinking or intercalating agents (*48*). The radiosensitivity of tumor cells is enhanced by neocarzinostatin, as is the incidence of "potentially lethal damage" owing to X-irradiation; the degree of enhancement is inversely proportional to the DNA repair capabilities of the particular cell line, even though neocarzinostatin inhibits all repair regardless of the prior capabilities of the line. This almost certainly indicates that neocarzinostatin prohibits repair of residual damage after irradiation, so that the less damage that has been repaired prior to neocarzinostatin addition, the more damaged a cell remains (*49,50*).

Enediynes act as antimitotic agents by inducing a temporary G2 delay in several cell lines (*51,52*). Cells remain blocked at G2M for some period of time, demonstrating a decreased mitotic index at 1 h, followed by an "overshoot" at 48 h after drug washout. In neural crest tumor cells, the antimitotic effect is irreversible, perhaps because the G2M block is lengthy enough to trigger an endogenous cellular program for differentiation, and in these cells, the differentiated state is a post mitotic one (*19,47,53*). Although poly-ADP ribosylation of chromatin protein increases in HeLa cells that undergo G2 delay after neocarzinostatin treatment, inhibitors of this chemical process do not universally inhibit the cell-cycle changes, and the order of potency of the inhibitors that work on both processes is not the same. This implies that these two events are not causally related (*51*).

Recently, apoptosis induction has been described after enediyne treatment of a variety of cell types (*15,54,55*). Stable designed enediynes appear to block apoptosis induction by reactive designed enediynes (*15*), implying that there is a receptor–ligand-like interaction involved in this process. However, this competitive inhibition is not universally seen with natural enediynes, such as calicheamicin (*56*). Although oligonucleosomal-length cleavage fragments could be produced simply by the direct cleavage of DNA by enediynes in the face of an intact nucleosomal structure, kinetic studies of the cleavage process, and its dependence on protein synthesis and the presence of an endonuclease in at least some cell types make it likely that direct cleavage by the enediynes triggers an endogenous program for cell death (*55*).

In some neural crest cells treated with enediynes, morphological differentiation results (*19,44,53*). The precise nature of the differentiation (e.g., whether neuron-like or Schwann cell-like) and the determination of whether a particular cell undergoes apoptosis or differentiation are functions of the endogenous properties of that cell, rather than the concentration or nature of the enediyne with which it is treated (*44*). Furthermore, even in cases where morphological differentiation along neural lines is apparent, the biochemical accompaniments of this morphological change do not always parallel normal differentiation (*57*).

Finally, in cell-culture studies, two distinct types of resistance to neocarzinostatin and related enediynes have been described. In one, enediyne resistance is part of a multidrug resistance phenotype, with overexpression of P-glycoprotein, and reversal of the phenomenon with verapamil (*58,59*). In the other, resistance to neocarzinostatin alone is seen, and is not accompanied by crossresistance to other enediynes or other chemotherapeutic agents. The precise mechanism underlying the latter phenomenon is not known (*58*).

4. ANTITUMOR EFFECTS OF NEOCARZINOSTATIN: PRECLINICAL AND CLINICAL STUDIES

Since early reports of efficacy of neocarzinostatin against L1210 leukemia in mice (*60*), this drug has been examined in several of Phase I and Phase II trials for a variety of hematological and solid malignancies. Because pharmacokinetic studies indicated that neocarzinostatin has a short serum half-life, undergoes rapid renal clearance, and is concentrated in the kidney and bladder, Sakamoto et al. (*5*) undertook a study of newly diagnosed patients with bladder carcinoma confined to the primary organ. Of these 30 patients, each of whom received three or four courses consisting of 1 mg

neocarzinostatin iv daily for 7 d followed by a 7-day "rest period," 2 attained complete response, 21 attained partial response, and 6 did not respond to therapy. In all cases, neocarzinostatin treatment was followed by transurethral resection of residual tumor. At followup (12–46 mo), 5 of the 30 patients had recurrences of their tumor. The published recurrence rate for bladder carcinoma with transurethral resection alone is 25–70% within 1 yr and 90% within 2 yr. Toxicity in all patients was reversible and tolerable. The most common problem was myelosuppression, which apparently remitted during the 7-d rest period, and exhibited cumulative severity, which limited the total number of courses of drug that could be given to any patient. Three of the patients treated had transient elevations in SGOT without related symptoms.

These encouraging results led to the undertaking of a Phase II study that included patients with metastatic bladder, prostate, or hepatic carcinoma (*4*). In contrast to the patients treated for local disease in an organ in which neocarzinostatin is known to attain high concentrations, only 1 of the 35 patients with metastatic disease (a patient with bladder carcinoma) had a partial response to treatment. Myelotoxicity was again the dose-limiting factor in these patients who received iv bolus therapy once daily for 5 d and repeated when their blood cell counts permitted. In addition, two patients sustained dose-limiting toxicity involving pulmonary fibrosis, which responded to steroid treatment and hepatic toxicity, respectively. One patient experienced anaphylaxis during a repeated course of the drug, and was successfully treated with supportive therapy and steroids. Subsequently, 122 patients with disseminated malignant melanoma were randomized to treatment with chlorozotocin, neocarzinostatin, or methyl-CCNU. Although toxicity of neocarzinostatin (thrombocytopenia, leukopenia, and one anaphylactic response) was said to be "tolerable" in this study, the therapeutic response was meager at best. Furthermore, in a Phase II trial of 53 patients with acute leukemia, chronic myelogenous leukemia (CML) in blast crisis, or a variety of solid tumors, only two of the patients with CML responded at all, and both of them relapsed by 6 wk following therapy (*7*). Thus, although there have been isolated reports since these larger trials of the efficacy of neocarzinostatin as a single, unmodified agent in the therapy of urinary tract malignancies (*61*), it appears that neocarzinostatin's role may be limited to therapy of tumors in particular sites where high local concentrations of the drug can safely be attained, and as a substrate for the development of more targeted agents and systems for drug delivery. In this regard, Maeda et al. (*8*) have developed a two-compartment theoretical model of neocarzinostatin as an intra-arterial agent for use in the therapy of malignant gliomas of the central nervous system. They cite the short serum half-life and long CSF half-life of the drug, its several orders of magnitude greater toxicity for glioma cells relative to normal glial elements, and its rapid clearance from the systemic circulation as evidence in favor of this strategy.

5. IMPROVING THE THERAPEUTIC INDEX OF ENEDIYNES

Problems with lack of efficacy in solid tumors, and chromophore-mediated bone marrow and protein-mediated anaphylactoid toxicity of neocarzinostatin have led to several types of attempts at improving the therapeutic index of the enediynes. One early effort to increase the delivery of neocarzinostatin to solid tumors began with the observation that, under angiotensin-induced hypertension, there appeared to be a

two-fold increase in neocarzinostatin delivery to tumors with no change in delivery to normal tissues (*62*). This turned out not to be practical, and more recent efforts in this regard include:

1. Conjugation of neocarzinostatin to a high-mol-wt polymer, which improves its pharmacokinetic properties and decreases the "exposure" of the protein to the immune system;
2. Conjugation of enediynes to monoclonal antibodies (MAb) to target their effects for particular tumor tissues;
3. Targeting of enediynes themselves or their activating (thiol) agents for particular tumor tissues; and
4. Synthesis of novel enediynes, which might have differential delivery to or activity in tumor cells relative to normal cells.

A brief discussion of each of these strategies follows.

The most widely studied modification of neocarzinostatin is the conjugation of this compound to a styrene-maleic acid copolymer (SMANCS; *63–68*). SMANCS demonstrates more rapid and more complete internalization by cells than neocarzinostatin (*64,65*), a finding that probably accounts in part for its improved efficacy against tumor lines. Furthermore, SMANCS appears to be a biologic response modifier, as well. The copolymer appears to elicit increased secretion of interferons γ and β (*66*), and to increase NK activity, macrophage cytostasis, cytotoxic T-lymphocyte activity, and delayed hypersensitivity to sheep erythrocytes (*68,69*). Conjugation of neocarzinostatin to a styrene-pyran copolymer perhaps leads to decreased bone marrow toxicity relative to either the native compound or SMANCS. This compound exhibits the same molar cell kill as neocarzinostatin, but has only 70% of the acute toxicity (demonstrated by spleen and bone marrow CFU-C assays) of the native compound, allowing higher doses to be used in the animal models in which it has been tried. It has been suggested that the decrease in toxicity is related to a more tumor-selective pattern of drug delivery, owing to differences between normal and neoplastic vasculature (*67*). In one study of hepatocellular carcinoma patients who were given intra-arterial SMANCS dissolved in the lipid lymphographic agent ethiodol, radiographic, histologic, and serologic (α-fetoprotein levels) evidence demonstrates promising antitumor activity of this preparation in this particular localized malignancy (*70*). A method has also been proposed for rescuing patients from the bone marrow toxicity of SMANCS by taking advantage of the more rapid uptake of this compound into tumors relative to normal tissues and by using Tiopronin (*N*-2-mercaptopropionyl glycine) to inactivate residual SMANCS in the serum (*71*). Combining local (ip or ia, depending on the tumor model) neocarzinostatin therapy with iv Tiopronin appears to be a promising approach in regionally confined tumors (*72,73*).

Targeting of tumor tisues for cytotoxic effects by exploiting tumor-specific antigens and MAb made against them has been attempted with conjugates to many cytotoxic agents. The enediynes are no exceptions in this regard. Much of this work has involved the conjugation of neocarzinostatin to the MAb A7. This conjugate demonstrates marked selectivity for tumor relative to normal tissue and, therefore, has a better therapeutic index than neocarzinostatin alone in several tumor models (*74*). Specific localization of drug has been shown for SC xenografts of colon carcinoma (*75,76*) and pancreatic carcinoma (*77,78*). This improvement in therapeutic index is thought to be not only a function of the specific binding of the conjugate to antigens

on tumor cells, but also the result of improved serum half-life (both decreased renal clearance and increased stability with a decreased likelihood of dissociation of chromophore from the protein), as well (*79*). Other promising attempts at antibody conjugation to neocarzinostatin have included thioether linkage to an MAb to astrocytoma cells (*80*). In addition, several studies have proposed conjugating neocarzinostatin to anti-idiotype antibodies in order to effect specific cytolysis of antisingle-strand DNA targeting or antibody-producing cells in patients with disorders, such as systemic lupus erythematosis (*81–83*). Recently, a series of calicheamicin analogs have been conjugated to MAb with increased magnitude and specificity of the cytotoxicity for these malignant cells (*17*). It is particularly interesting that this study demonstrates that the structure–activity relationship obtained for a series of native calicheamicin analogs does not predict the relative activities of the MAb conjugates of these compounds.

Targeting of the toxicity of enediynes has also been attempted using strategies other than antibody conjugation. Taking advantage of the transferrin receptors on leukemia cells, Kohgo and colleagues (*84*) have improved the tumor cell uptake and in vitro efficacy of neocarzinostatin for K562 cells by conjugating neocarzinostatin to transferrin. The optimal ratio of transferrin to neocarzinostatin in such conjugates is 4.0. Greater proportions of transferrin compromise the DNA cleaving efficacy of the drug; greater proportions of neocarzinostatin compromise the binding of the conjugate to the transferrin receptor. The internalization of the transferrin·4(neocarzinostatin) conjugate proceeds at the same rate as transferrin internalization by these cells (*85*). Targeting has also been achieved in neuroblastoma cells by sulfhydryl loading of these cells using the neurotransmitter analog 6-mercaptodopamine prior to treatment with neocarzinostatin (*86*). Since neuroblastoma cells have an active dopamine uptake system, they concentrate 6-mercaptodopamine against a concentration gradient. Most normal cells that actively concentrate dopamine are postmitotic, and so are not readily affected by neocarzinostatin. Neocarzinostatin is therefore selectively activated and effective in neuroblastoma cells, and cytolysis can be effected at much lower concentrations than would ordinarily be required.

Efforts to design or engineer enediynes have led to the production of some that appear to have selectivity for tumor cells relative to normal bone marrow progenitors (*14*). The mechanism of this selectivity is not clear, but it raises the possibility that such enediynes could be used as therapeutic agents with tolerable toxicity, without the need for conjugation or coupling of these compounds to targeting molecules.

6. CONCLUSIONS

Enediynes have been shown to exhibit antineoplastic activity in a number of tissue-culture and animal models. To the extent that their toxicity is tolerable, they presently have clinical potential in particular localized tumors with pharmacological or anatomical (i.e., site-specific delivery) circumstances that permit attainment of tumoricidal concentrations of these drugs. Harnessing the cytotoxic activity and the known chemical mechanism of these drugs for general clinical use will require improvement of their therapeutic index. Approaches that show promise in this regard include increasing the selectivity of drug delivery and/or drug activation, and selectively rescuing normal tissues from enediyne toxicity.

REFERENCES

1. Ishida N, Miyazaki K, Kumagai K, Rikumaru M. Neocarzinostatin, an antitumor antibioticum of high molecular weight. *J Antibiot* 1965; 18:68-76.
2. Ebina T, Ohtsuki K, Seto M, Ishida N. Specific G_2 block in HeLa-S3 cells by neocarzinostatin. *Eur J Cancer* 1975; 11:155-158.
3. Goldberg IH. Mechanism of neocarzinostatin action: role of DNA microstructure in determination of chemistry of bistranded oxidative damage. *Accounts of Chem Res* 1991; 24:191-196.
4. Natale RB, Yagoda A, Watson RC, Stover DE. Phase II trial of neocarzinostatin in patients with bladder and prostatic cancer. *Cancer* 1980; 45:2836-2842.
5. Sakamoto S, Ogata J, Ikegami K, Maeda H. Chemotherapy for bladder cancer with neocarzinostatin: evaluation of systemic administration. *Eur J Cancer* 1980; 16:103-113.
6. Von Hoff DD, Amato DA, Kaufman JH, Falkson G, Cunningham TJ. Randomized trial of chlorozotocin, neocarzinostatin, or methyl-CCNU in patients with malignant melanoma. *Am J Clin Oncol (CCT)* 1984; 7:135-139.
7. McKelvey EM, Murphy W, Zander A, Bodey GP. Neocarzinostatin: report of a phase II clinical trial. *Cancer Treatment Rep* 1981; 65:699-701.
8. Maeda H, Sano Y, Takeshita J, Iwai Z, Kosaka H, Marubayashi T, Matsukado Y. A pharmacokinetic simulation model for chemotherapy of brain tumor with an antitumor protein antibiotic, neocarzinostatin. *Cancer Chemother Pharmacol* 1981; 5:243-249.
9. Meienhofer J, Maeda H, Glaser CB, Czombos J, Kuromizu K. Primary structure of neocarzinostatin, an antitumor protein. *Science* 1972; 178:875,876.
10. Napier MA, Holmquist B, Strydom DJ, Goldberg IH. Neocarzinostatin: spectral characterization and separation of a non-protein chromophore. *Biochem Biophys Res Commun* 1979; 89: 635-642.
11. Dasgupta D, Goldberg IH. Mode of reversible binding of neocarzinostatin chromophore to DNA: base sequence dependency of binding. *Nucleic Acids Res* 1986; 14:1089-1105.
12. Kappen LS, Goldberg IH. Mechanism of the effect of organic solvents and other protein denaturants on neocarzinostatin activity. *Biochemistry* 1979; 18:5647-5653.
13. Kappen LS, Napier MA, Goldberg IH. Roles of chromophore and apo-protein in neocarzinostatin action. *Proc Natl Acad Sci USA* 1980; 77:1970-1974.
14. Nicolaou KC, Dai W-M, Tsay S-C, Estevez VA, Wrasidlo W. Designed enediynes: a new class of DNA-cleaving molecules with potent and selective anticancer activity. *Science* 1992; 256: 1172-1178.
15. Nicolaou KC, Stabila P, Esmaeli-Azad B, Wrasidlo W, Hiatt A. Cell-specific regulation of apoptosis by designed enediynes. *Proc Natl Acad Sci USA* 1993; 90:3142-3146.
16. Lee SH, Goldberg IH. Role of epoxide in neocarzinostatin chromophore stability and action. *Mol Pharmacol* 1988; 33:396-401.
17. Hinman LM, Hamann PR, Wallace R, Menendez AT, Durr FE, Upeslacis J. Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. *Cancer Res* 1993; 53:3336-3342.
18. Schonlau F, Kohnlein W, Garnett MC. The acidic groups of the neocarzinostatin protein play an important role in its biological activity. *Mol Pharmacol* 1994; 45:1268-1272.
19. Schor NF. Neocarzinostatin induces neuronal morphology of mouse neuroblastoma in culture. *J Pharmacol Exp Ther* 1989; 249:906-910.
20. Kuroda Y, Sasaki T, Hoshino F. Unusual survival kinetics of neocarzinostatin-treated HeLa cells: its relation to the drug-inactivation. *J Radiat Res* 1991; 32:191-201.
21. Dasgupta D, Auld DS, Goldberg IH. Cryospectrokinetic evidence for the mode of reversible binding of neocarzinostatin chromophore to poly(deoxyadenylic-thymidylic acid). *Biochemistry* 1985; 24:7049-7054.
22. Poon R, Beerman TA, Goldberg IH. Characterization of DNA strand breakage *in vitro* by the antitumor protein neocarzinostatin. *Biochemistry* 1977; 16:486-493.
23. Dedon PC, Goldberg IH. Sequence-specific double-strand breakage of DNA by neocarzinostatin involves different chemical mechanisms within a staggered cleavage site. *J Biol Chem* 1990; 265:14,713-14,716.

24. Meschwitz SM, Schultz RG, Ashley GW, Goldberg IH. Selective abstraction of ^2H from C-1' of the C residue in AGC·ICT by the radical center at C-2 of activated neocarzinostatin chromophore: structure of the drug/DNA complex responsible for bistranded lesion formation. *Biochemistry* 1992; 31:9117-9121.
25. McAfee SE, Asley GW. Modulation of neocarzinostatin-mediated DNA double strand damage by activating thiol: deuterium isotope effects. *Nucleic Acids Res* 1992; 20:805-809.
26. Povirk LF, Goldberg IH. Stoichiometric uptake of molecular oxygen and consumption of sulfhydryl groups by neocarzinostatin chromophore bound to DNA. *J Biol Chem* 1983; 258: 11,763-11,767.
27. Povirk LF, Goldberg IH. Detection of neocarzinostatin chromophore-deoxyribose adducts as exonuclease-resistant sites in defined-sequence DNA. *Biochemistry* 1985: 24:4035-4040.
28. Zein N, Sinha AM, McGahren WJ, Ellestad GA. Calicheamicin γ^I_1: an antitumor antibiotic that cleaves double-stranded DNA site specifically. *Science* 1988; 240: 1198-1201.
29. Zein N, Poncin M, Nilakantan R, Ellestad GA. Calicheamicin γ^I_1 and DNA: molecular recognition process responsible for site-specificity. *Science* 1989; 244:697-699.
30. Drak J, Iwasawa N, Danishefsky S, Crothers DM. The carbohydrate domain of calicheamicin γ^I_1 determines its sequence specificity for DNA cleavage. *Proc Natl Acad Sci USA* 1991; 88: 7461-7468.
31. Walker S, Landovitz R, Ding WD, Ellestad GA, Kahne D. Cleavage behavior of calicheamicin γ^I and calicheamicin T. *Proc Natl Acad Sci USA* 1992; 89:4608-4612.
32. Uesugi M, Sugiura Y. New insights into sequence recognition process of esperamicin A_1 and calicheamicin γ^I_1: origin of their selectivities and "induced fit" mechanism. *Biochemistry* 1993; 32:4622-4627.
33. Uesawa Y, Kuwahara J, Sugiura Y. Light-induced DNA cleavage by esperamicin and neocarzinostatin. *Biochem Biophys Res Commun* 1989; 164:903-911.
34. Uesawa Y, Sugiura Y. Heat-induced DNA cleavage by esperamicin antitumor antibiotics. *Biochemistry* 1991; 30:9242-9246.
35. Shiraki T, Sugiura Y. Visible light induced DNA cleavage by the hybrid antitumor antibiotic dynemicin A. *Biochemistry* 1990; 29:9795-9798.
36. Sugiura Y, Arakawa T, Uesugi M, Shiraki T, Ohkuma H, Konishi M. Reductive and nucleophilic activation products of dynemicin A with methyl thioglycolate. A rational mechanism for DNA cleavage of the thiol-activated dynemicin A. *Biochemistry* 1991; 30:2989-2992.
37. Sugiura Y, Shiraki T, Konishi M, Oki T. DNA intercalation and cleavage of an antitumor antibiotic dynemicin that contains anthracycline and enediyne cores. *Proc Natl Acad Sci USA* 1990; 87:3831-3835.
38. Wender PA, Kelly RC, Beckham S, Miller BL. Studies on DNA-cleaving agents: computer modeling analysis of the mechanism of activation and cleavage of dynemicin-oligonucleotide complexes. *Proc Natl Acad Sci USA* 1991; 88:8835-8839.
39. Cardozo MG, Hopfinger AJ, Molecular mechanics and molecular dynamics studies of the intercalation of dynemicin-A with oligonucleotide models of DNA. *Mol Pharmacol* 1991; 40: 1023-1028.
40. Sakata N, S-Tsuchiya K, Moriya Y, Hayashi H, Hori M. Aminopeptidase activity of an antitumor antibiotic, C-1027. *J Antibiot* 1992; 45:113-117.
41. Matsumoto T, Okuno Y, Sugiura Y. Specific interaction between a novel enediyne chromophore and apoprotein in macromolecular antitumor antibiotic C-1027. *Biochem Biophys Res Commun* 1993; 195:659-666.
42. Zein N, Colson KL, Leet JE, Schroeder DR, Solomon W, Doyle TW, Casazza AM. Kedarcidin chromophore: an enediyne that cleaves DNA in a sequence-specific manner. *Proc Natl Acad Sci USA* 1993; 90:2822-2826.
43. Xu Y-J, Zhen Y-S, Goldberg IH. C1027 chromophore, a potent new enediyne antitumor antibiotic, induces sequence-specific double-strand DNA cleavage. *Biochemistry* 1994; 33:5947-5954.
44. Hartsell TL, Schor NF. Neuroblastoma cell type-specific apoptosis and morphological differentiation induced by enediyne agents. *Soc Neurosci Abst* 1994; 20:687.
45. DeGraff WG, Mitchell JB. Glutathione dependence of neocarzinostatin cytotoxicity and mutagenicity in Chinese hamster V-79 cells. *Cancer Res* 1985; 45:4760-4762.
46. Ozawa S, Inaba M. Kinetic analysis of the in vitro cell-killing action of neocarzinostatin. *Cancer Chemother Pharmacol* 1989; 23:279-282.

47. Will P, Guger KA, Schor NF. Effects of neocarzinostatin upon the development of tumors from murine neuroblastoma cells. *Cancer Chemother Pharmacol* 1994; 35:115–120.
48. Terado T, Kimura H, Ikebuchi M, Hill C, Aoyama T. The enhancement of cell lethality and changes in production and repair of DNA damage by hypertonic treatment after exposure to X rays, bleomycin, and neocarzinostatin. *Radiat Res* 1993; 135:189–196.
49. Antoku S, Kura S. Enhancement of radiosensitivity of cultured mammalian cells by neocarzinostatin. I. Inhibition of the repair of sublethal damage. *Int J Radiat Biol* 1990; 58:613–622.
50. Antoku S, Kura S. Enhancement of radiosensitivity of cultured mammalian cells by neocarzinostatin. II. Fixation of potentially lethal damage. *Int J Radiat Biol* 1990; 58:623–632.
51. Iseki S, Mori T. Effect of poly(adenosine diphosphate-ribose) polymerase inhibitors on neocarzinostatin-induced G_2 delay in HeLa-S3 cells. *Cancer Res* 1985; 45:4224–4228.
52. Xu Y-J, Li D-D, Zhen Y-S. Mode of action of C-1027, a new macromolecular antitumor antibiotic with highly potent cytotoxicity, on human hepatoma BEL-7402 cells. *Cancer Chemother Pharmacol* 1990; 27:41–46.
53. Falcione M, Milligan KD, Schwartz MC, Schor NF. Prevention of neocarzinostatin-induced cell death and morphologic change in SK-N-SH human neuroblastoma cells by continuous exposure to nerve growth factor. *Biochem Pharm* 1993; 46:731–738.
54. Hartsell TL, Schor NF. Treatment with neocarzinostatin induces apoptosis in cultured neuroblastoma cells. *Soc Neurosci Abst* 1993; 19:635.
55. Hartsell TL, Yalowich JC, Ritke M, Martinez AJ, Schor NF. Induction of apoptosis in murine and human neuroblastoma cell lines by the enediyne natural product neocarzinostatin. *J Pharmacol Exp Therap* 1995; 275:479–485.
56. Hartsell TL, Hinman LM, Hamann PR, Schor NF. Determinants of the response of neuroblastoma cells to DNA damage: The roles of pretreatment cell morphology and chemical nature of the damage. *J Pharmacol Exp Therap* 1996; 277:1158–1166.
57. Lowengrub JA, Schor NF. Neuronal "differentiation" of murine neuroblastoma cells induced by neocarzinostatin: Neural cell adhesion molecules. *Brain Res* 1993; 613:123–131.
58. Rauscher FJ III, Beerman TA, Baker RM. Characterization of auromomycin-resistant hamster cell mutants that display a multidrug resistance phenotype. *Mol Pharmacol* 1990; 38:198–206.
59. Miyamoto Y, Maeda H. Enhancement of verapamil of neocarzinostatin action on multidrug-resistant Chinese hamster ovary cells: possible release of nonprotein chromophore in cells. *Jpn J Cancer Res* 1991; 82:351–356.
60. Bradner WT, Hutchison DJ. Neocarzinostatin (NSC-69856): An antitumor antibiotic effective against ascitic leukemia L1210 in mice. *Cancer Chemother Rep* 1966; 50:79–84.
61. Satake I, Tari K, Yamamoto M, Nishimura H. Neocarzinostatin-induced complete regression of metastatic renal cell carcinoma. *J Urol* 1985; 133:87–89.
62. Abe I, Hori K, Saito S, Tanda S, Li Y, Suzuki M. Increased intratumor concentration of fluorescein-isothiocyanate-labeled neocarzinostatin in rats under angiotensin-induced hypertension. *Jpn J Cancer Res (Gann)* 1988; 79:874–879.
63. Maeda H, Ueda M, Morinaga T, Matsumoto T. Conjugation of poly(styrene-*co*-maleic acid) derivatives to the antitumor protein of neocarzinostatin: pronounced improvements in pharmacological properties. J Med Chem 1985; 28:455–461.
64. Oda T, Maeda H. Binding to and internalization by cultured cells of neocarzinostatin and enhancement of its actions by conjugation with lipophilic styrene-maleic acid copolymer. *Cancer Res* 1987; 47:3206–3211.
65. Oda T, Sato F, Maeda H. Facilitated internalization of neocarzinostatin and its lipophilic polymer conjugate, SMANCS, into cytosol in acidic pH. *JNCI* 1987; 79:1205–1211.
66. Suzuki F, Pollard RB, Maeda H. Stimulation of non-specific resistance to tumors in the mouse using a poly(maleic-acid-styrene)-conjugated neocarzinostatin. *Cancer Immunol Immunother* 1989; 30:97–104.
67. Yamamoto H, Miki T, Oda T, Hirano T, Sera Y, Akagi M, Maeda H. Reduced bone marrow toxicity of neocarzinostatin by conjugation with divinyl ether-maleic acid copolymer. *Eur J Cancer* 1990; 26:253–260.
68. Suzuki F, Matsumoto K, Schmitt DA, Pollard RB, Maeda H. Immunomodulating activities of orally administered SMANCS, a polymer-conjugated derivative of the proteinaceous antibiotic neocarzinostatin, in an oily formulation. *Int J Immunopharmacol* 1993; 15:175–183.
69. Suzuki F, Pollard RB, Uchimura S, Munakata T, Maeda H. Role of natural killer cells and

macrophages in the nonspecific resistance to tumors in mice stimulated with SMANCS, a polymer-conjugated derivative of neocarzinostatin. *Cancer Res* 1990; 50:3897-3904.
70. Konno T, Maeda H, Iwai K, Tashiro S, Maki S, Morinaga T, Mochinaga M, Hiraoka T, Yokoyama I. Effect of arterial administration of high-molecular-weight anticancer agent SMANCS with lipid lymphographic agent on hepatoma: a preliminary report. *Eur J Cancer Clin Oncol* 1983; 19:1053-1065.
71. Oda T, Yamamoto H, Miki T, Maeda H. Differential neutralizing effect on tiopronin on the toxicity of neocarzinostatin and SMANCS: a new rescue cancer chemotherapy. *Jpn J Cancer Res* 1989; 80:394-399.
72. Iwamoto Y, Kuroiwa T, Aoki K, Baba T. "Two-route chemotherapy" using high-dose intra-arterial neocarzinostatin and systemic tiopronin, its antidote, for rat limb tumor. *Cancer Chemother Pharmacol* 1986; 17:247-250.
73. Hasuda K, Kobayashi H, Kuroiwa T, Aoki K, Taniguchi S., Baba T. Efficacy of two-route chemotherapy using intraperitoneal neocarzinostatin and its antidote, intravenous tiopronin, for peritoneally disseminated tumors in mice. *Jpn J Cancer Res* 1989; 80:283-289.
74. Yamaguchi T, Tsurumi H, Kitamura K, Otsuji E, Miyagaki T, Kotani T, Takahashi T. Production, binding and cytotoxicity of human/mouse chimeric monoclonal antibody-neocarzinostatin conjugate. *Jpn J. Cancer Res* 1993; 84:1190-1194.
75. Takashina K-I, Kitamura K, Yamaguchi T, Noguchi A, Noguchi A, Tsurumi H, Takahashi T. Comparative pharmacokinetic properties of murine monoclonal antibody A7 modified with neocarzinostatin, dextran, and polyethylene glycol. *Jpn J. Cancer Res* 1991; 82:1145-1150.
76. Yamaguchi T, Tsurumi H, Kotani T, Yamaoka N, Otsuji E, Kitamura K, Takahashi T. *In vivo* efficacy of neocarzinostatin coupled with Fab human/mouse chimeric monoclonal antibody A7 against human colorectal cancer. *Jpn J. Cancer Res* 1994; 85:167-171.
77. Otsuji E, Yamaguchi T, Yamaoka N, Kitamura K, Yamaguchi N, Takahashi T. Intratumoral administration of neocarzinostatin conjugated to monoclonal antibody A7 in a model of pancreatic cancer. *J Surg Oncol* 1993; 53:215-219.
78. Otsuji E, Yamaguchi T, Yamaoka N, Taniguchi K, Kato M, Kotani T, Kitamura K, Takahashi T. Biodistribution of neocarzinostatin conjugated to chimeric Fab fragments of the monoclonal antibody A7 in nude mice bearing human pancreatic cancer xenografts. *Jpn J. Cancer Res* 1994; 85:530-535.
79. Gottschalk U, Garnett MC, Ward RK, Mailbucher A, Kohnlein W. Increased serum stability and prolonged biological half-life of neocarzinostatin covalently bound to monoclonal antibodies. *J Antibiot* 1991; 44:1148-1153.
80. Kondo S, Nakatsu S, Sakahara H, Kobayashi H, Konishi J, Namba Y. Antitumor activity of an immunoconjugate composed of anti-human astrocytoma monoclonal antibody and neocarzinostatin. *Eur J Cancer* 1993; 29A:420-423.
81. Sasaki T, Muryoi T, Takai O, Tamate E, Ono Y, Koide Y, Ishida N, Yoshinaga K. Selective elimination of anti-DNA antibody-producing cells by antiidiotypic antibody conjugated with neocarzinostatin. *J Clin Invest* 1986; 77:1382-1386.
82. Sasaki T, Tamate E, Muryoi T, Takai O, Yoshingaga K. In vitro manipulation of human anti-DNA antibody production by anti-idiotypic antibodies conjugated with neocarzinostatin. *J Immunol* 1989; 142:1159-1165.
83. Harata N, Sasaki T, Osaki H, Saito T, Shibata S, Muryoi T, Takai O, Yoshinaga K. Therapeutic treatment of New Zealand mouse disease by a limited number of anti-idiotypic antibodies conjugated with neocarzinostatin. *J Clin Invest* 1990; 86:769-776.
84. Kohgo Y, Kondo H, Kato J, Sasaki K, Tsuchima N, Nishisato T, Hirayama M, Fujikawa K, Shintani N, Mogi Y, Niitsu Y. Kinetics of internalization and cytotoxicity of transferrin-neocarzinostatin conjugate in human leukemia cell line, K562. *Jpn J. Cancer Res* 1990; 81:91-99.
85. Sasaki T, Kohgo Y, Kato J, Kondo H, Niitsu Y. Intracellular metabolism and cytotoxicity of transferrin-neocarzinostatin conjugates of differing molar ratios. *Jpn J. Cancer Res* 1993; 84: 191-196.
86. Schor NF. Targeted enhancement of the biological activity of the antineoplastic agent, neocarzinostatin: Studies in murine neuroblastoma cells. *J Clin Invest* 1992; 89:774-781.

12 Matrix Metalloproteinase Inhibitors

William G. Stetler-Stevenson, MD, PhD

CONTENTS

1. EXTRACELLULAR MATRIX DEGRADATION IN CANCER PROGRESSION

1.1. Historical Perspective

The contribution of extracellular matrix turnover to the invasive phenotype of cancer cells has long been recognized. Hippocrates (460–370 BC), who described invading tendrils of tumor tissue and the resulting destruction of bone and soft tissue, ascribed this behavior to an imbalance of the "Four Humors" resulting in a local excess of one of these, which he called "black bile" (*1*). This theory was later extended by Galen (131–203 AD), who proposed that the "black bile" was concentrated in areas of tumor invasion (*2*). Our modern view of the process of cancer invasion and metastasis formation is remarkably similar to these early hypotheses, except that we recognize various lytic enzymes as major components of Hippocrates' "black bile." Furthermore, Hippocrates' concept of a localized imbalance of "Humors" is essentially identical to our current understanding that extracellular matrix degradation and tumor cell migration are dependent on a critical balance between activated proteases and their endogenous inhibitors.

The current view on the role of proteases in cancer metastasis has been refined considerably over the last 20 years. The function of proteases in cancer invasion is now replete with many details, and the role of specific classes of proteases has been defined.

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

As a result of these studies, several specific proteases have been identified as potential therapeutic targets for novel cancer therapy. Members of the matrix metalloproteinases are one such group. The first members of this family of proteases were the collagenases that were identified through their ability to degrade the collagen triple helix selectivity. However, the role of these proteolytic activities in cancer progression was not appreciated for many years.

In the 1960s and early 1970s, investigators frequently noted the association of a variety of protease activities with invasive tumor cells, including both lysosomal and neutral protease activities (*3–9*). It had long been recognized that native collagen in fibrillar form was extremely resistant to protease action at neutral pH. Collagenases that selectively degrade these fibrillar collagens within the triple helical domain were initially identified in resorbing tadpole tail and in mammalian tissues showing evidence of collagen breakdown (*10,11*). In the late 1960s and early 1970s, collagen-degrading activities were reported in association with both epithelial and mesenchymal malignancies. These included basal cell carcinomas of the skin, melanoma, squamous cell carcinomas, soft tissue sarcomas, and breast cancer tissues (*12–20*). Collagenase activities were also obtained from tumor explants. These collagenases were poorly characterized, and it was difficult to determine if they originated from tumor cells or host response. Furthermore, the correlation between these collagenase activities and the metastatic potential of the tumor was, at best, only broadly demonstrated.

In 1977, Lance Liotta and colleagues used the T241 mouse fibrosarcoma model to demonstrate that tumor cells obtained from the venous effluent of a primary tumor possessed significantly higher basement membrane-degrading and type I collagen matrix-degrading activity when compared to cells of the primary tumor (*21*). This suggested that metastasizing tumor cells form a distinct subpopulation within the primary tumor and that these cells possessed enhanced invasive potential represented in part by extracellular matrix-degrading protease activity. In that same year, Kuettner and colleagues (*22*) published a report that described the production of collagenase activity by human osteosarcoma and mammary carcinoma cell lines. This activity was inhibited by a cartilage-derived protein of low molecular weight. Since cartilage is rarely invaded by neoplasms, the authors concluded that the activity of the collagenase and, subsequently, its regulation by the cartilage-derived inhibitor played an important role in tumor cell invasion. These two studies represent a turning point in the view of investigators toward the role of proteases in cancer progression. For the first time, the research focused on degradation of specific components of the extracellular matrix, and this activity was correlated with the aggressive behavior of subpopulations of tumor cells. They are also the first indication that inhibition of collagenase activity might be a useful therapeutic strategy for blocking the spread of tumor cells.

1.2. Basement Membrane as a Barrier to Tumor Invasion

Liotta and colleagues combined the results of these initial experiments with the observation that destruction of basement membranes is often associated with tumor invasion, to formulate a working hypothesis that tumor cells selectively degrade components of the basement membrane (*23*). Concurrent investigations by other laboratories analyzed the molecular components of the basement membrane and characterized collagen-degrading enzymes from a variety of sources. Type IV collagen was shown to be a major structural component of basement membranes and distinct from

the fibrillar collagens previously identified (*24–26*). Specific type IV collagen-degrading activity was first described by Liotta et al. in 1979 (*23*). This activity was derived from the culture media of the T241 metastatic murine fibrosarcoma cell line, and was shown to degrade type IV collagen selectively, generating specific N-terminal 1/4 and C-terminal 3/4 cleavage products. This activity had a molecular mass of approx 65–72 kDa, pH optima around 7.0, and was sensitive to chelating agents suggesting that it was also a neutral metalloproteinase activity. However, the relationship of this type IV collagenase to dermal fibroblast collagenase was not appreciated for several years. Primary sequence information on this interstitial collagenase and on the tumor type IV collagenase would subsequently reveal that these enzymes constituted the first members of a new family of proteases that has become known as the matrix metalloproteinase (MMP) family (*27,28*). The mammalian interstitial collagenase is recognized as the first member of this family and is often referred to as MMP-1 (*27,28*). The 72-kDa type IV collagenase, first isolated by Liotta, is recognized as the second member and is referred to as MMP-2. Current terminology also refers to this protease as gelatinase A in recognition of the potent gelatinolytic activity that this protease possesses (*27*). Several additional members of this family would also later be identified through their overexpression and association with tumor cells (i.e., transin [rat homolog of human stromelysin-1] [*29*], stromelysin-3 [*30*], and collagenase-3 [*31*]).

Subsequent experiments demonstrated a direct correlation between type IV collagen-degrading activity and metastatic potential of several murine tumor cell lines (*32*). These findings suggested that the basement membrane posed a significant barrier for tumor cell invasion. This concept formed the basis of many studies that attempted to characterize the interaction of tumor cells with the extracellular matrix and formed the basis of the three-step hypothesis of tumor cell interaction with the basement membrane (*33*). This hypothesis defines the invasive phenotype as consisting of three distinct phases: attachment of tumor cells to the basement membrane or extracellular matrix, creation of a proteolytic defect in the basement membrane, and migration of the tumor cells into this defect (*34*). Although initially presented over 10 years ago, this three-step model remains a useful framework for understanding the extracellular matrix–cell interactions that occur during successful cell invasion, whether pathologic or physiologic (*34*). This model highlights tumor cell-mediated extracellular matrix proteolysis as a central feature of tumor invasion and metastasis. It also presents a framework that can be used to test the role of various proteases in cancer invasion.

Subsequent investigations led to the development of in vitro invasion assays, which use either native (*35*) or reconstituted basement membranes (*36,37*). These assays have been useful in demonstrating the requirement of MMP activity during tumor cell invasion. In addition they have also been useful in screening metalloproteinase inhibitors prior to actual in vivo animal testing in either lung colonization or spontaneous metastasis models of tumor progression.

2. THE MMP AND TISSUE INHIBITOR OF METALLOPROTEINASE FAMILIES

2.1. MMPs

Investigation of the biochemistry and molecular biology of chronic diseases, such as arthritis and cancer, has revealed a critical role for extracellular matrix remodeling.

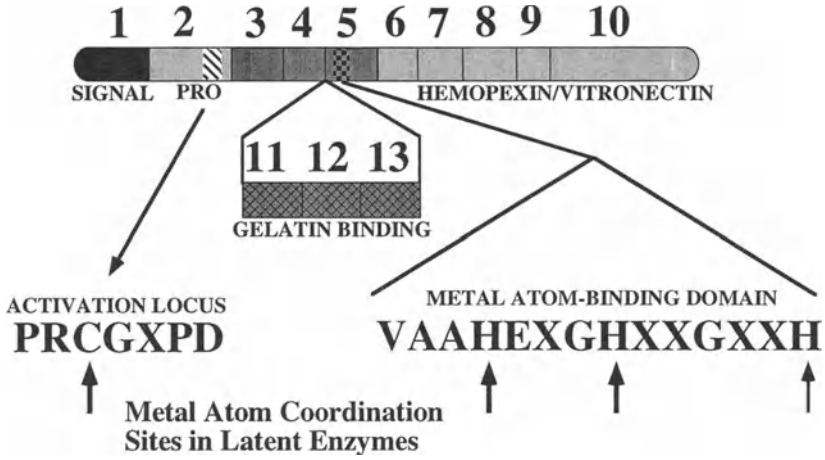

Fig. 1. Domain structure of the matrix metalloproteinases. General structure of a prototypic member of the MMP family is depicted. The exons encoding the full-length protein are represented as 1–10. These include a signal sequence, encoded in exon 1, for extrusion into the endoplasmic reticulum, as well as a prosegment, encoded in exon 2, that contains a highly conserved peptide sequence responsible for the lack of protease activity in the proenzyme. This prosegment contains a unpaired cysteine residue that coordinates the zinc atom of the active site resulting in latency. Exons 4, 5, and 6 encode for the catalytic domain that contains a signature metal atom binding domain that defines this family of proteases. The involvement of these three histidyl residues in zinc atom binding has been confirmed by X-ray diffraction analysis. The gelatinases contain a disruption of the catalytic domain represented by the insertion of three exons immediately upstream of the sequence encoding the metal-atom binding domain. These three exons encode for type II gelatin binding motifs originally observed in fibronectin. The carboxyl region of the MMP family members is the least conserved region. Many members of this protease family show sequence homology to hemopexin and/or vitronectin in this region as illustrated.

These studies also identified several unique matrix-degrading proteases overexpressed in tumor cells that are now known to be members of the MMP family. These are a group of related zinc metallo-endopeptidases with neutral pH optima, which collectively are capable of degrading most, if not all, of the structural components of the extracellular matrix, such as collagens, proteoglycans, and glycoproteins, like fibronectin and laminin (for general review of these enzymes, *see 28,38–40*). These enzymes share a number of common structural domains that identify members of this protease family (Fig. 1). The currently recognized members of the MMP family and their recognized substrates are listed in Table 1. These proteases are subgrouped according to substrate preference (*28,38,39*). The interstitial collagenases, which degrade fibrillar collagens, include fibroblast interstitial collagenase and neutrophil collagenase. The stromelysins have a broad range of substrates, including proteoglycans, glycoproteins, and nonhelical collagens, as well as other latent MMPs resulting in activation. This group includes stromelysin-1, stromelysin-2, and matrilysin. Stromelysin-3 is the newest member of this group and is included primary because of sequence homology, although its substrate specificity is not well defined.

The gelatinase subgroup possesses potent gelatin-degrading activity as well as selective activity against a variety of collagens, such as types IV, V, VII, X, and XI (*28*). This group also degrades other matrix components, such as elastin, fibronectin, and

Table 1
Matrix Metalloproteinase Family Members

Subgroup	*E.C. nomenclature/ other designations*	*MMP number*	*Molecular species*	*Substrates*
Interstitial collagenases				
	Interstitial collagenase (fibroblast type)	MMP-1	52, 57 kDa	Fibrillar collagens (III> > I)
	PMN collagenase	MMP-8	75 kDa	Fibrillar collagens (I> > III)
	Collagenase-3	MMP-13	54 kDa	Fibrillar collagen
Stromelysins				
	Stromelysin-1	MMP-3	52, 58 kDa	LMN, FBN core protein, other MMP nonhelical collagen
	Stromelysin-2	MMP-10	58 kDa	Same as MMP-3
	Stromelysin-3	MMP-11	29 kDa	α-1-Antitrypsin
	Matrilysin	MMP-7	28 kDa	Similar to MMP-3
Gelatinases				
	Gelatinase-A, 72 kDa type IV collagenase	MMP-2	72 kDa	Gel, col IV, col V FBN, VN, Elastin, col I, cell surface?
	Gelatinase-B, 92 kDa type IV collagenase	MMP-9	92 kDa	Gel, col IV, col V
Other				
	Metalloelastase	MMP-12	53 kDa	Elastin
	Membrane-type MMP	MMP-14	66 kDa	Progelatinase A

vitronectin. Gelatinase A, which is the same as the original 72-kDa type IV collagenase isolated by Liotta and colleagues, as well as gelatinase B, are the current members of this group. Newer members of the MMP family that are less well characterized include macrophage metalloelastase (MMP-12) (*41*) and collagenase-3 (MMP-13) whose expression appears to be selectively associated with human breast cancer (*31*). The newest member of this family contains a transmembrane domain and is referred to as membrane type MMP or MT-MMP (*42*). Some distinguishing features and known substrate specificities of these proteases are briefly outlined in Table 1.

These enzymes are characteristically produced and secreted into the extracellular milieu as proenzymes (*28,38,39*). MT-MMP is the obvious exception to this in that this enzyme is membrane-associated and thus not secreted, but it does appear to be initially produced as a proenzyme. The latency of the proenzyme form of all MMPs is the result of interaction of a highly conserved cysteine of the amino-terminal pro-domain with the zinc atom at the active site (*43*). The cysteine switch hypothesis states that disruption of this interaction results in proenzyme activation. This family of proteases is further defined by the fact that they are selectively inhibited by a group of endogenous inhibitors known as the tissue inhibitors of metalloproteinases or (TIMPs). The balance between the level of activated MMPs and available TIMPs determines the net MMP activity and is therefore a pivotal determinant of extracellular matrix turnover.

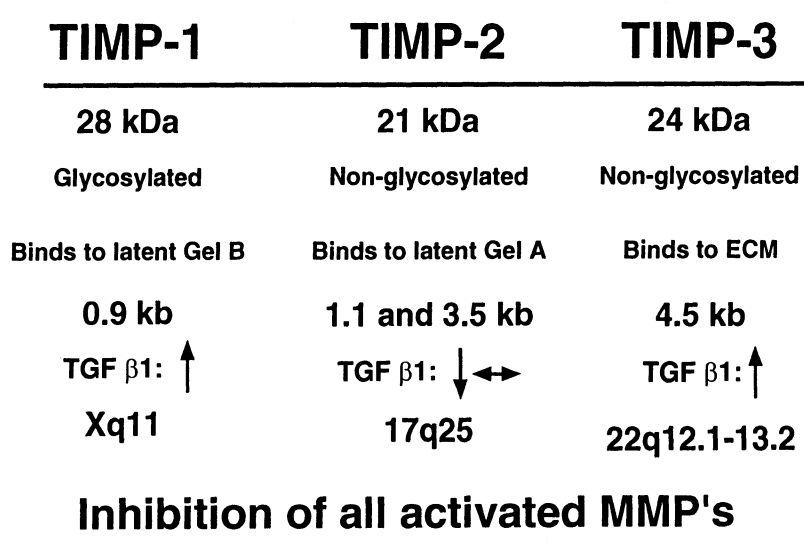

Fig. 2. Comparison of TIMP family members. The three currently recognized members of the TIMP family are compared and contrasted. Features compared are: molecular mass of the mature protein, glycosylation, unique binding properties, transcript sizes, modulation of gene expression by the action of TGFβ1, and chromosome location of the human gene. Finally, the activity that defines this group of inhibitors is their ability to inhibit all activated members of the MMP family.

2.2. TIMPs

Currently, there are three well-defined members of the TIMP family of inhibitors that share approx 40% homology at the amino acid level (*28,40*). These are TIMP-1, TIMP-2, and TIMP-3 (Fig. 2). The TIMPs bind with high affinity and 1:1 stoichiometry to active MMPs resulting in the loss of protease activity. All three proteins of this family contain 12 highly conserved cysteine residues. In TIMP-1 and TIMP-2, these cysteine residues have been shown to form disulfide bridges generating six peptide loops and two peptide knots. The exact mechanism of TIMP-mediated inhibition of MMP activity is currently not known.

3. THE ROLE OF MMPs IN CANCER

3.1. From Correlation to Causality

The rapid growth of information on new members of the MMP family has resulted in an explosion of studies on the role of specific MMPs in cancer cell invasion and metastasis formation. Many studies have assessed MMP expression at either the protein or nucleic acid level, and correlated this expression with invasive ability or metastatic potential of the tumor (*34*). These studies, performed both in vitro and in vivo using a variety of models, consistently demonstrate a direct correlation between the overexpression of MMPs, and increased invasive capacity or metastatic potential of a variety of tumor cell lines.

Similar techniques have been employed to study MMP expression directly in human tumor tissues. A direct correlation between MMP expression and the invasive phenotype of human tumor cells has been observed in lung, prostate, stomach, colon, breast,

Protease	IC-1	Gel A	St-1	Matr	Gel B	St-2	St-3
Tumor Type	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9	MMP-10	MMP-11
Breast		X			X		X
Prostate		X		X	X		
Ovary		X					
Lung	X	X					X
Colo-rectal	X	X		X	X		X
Gastric		X		X			
Thyroid		X					
Liver		X					
Oral (Squamous)	X	X	X	X			
Salivary gland		X					

Fig. 3. Expression of MMPs in human tumor tissues. The results of immunohistochemical and *in situ* hybridization analysis of MMP expression in human tumor tissues are summarized graphically. The results demonstrate the frequent detection of gelatinase A, matrilysin (MMP-7), and gelatinase B in a variety of tumor types. The abbreviations used are IC-1 for interstitial collagenase, Gel A for gelatinase A, St-1 for stromelysin-1, Matr for matrilysin, Gel B for gelatinase B, St-2 for stromelysin-2, and St-3 for stromelysin-3.

ovary, thyroid, and oral squamous cell cancers. Figure 3 summarizes these studies using human tumor tissues. These studies show that many members of the MMP family are overexpressed in human tumor tissue. Although gelatinase A is expressed in all tumor types examined, no single MMP is consistently overexpressed in every single tumor of a given histopathologic classification. This fact probably reflects tumor cell heterogeneity, possible variable MMP expression with tumor progression, as well as differential expression in response to changing extracellular matrices that are encountered during tumor progression.

Three members of the MMP family are overexpressed at a high frequency in many human tumor types. These include stromelysin-3, gelatinase A, and gelatinase B. Stromelysin-3 expression was initially observed in human breast cancer tissues where the level of expression correlated with tumor progression (*30*). More recent studies have demonstrated expression of stromelysin-3 mRNA in a high percentage of primary tumors in the lung (*44*), colon (*45*), squamous cell cancers of the head and neck (*46*), and as basal cell cancers of the skin (*47*). Although stromelysin-3 shows a very strong correlation with the presence of invasive carcinoma, the substrate for this protease remained elusive until recently. This enzyme has now been demonstrated to possess potent degrading activity against a member of the serpin family (**ser**ine **p**rotease **in**hibitor), α-1 proteinase inhibitor (*48*). This suggests that MMPs may cooperate with other proteases systems to facilitate matrix turnover.

The specific protease characterized by Liotta as a type IV collagenase of tumor cells is now referred to as gelatinase A. Studies have demonstrated the overexpression of this enzyme in many human tumor tissues, including breast, colon, thyroid, lung, gastric, prostate, and ovarian cancers (*34*). A recent study has demonstrated that

expression of pro-MMP-2 in human oral squamous cell carcinomas is associated with an increased frequency of lymph node metastases (*49*). Those authors suggest that this marker may be useful in evaluating the malignant potential of individual oral squamous cell tumors.

Frequently, the expression of progelatinase A is observed in human tumor tissues prior to the development of frankly invasive carcinoma (i.e., severe dysplasia and/or carcinoma *in situ* (*50*). This suggests that expression of this proenzyme may be necessary, but is not sufficient for acquisition of the invasive phenotype, and that regulation of progelatinase A activation is a key step during tumor cell invasion.

Recent studies on human tumor cell lines and tumor tissues confirm this hypothesis. Researchers have used the technique of gelatin zymography to assess the contribution of the activated (62-kDa) form of gelatinase A to the invasive phenotype of human breast and nonsmall-cell lung cancer (*51–53*). These studies demonstrate that although tumors from these tissues may express both progelatinase A and B, only a few tumors expressed small amounts of the activated form of gelatinase B. In contrast, the fraction of total gelatinase A present in the activated (62-kDa) form was consistently elevated in malignant disease and correlated with the tumor grade. Analysis of breast cancer cell lines demonstrates a correlation between the ability to activate progelatinase A with the invasive potential (*54*). Microdissection of human colorectal tumors and analysis of these microdissected tissues by zymography demonstrate a selective increase in the activated 62-kDa form of gelatinase A in the invasive components of these tumors (*55*).

These studies highlight the limitations inherent in the measurement of MMP overexpression by both immunohistochemistry and nucleic acid hybridization techniques. A key determinant of invasive behavior is MMP activation. For these enzymes, which are all secreted in the proenzyme form, overproduction of proenzyme and enzyme activity are not equivalent. Future experiments should focus on rigorously correlating tumor stage with levels of active enzyme or direct measurements of enzyme activity. However, the latter is clearly more difficult owing to the presence of endogenous inhibitors.

The correlative evidence presented above clearly suggests that the action of selected MMPs is required during tumor invasion and progression. These correlative data are strongly supported by studies modulating endogenous MMP inhibitors (i.e., TIMPs) to block tumor invasion and metastasis. These studies unequivocally demonstrate a causal role for MMPs in tumor invasion. Numerous studies have correlated low TIMP expression with enhanced invasive and metastatic properties in both murine and human tumor cell lines (*56,57*). TIMP-1 has been shown to inhibit metastasis in vivo in animal models using B16F10 murine melanoma cells or *ras*-transfected rat embryo fibroblasts (*58–60*). TIMP-2 transfection and overexpression in *ras*-transfected rat embryo fibroblast resulted in loss of lung colonizing ability following iv injection of these cells, but did not completely block the formation of pulmonary metastases from primary tumors following sc inoculation of tumor cells (*58,61*). Transfection and overexpression of TIMP-2 have also been shown to inhibit the growth of primary tumors following sc inoculation of *scid* mice with human melanoma cells, but again did not prevent metastasis formation (*61*). More recently, the ratio of metalloproteinase activity and TIMP-2 has been shown to be a critical determinant in the adhesion and spreading of human melanoma cells (*62*). Alteration of cell adhesion and spreading in turn alters the ability of tumor cells to migrate and invade. These are cell func-

tions that are essential for the fully competent metastatic phenotype. These experiments using TIMPs have defined the requirement for functional MMP activity during tumor cell invasion. This suggest that targeting the activity of these metalloproteinases may provide a clinically useful mechanism for blocking the local invasion and possibly the metastatic spread of cancer cells.

3.2. Angiogenesis

Endothelial cell invasion during angiogenesis shares a number of functional similarities with invasive tumor cells. This includes crossing a basement membrane connective tissue barrier and the production of MMPs (*63,64*). The requirement for MMP activity during angiogenesis has been demonstrated in several systems. TIMP-1 blocks basic fibroblast growth factor (bFGF)-induced endothelial cell invasion on human amnion membranes (*65*). Both TIMP-1 and TIMP-2 block polyamine-stimulated angiogenesis in the chick chorioallantoic membrane assay (*66*). More recently, Albini and colleagues demonstrated that TIMP-2 can inhibit the angiogenic response induced by Kaposi's sarcoma cell-conditioned media in vivo (*67*).

Schnaper and colleagues have shown that a critical balance of MMPs and TIMP influences endothelial cell morphogenesis in an in vitro model of angiogenesis (*68*). In these experiments, the addition of exogenous TIMPs inhibits endothelial tube formation on a reconstituted basement membrane matrix. This effect was also observed following treatment of the endothelial cell cultures with gelatinase A-neutralizing antibodies. Addition of exogenous purified gelatinase A resulted in enhanced tube formation, but this effect was reversed at excess gelatinase A concentrations. As has been observed with tumor cells for both MMPs and other protease systems, excessive protease activity can be detrimental to invasive behavior of endothelial cells (*62,69*).

Moses et al. have identified a cartilage-derived inhibitor (CDI) of angiogenesis that has collagenase-inhibitory activity and demonstrates amino acid sequence homology to the TIMPs (*70*). The relationship of this factor to the cartilage-derived collagenase inhibitor originally described by Kuettner and colleagues (*22*) is not known. CDI has been shown to inhibit endothelial cell migration and proliferation in response to bFGF, a major angiogenic growth factor produced by human tumors. Recent studies have also shown that TIMP-2 can inhibit the bFGF-induced proliferation of human microvascular endothelial cells (*71*). This effect was not seen with TIMP-1 or synthetic MMP inhibitors, such as Batimastat (*vide infra*). This identified a unique biological activity of TIMP-2 that is not related to metalloproteinase inhibitory activity. Furthermore, this implies that TIMP-2 may have additional actions that limit neovascularization associated with solid tumor growth and metastasis in vivo.

These findings suggest that the use of either endogenous or synthetic MMP inhibitors to block tumor cell invasion and metastasis may also disrupt the tumor-induced angiogenesis that is required for tumor growth and metastasis. The use of such agents as TIMP-2 may have additional biological activities that could be exploited clinically, i.e., inhibition of bFGF-induced endothelial cell growth.

4. CHANGING RATIONALE FOR TARGETING MMPs IN HUMAN CANCER

As outlined previously, the MMPs are clearly implicated in the processes of tumor cell invasion and angiogenesis. These processes of tumorigenesis and tumor-induced

angiogenesis are interdependent. Elaboration of angiogenic factors and induction of an angiogenic response is a phenotype clearly associated with more aggressive tumors (*72,73*). In turn, formation of new blood vessels permits the expansion of tumor foci (primary or metastatic) in three dimensions. Following vascularization, tumor foci undergo rapid local expansion and acquire an enhanced metastatic potential that correlates directly with the degree of vascularization of the primary tumor (*74–76*). Thus, tumor invasion, growth, and metastasis are closely linked to tumor-induced angiogenesis, and both seem to require MMP activity. This suggests that targeting these enzymes in cancer therapy may provide synergistic actions that result in unique therapeutic benefits.

As originally formulated, the strategy of inhibiting MMP activity was directed at preventing metastasis formation. This concept originally met with considerable resistance, because it is well known in the clinic that the majority of cancer patients have metastases at the time of diagnosis of their primary tumor. Thus, the initial presentation of the cancer patient usually occurs at a later stage in the disease when tumor cell invasion and dissemination have already occurred. However, several factors now indicate that inhibition of MMP activity, as therapeutic targets in cancer therapy, may have additional, previously unappreciated therapeutic consequences that are beneficial to the cancer patient. The most compelling of these is the possible use of these agents as cytostatic agents, which would prevent the growth of both primary tumor and metastatic foci. This may be a result of direct action of these agents on the tumor cells or a secondary effect through inhibition of MMP activity required for release of growth factors sequestered in the extracellular matrix or inhibition of tumor-induced angiogenesis required for tumor growth.

The conventional cytotoxic approach to cancer chemotherapy has had limited success in the treatment of advanced solid tumors, and recently the development of new cytotoxic strategies and agents has slowed. This trend, combined with the frequent development of drug resistance to known cytotoxic agents, has led many to explore targets that may lead to the development of a new type of chemotherapy, tumor cell cytostatic agents or "tumoriostatic" agents (Fig. 4). As described previously, endogenous MMP inhibitors, TIMPs, have been shown to inhibit the growth of the primary tumors in experimental models as well as block growth factor stimulation of endothelial cells. Thus, the MMPs are definitely within this class of therapeutic targets that engender cytostatic therapy, as are adhesion molecules, signal transduction pathways, growth factors, and angiogenic responses. The hope is that by the use of selective inhibitors for these targets, one can achieve a halt in tumor progression without significant toxicity. The potential utility of this cytostatic approach to cancer chemotherapy is best exemplified by the recent use of tamoxifen to treat invasive breast cancer (*77,78*).

Alternatively, the cytostatic strategy could be used as an adjuvant to conventional cytotoxic therapy to prevent regrowth of tumor between cycles of tumor cell killing. One could envisage alternating cycles of cytotoxic and cytostatic therapy. This could prevent tumor regrowth between cycles of cytotoxic agents. Enhancement of cytotoxic therapies that rely on active tumor cell replication might be achieved by initiating cytotoxic therapy immediately prior to release from cytostatic therapy. This could result in improved reduction of tumor burden, which would be amendable to continued cytostatic therapy (*see* Fig. 4). By limiting the size of the tumor cell population

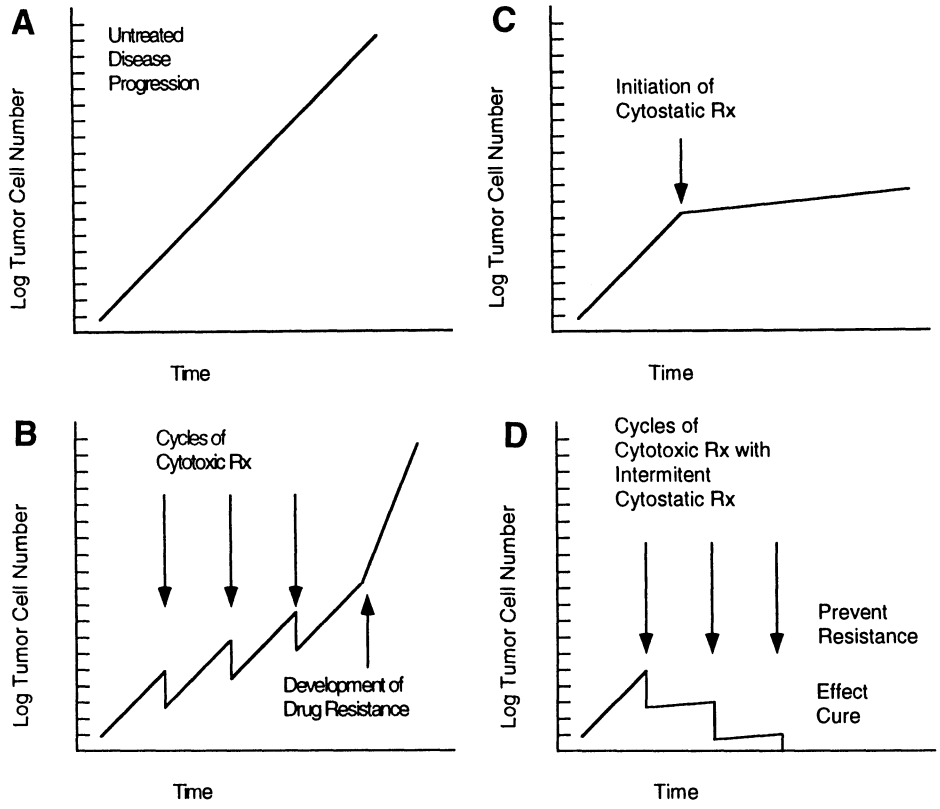

Fig. 4. Cancer therapy paradigms: cytotoxic, cytostatic, and combined therapy. Cancer treatment paradigms are presented in terms of total tumor cell number. **(A)** Untreated cancer. The exponential growth of the early primary is maintained by metastasis formation resulting in exponential increase in tumor burden. **(B)** Cytotoxic therapy. Conventional cytotoxic therapy results in initial reduction of tumor cell burden with eventual regrowth. There is an overall reduction in the rate of tumor cell accumulation, but repeated exposure to cytotoxic agents leads to eventual development of drug resistance and treatment failure. **(C)** Cytostatic therapy. Initiation and continuation of cytostatic therapy with no cytotoxicity prevent further growth of primary tumor and/or metastatic foci. Chronic long-term treatment greatly reduces or eliminates tumor cell accumulation. **(D)** Combined cytotoxic and cytostatic therapy. Administration of cytotoxic agent reduces tumor cell burden, and regrowth is prevented by addition of cytostatic agent. Cytostatic agents are then removed shortly before initiating a second cycle of cytotoxic therapy for further reduction of tumor cell burden. Repeated alternating cycles of cytotoxic and cytostatic therapy could more effectively reduce tumor burden by preventing tumor regrowth between cycles of cytotoxic therapy. Combined therapy may also help prevent development of drug resistance to cytotoxic therapy by limiting the tumor cell population exposed to such agents. The potential clinical utility of combination therapy utilizing an MMP inhibitor and a conventional cytotoxic agent (cyclophosphamide) was recently demonstrated using a murine Lewis lung carcinoma model (*110*).

exposed to the cytotoxic agents, this approach could also help prevent the development of drug resistance. If proven effective at blocking tumor cell spread that is the hallmark of malignant transformation, agents that are well tolerated should also be tested as chemopreventive agents. However, all of these possibilities await the identification of suitable MMP inhibitor(s).

5. INHIBITORS OF MMPs FOR CANCER THERAPY

5.1. Overview

Successful pharmacological targeting of metalloproteinase activity is best exemplified by the development of angiotensin-converting enzyme (ACE) inhibitors to block conversion of angiotensin I to angiotensin II in the treatment of refractory hypertension (*79–81*). This effort led to the identification of Captopril and the prodrug Enalapril, which have become highly successful in the treatment of hypertension and congestive heart failure (*79,81*). This success demonstrates that mechanism-based inhibitors for metalloproteinases can be developed and exploited in the clinical setting. This same strategy has been utilized for the development of MMP inhibitors, since substrate analogs have been modified to target reactive moieties selectively at the active site of MMPs.

5.2. Substrate Analog Inhibitors of MMPs

As mentioned, the most conventional strategy for the development of protease inhibitors has been the substrate analog approach. In this strategy, a peptide analog resembling the native substrate is coupled with either an nonhydrolyzable functionality that replaces the scissile peptide bond or a zinc atom-coordinating or chelating moiety (Fig. 5). This is used as a starting point to synthesize a series of compounds with substitutions at different positions. This strategy designs inhibitors around two regions that interact with the active site of MMPs (*see* Fig. 5). The functionality that interacts with the active site zinc atom is the first region. The second region concerns the design of amino acid side chains on either the amino-terminal (P residues) side or the carboxy-terminal side (P′ residues) of the scissile bond. These residues interact with the active site pocket and thus can give the inhibitor the potential to target a specific MMP. Rational drug design systematically explores the structure–activity relationship of these inhibitors following substitution of various functionalities for the amino acid side chains throughout the parent compound.

This substrate analog approach has been the most widely adopted for the development of MMP inhibitors. In the 1980s, a number of pharmaceutical companies became interested in MMP inhibitors with the specific aim of treating the joint destruction associated with rheumatoid arthritis. These efforts were successful in developing a series of MMP inhibitors. Inhibitors based on a thiol, carboxylic, and phosphinic acid, as well as hydroxamate strategies were developed and explored. The most potent inhibitors are those designed to mimic the carboxy-terminal portion of the substrate and incorporate a hydroxamate moiety attached to the residue at the P_1' position (Fig. 5B). The evolution of these efforts has been described elsewhere and is beyond the scope of this chapter (*82–84*). These efforts have resulted in the first generation of MMP inhibitors that are or are about to be tested in phase 1 clinical trials for cancer therapy.

Batimastat, or BB-94 as it had been referred to previously, is a hydroxamic acid analog ([4-{*N*-hydroxyamino}-2*R*-isobutyl-3*S*-{thiophen-2-ylthiomethyl}-succinyl]-L-phenylalanine-*N*-methylamide) with broad-spectrum MMP inhibitory activity, but little activity against other unrelated metalloproteinases (*85*). Batimastat inhibits the metastatic spread of tumor cells in a number of animal models using either murine

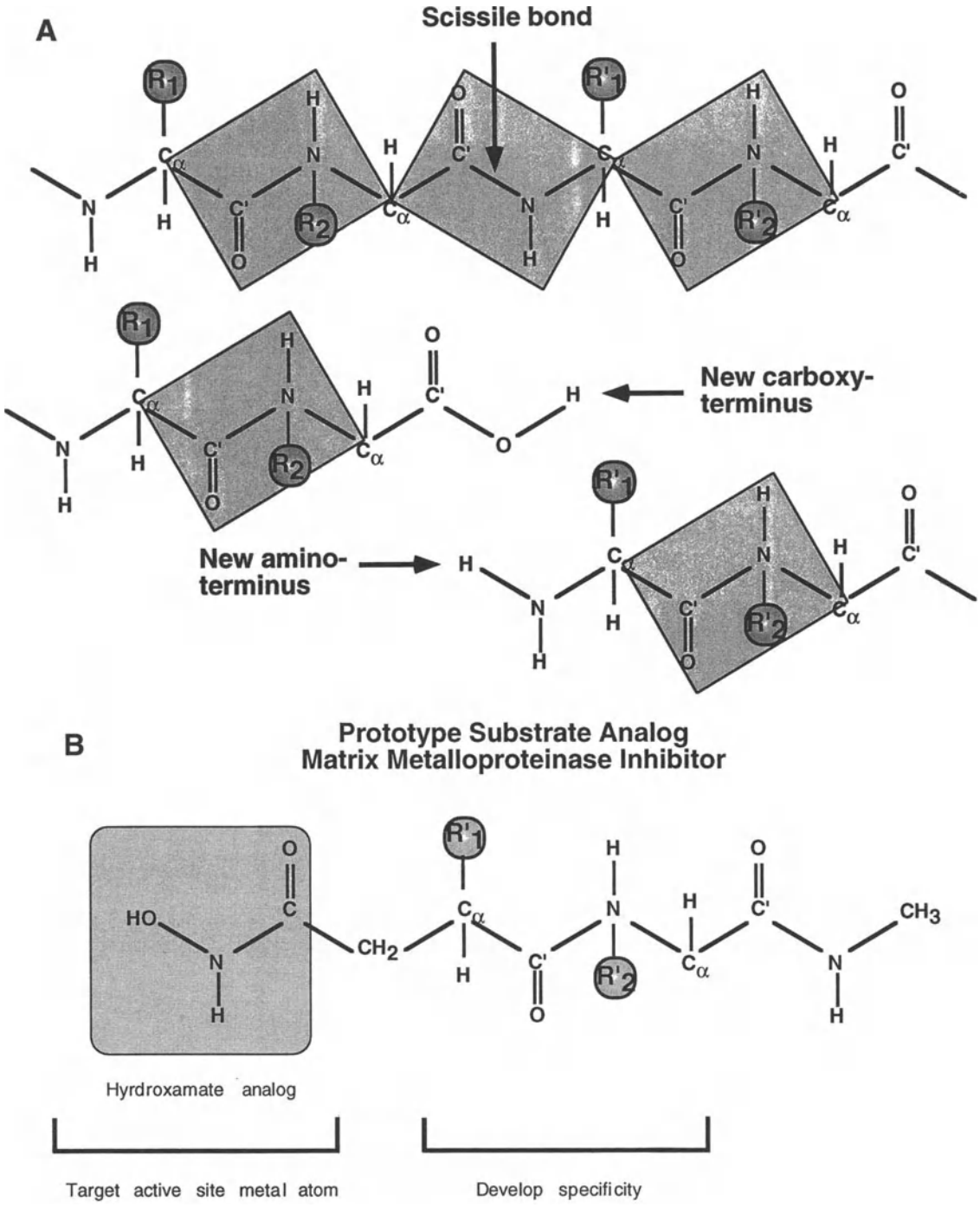

Fig. 5. MMP inhibitor development strategy. **(A)** Structure of the protease substrate. The peptide backbone structure of a protein is illustrated to show the target scissile bond and the nomenclature conventions used to denote amino acid side chains relative to the cleavage site. **(B)** Substrate analog MMP inhibitor. The structural basis for the hydroxamate analog inhibitors currently under development is illustrated. These are derived from the carboxyl side of the putative substrate and include a hydroxamic acid derivative for chelating the active site zinc atom. Potent synthetic MMP inhibitors based on this strategy have been identified, and are in early clinical trials or preclinical development.

B16 melanoma cells, human ovarian cancer cells, or human colon cancer xenografts (*53,86*). Somewhat unexpectedly, Batismastat also inhibits the growth of the primary tumor, although it is not directly cytotoxic to human tumor cells. Histologic examination of the primary tumor tissues in these experiments suggested that Batimastat treatment resulted in formation of fibrous tissue encapsulation and production of avascular stroma. Inhibition of primary tumor growth has also been observed with other hydroxmate-type, metalloproteinase inhibitors. These include BE16627B (L-*N*-[*N*-hydroxy-2-isobutyl-succinyamoyl]-seryl-L-valine), isolated from microbial cultures (*87,88*), and GM6001 (*N*-[2*R*-2-{hydroxamido-carbonymethyl}-4-methylpentanoyl]-L-tryptophan methylamide. The inhibitor BE16627B selectively blocks the in vivo growth of MMP-overproducing human HT1080 fibrosarcoma cells, but does not alter the growth of human colorectal carcinoma cells, HCT116, which secrete only low amounts of MMPs (*87*). GM6001, also known as Galardin, has been reported to prolong survival in C57/B16 mice with experimental B16/F10 murine melanoma metastases (*89*).

These findings imply that MMP inhibitors may either alter the angiogenic response required for normal tumor growth and invasion or suppress the proteinase-dependent release of sequestered growth factors from tumor stroma. Taraboletti et al. (*90*) have shown that Batimastat reduces the growth of experimental hemangiomas by blocking recruitment of normal endothelial cells to these tumors. These effects of Batimastat on endothelial cells suggest that the observed retardation of tumor growth may be owing, at least in part, to alteration of the angiogenic response. Recently, GM 6001 has been shown to inhibit directly the angiogenic response to tumor extract in the rat corneal pocket assay (*91*).

Batimastat is currently in clinical trials for the treatment of malignant effusions associated with thoracic and peritoneal neoplasms (*85*). The low solubility and poor oral bioavailability are severe limitations for the formulation and delivery of this compound. However, second-generation compounds with better pharmaceutical properties are currently in preclinical development and should begin clinical trials in the near future. One such compound is BB-2516, also known as Marimastat, which has oral bioavailability, entered phase I clinical trials in the US in mid-1995 (*92*), and should start early phase II trials by mid-1996.

The substrate analog approach also affords the potential for identifying selective inhibitors for various members of the MMP family based on differences in substrate specificity between these family members. The successful clinical application of an MMP inhibitor may require that it have selective activity and not inhibit other metalloproteinases essential for normal tissue homeostasis. Currently, the need for inhibition of selective subgroups of MMPs or specific members of the MMP family is not clear. The development of selective inhibitors would also provide excellent experimental tools for evaluating the role of various members of the MMP family in both physiologic connective tissue remodeling as well as various pathologic conditions.

Noting the specificity and facility of various MMPs on autoproteolytic processing following activation, Docherty and colleagues at Celltech developed a potent and selective inhibitor for the gelatinases (*93*). These investigators observed that progelatinase A undergoes rapid autoproteolytic removal of the prodomain to generate an active enzyme species with a tyrosine residue as the new amino-terminus. This group developed inhibitors using tyrosine analogs in the P_1' position. This has resulted in

the development of the most potent and selective synthetic MMP inhibitor yet characterized. These compounds are again hydroxamate-based inhibitors with tyrosine analogs at the P_1' position and cyclohexyl moiety at the P_2' position (*84,93*). These inhibitors are not yet available for animal testing or clinical trials in cancer patients.

Recent studies have reported the structural characterization of members of the MMP family by X-ray diffraction or nuclear magnetic resonance spectroscopy. These include human fibroblast collagenase, human neutrophil collagenase, stromelysin, and matrilysin (*94–99*). The information represented in these studies will be useful for the development of the next generation of inhibitors that can selectively inhibit individual members of the closely related MMPs.

5.3. *Future Directions*

Several alternative strategies for inhibition of MMPs also exist and could be exploited for the development of inhibitors. These have received far less attention and support than the substrate analog approach described above. They include inhibitors based on the cysteine switch mechanism in which profragment analogs are targeted to the active site. Other alternatives include TIMP analogs and manipulation of endogenous TIMP production at the tumor site. This last alternative is currently being attempted using a gene therapy approach (Stetler-Stevenson et al., unpublished).

As described previously, all members of the MMP family are secreted in latent form with a profragment that contains a highly conserved sequence responsible for maintenance of latency (*100–102*). This sequence contains a cysteine residue that is thought to coordinate the Zn atom of the active site. This hypothesis is supported by the observation that substitutions in this sequence result in rapid autoactivation of MMPs (*103*). Furthermore, the linear synthetic peptide sequence (MRKPRCGN/VPDV) containing cysteine, but not the conservative substitution of serine for cysteine (MRKPRSGN/VPDV), acts as an inhibitor for gelatinase A (IC_{50}, 8 μM) and stromelysin (IC_{50}, 117 μM) (*101*). This peptide inhibits the MMP-dependent in vitro invasion of tumor cells through a reconstituted basement membrane (*104*). It has also been shown to block neurite outgrowth on reconstituted extracellular matrix, as well as endothelial cell invasion in models of Kaposi's sarcoma (*105,106*).

Recent studies have shown that isocysteine could be substituted for cysteine in these peptides and that other amino acid residues are also essential for inhibitory activity (*107–109*). Introduction of a substituted tyrosine residue at a position located two amino acid residues on the C-terminal side of the cysteine or isocysteine produced a potent inhibitor of stromelysin (IC_{50}, 3 μM). Further exploration of this inhibitor strategy is warranted. Possible combination with other more stable, zinc-chelating strategies could possibly produce a new class of MMP inhibitors.

Little is known about the mechanism through which TIMPs inhibit the MMPs. Structural studies have begun to characterize the domains of the TIMPs that may be involved. Linear peptide analogs of TIMP sequences have had little success in mimicking TIMPs metalloproteinase inhibitory activity. This suggests that the inhibitory action of TIMPs on these proteases is the result of several peptide domains or amino acid side chains that require the correct three-dimensional spatial orientation that is the result of a specific tertiary structure of these proteins. The answer to the mechanism of TIMP action on MMPs will require structural studies on TIMP–MMP complexes.

6. SUMMARY

Malignant tumor cell invasion is now viewed as dysregulated physiologic invasion (*34*). Investigators have started to define the molecular events involved in the process of tumor cell invasion and have found similarities to events involved in angiogenesis, wound healing, and embryonic development. These physiologic invasive processes are strictly regulated both temporally and spatially, and are responsive to negative stimuli that limit these events. Tumor cell invasion and metastasis is also strictly regulated, but is clearly not responsive to negative regulatory signals. In particular, MMP activity is a common denominator for these processes. Regulation of these enzymes is disrupted in tumor cells as evidenced by studies demonstrating transcriptional activation of MMPs and enhanced MMP activation in tumor tissues. Animal models of tumor invasion and metastasis demonstrate that abrogation of MMP activity through the use of synthetic, low/mol/wt, substrate analog inhibitors can effectively block tumor dissemination and also limit primary tumor growth. The suppression of tumor growth has been indirectly linked to suppression of tumor-induced angiogenesis by these MMP inhibitors. This indicates that MMP inhibitors may disrupt a number of key processes involved in cancer progression. The encouraging results of the preclinical testing with these inhibitors, coupled with the strong theoretical basis for this MMP inhibitor approach, lead one to conclude that these inhibitors are a promising new direction in cancer therapy.

ACKNOWLEDGMENTS

The author wishes to thank Mark E. Sobel, Marta L. Corcoran, and David E. Kleiner, Jr., for their critical reading of the manuscript and helpful discussions. The author also thanks Lance Liotta for continued support and encouragement.

REFERENCES

1. Adams F. *The Genuine Works of Hippocrates.* London: Sydeenham Society, 1849.
2. Osler W. *The Evolution of Modern Medicine.* New Haven: Yale University Press, 1921.
3. Koono M, Ushijima K, Hayashi H. Studies on the mechanisms of invasion in cancer. 3. Purification of a neutral protease of rat ascites hepatoma cell associated with production of chemotactic factor for cancer cells. *Int J Cancer* 1974; 13:105–115.
4. Koono M, Katsuya H, Hayashi H. Studies on the mechanisms of invasion in cancer. IV. A factor associated with release of neutral protease of tumor cell. *Int J Cancer* 1974; 13:334–342.
5. Pfleiderer A Jr. Histochemical study of endometrial carcinoma. Systemic investigations of enzymes in infiltrating regions of tumors and interrelations with infiltrated connective tissue. *Am J Obstet Gynecol* 1969; 104:823–828.
6. Sinha B, Goldenberg GJ. The effect of trypsin and neuramidase on the circulation and organ distribution of tumor cells. *Cancer* 1974; 34:1956–1961.
7. Sylven B, Bois-Svenson I. Protein content and enzymatic assays of intersitial fluid from some normal tissues and transplanted mouse tumors. *Cancer Res* 1960; 20:239–248.
8. Sylven B, Bois-Svensson I. On the chemical pathology of interstitial fluid. I. Proteolytic activities in transplanted mouse tumors. *Cancer Res* 1965; 25:458–468.
9. Sylven B. Lysosomal enzyme activity in the interstitial fluid of solid mouse tumour transplants. *Eur J Cancer* 1968; 4:463–474.
10. Nagai Y, Lapiere CM, Gross J. Tadpole collagenase. Preparation and purification. *Biochemistry* 1966; 5:3123–3130.
11. Harris ED, Krane SM. Collagenases. *New Engl J Med* 1974; 291:557–563, 605–609, 652–661.

12. Dresden MH, Hellman SA, Schmidt JD. Collagenolytic enzymes in human neoplasms. *Cancer Res* 1972; 32:993-996.
13. Harris ED Jr., Faulkner CS, Wood S Jr. Collagenase in carcinoma cells. *Biochem Biophys Res Commun* 1972; 48:1247-1253.
14. Carter RL. Metastatic potential of malignant tumours. *Invest Cell Pathol* 1978; 1:275-286.
15. Hashimoto K, Yamanishi Y, Maeyens E. Collagenlytic activities of squamous cell carcinoma of the skin. *Cancer Res* 1973; 33:2790-2801.
16. Robertson DM, Williams DC. *In vitro* evidence of neutral collagenase activity in an invasive mammalian tumour. *Nature* 1969; 221:259,260.
17. Strauch L. The role of Collagenase in tumor invasion. In: Tarin D., ed. *Tissue Interactions in Carcinogenesis.* London: Acedemic. 1972:399-433.
18. Taylor AC, Levy BM, Simpson JW. Collagenlytic activity of sarcoma tissues in culture. *Nature* 1970; 228:366,367.
19. Yamanishi Y, Dabbous MK, Hashimoto K. Effect of Collagenolytic enzymes in basal cell epithelioma of the skin on reconstituted collagen and physical properties and kinetics of the crude enzyme. *Cancer Res* 1972; 32:2551-2560.
20. Yamanishi Y, Maeyens E, Dabbous MK. Collagenlytic activity in malignant melanoma: physiochemical studies. *Cancer Res* 1973; 33:2507-2512.
21. Liotta LA, Kleinerman J, Catanzaro P, Rynbrandt D. Degradation of basement membrane by murine tumor cells. *J Natl Cancer Inst* 1977; 58:1427-1431.
22. Kuettner KE, Soble L, Croxen RL, Marczynska B, Hiti J, Harper E. Tumor cell collagenase and its inhibition by a cartilage-derived protease inhibitor. *Science* 1977; 196:653,654.
23. Liotta LA, Abe S, Robey PG, Martin GR. Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor. *Proc Natl Acad Sci USA* 1979; 76:2268-2272.
24. Kleinman HK, McGarvey JL, Liotta LA, Robey PG, Tryggvason K, Matrin GR. Isolation and characterization of type IV porcollagen, laminin and heparan sulfate from the EHS sarcoma. *Biochemistry* 1981; 21:6188-6193.
25. Timpl RA, Dziadek M. Structure, development and molecular pathology of basement membranes. *Int Rev Exp Pathol* 1986; 29:1-112.
26. Yurchenco PD. Assembly of basement membranes. *Ann NY Acad Sci* 1990; 580:195-213.
27. Nagase J, Barrett AJ, Woessner JF. Nomenclature and Glossary of the Matrix Metalloproteinases. *Matrix* 1992; suppl no 1:421,424.
28. Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 1993; 4:197-250.
29. Matrisian LM, Bowden GT, Krieg P, Fürstenberger G, Briand J-P, Leroy P, Breathnach R. The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. *Proc Natl Acad Sci* 1986; 83:9413-9417.
30. Bassett P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, Podhajcer OL, Chenard MP, Rio MC, Chambon P. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinoma. *Nature* 1990; 348:699-704.
31. Freije JM, Diez-Itza I, Balbin M, Sanchez LM, Blasco R, Tolivia J, Lopez-Otin C. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J Biol Chem* 1994; 269:16,766-16,773.
32. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* 1980; 284:67,68.
33. Liotta LA, Rao CN, Barsky SF. Tumor invasion and the extracellular matrix. *Lab Invest* 1983; 49:636-649.
34. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu Rev Cell Biol* 1993; 9:541-573.
35. Mignatti P, Robbins E, Rifkin DB. Tumor invasion through the human amnion membrane: requirement for a proteinase cascade. *Cell* 1986; 47:487-498.
36. Hendrix MJ, Gehlsen KR, Wagner HN Jr., Rodney SR, Misiorowski RK, Meyskens FL Jr. In vitro quantification of melanoma tumor cell invasion. *Clin Exp Metastasis* 1985; 3:221-233.
37. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN. A rapid in vitro assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987; 47:3239-3245.

38. Matrisian LM. The matrix-degrading metalloproteinases. *BioEssays* 1992; 14:455-462.
39. Kleiner DE Jr., Stetler-Stevenson WG. Structural biochemistry and activation of matrix metalloproteases. *Curr Opinion Cell Biol* 1993; 5:891-897.
40. Ray JM, Stetlerstevenson WG. The role of matrix metalloproteases and their inhibitors in tumor invasion, metastasis and angiogenesis. *Eur Respir J* 1994; 7:2062-2072.
41. Shapiro SD, Kobayashi DK, Ley TJ. Cloning and characterization of a unique elastolytic metalloproteinase produced by human alveolar macrophages. *J Biol Chem* 1993; 268:23,824-23,829.
42. Sato J, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M. A matrix metalloproteinase expressed on the surface of invasive tumor cells. *Nature* 1994; 370:61-65.
43. Springman EB, Angleton EL, Birkedalhansen H, Vanwart HE. Multiple modes of activation of latent human fibroblast collagenase—evidence for the role of a cys-73 active-site zinc complex in latency and a cysteine switch mechanism for activation. *Proc Natl Acad Sci* 1990; 87: 364-368.
44. Urbanski SJ, Edwards DR, Maitland A, Leco KJ, Watson A, Kossakowska AE. Expression of metalloproteinases and their inhibitors in primary pulmonary carcinomas. *Br J Cancer* 1992; 66:1188-1194.
45. Urbanski SJ, Edwards DR, Hershfield N, Huchcroft SA, Shaffer E, Sutherland L, Kossakowska AE. Expression pattern of metalloproteinases and their inhibitors changes with the progression of human sporadic colorectal neoplasia. *Diagn Mol Pathol* 1993; 2:81-89.
46. Muller D, Wolf C, Abecassis J, Millon R, Engelmann A, Bronner G, Rouyer N, Rio MC, Eber M, Methlin G, et al. Increased stromelysin 3 gene expression is associated with increased local invasiveness in head and neck squamous cell carcinomas. *Cancer Res* 1993; 53:165-169.
47. Majmudar G, Nelson BR, Jensen TC, Voorhees JJ, Johnson TM. Increased expression of stromelysin-3 in basal cell carcinomas. *Mol Carcinog* 1994; 9:17-23.
48. Pei D, Majmudar G, Weiss SJ. Hydrolytic inactivation of a breast carcinoma cell-derived serpin by human stromelysin-3. *J Biol Chem* 1994; 269:25,849-25,855.
49. Kusukawa J, Sasaguri Y, Shima I, Kameyama T, Morimatsu M. Expression of matrix metalloproteinase-2 related to lymph node metastasis of oral squamous cell carcinoma. A clinicopathologic study. *Am J Clin Pathol* 1993; 99:18-23.
50. Monteagudo C, Merino MJ, San-Juan J, Liotta LA, Stetler-Stevenson WG. Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. *Am J Pathol* 1990; 136:585-592.
51. Brown PD, Bloxidge RE, Stuart NS, Gatter KC, Carmichael J. Association between expression of activated 72-kilodalton gelatinase and tumor spread in non-small-cell lung carcinoma. *J Natl Cancer Inst* 1993; 85: 574-578.
52. Brown PD, Bloxidge RE, Anderson E, Howell A. Expression of activated gelatinase in human invasive breast carcinoma. *Clin Exp Metastasis* 1993; 11:183-189.
53. Davies B, Brown PD, East N, Crimmin MJ, Balkwill FR. A synthetic matrix metalloproteinase inhibitor decreases tumor burden and prolongs survival of mice bearing human ovarian carcinoma xenografts (published erratum appears in *Cancer Res* Aug 1, 1993; 53 [15]:3652). *Cancer Res* 1993; 53:2087-2091.
54. Azzam HS, Arand G, Lippman ME, Thompson EW. Association of MMP-2 activation potential with metastatic progression in human breast cancer cell lines independent of MMP-2 production. *J Natl Cancer Inst* 1993; 85:1758-1764.
55. Emmert-Buck MR, Roth MJ, Zhuang Z, Campo E, Rozhin J, Sloane BF, Liotta LA, Stetler-Stevenson WG. Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumor regions of human colon cancer samples. *Am J Pathol* 1994; 145:1285-1290.
56. Testa JE. Loss of the metastatic phenotype by a human epidermoid carcinoma cell line, HEp-3, is accompanied by increased expression of tissue inhibitor of metalloproteinase 2. *Cancer Res* 1992; 52:5597-5603.
57. Ponton A, Coulombe B, Skup D. Decreased expression of tissue inhibitor of metalloproteinases in metastatic tumor cells leading to increased levels of collagenase activity. *Cancer Res* 1991; 51:2138-2143.
58. DeClerck YA, Perez N, Shimada H, Boone TC, Langley KE, Taylor SM. Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. *Cancer Res* 1992; 52:701-708.

59. Khokha R, Zimmer MJ, Graham CH, Lala PK, Waterhouse P. Suppression of invasion by inducible expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) in B16-F10 melanoma cells. *J Natl Cancer Inst* 1992; 84:1017–1022.
60. Khokha R. Suppression of the tumorigenic and metastatic abilities of murine B16-F10 melanoma cells in vivo by the overexpression of the tissue inhibitor of the metalloproteinase-1. *J Natl Cancer Inst* 1994; 86:299–304.
61. Montgomery AM, Mueller BM, Reisfeld RA, Taylor SM, DeClerck YA. Effect of tissue inhibitor of the matrix metalloproteinases-2 expression on the growth and spontaneous metastasis of a human melanoma cell line. *Cancer Res* 1994; 54:5467–5473.
62. Ray JM, Stetler-Stevenson WG. Gelatinase-a activity directly modulates melanoma cell-adhesion and spreading. *Embo J* 1995; 14:908–917.
63. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991; 64:327–336.
64. Braunhut SJ, Moses MA. Retinoids modulate endothelial cell production of matrix-degrading proteases and tissue inhibitors of metalloproteinases (TIMP). *J Biol Chem* 1994; 269: 13,472–13,479.
65. Mignatti P, Tsuboi R, Robbins E, Rifkin DB. In vitro angiogenesis on the human amniotic membrane: requirement for basic fibroblast growth factor-induced proteinases. *J Cell Biol* 1989; 108:671–682.
66. Takigawa M, Nishida Y, Suzuki F, Kishi J, Yamashita K, Hayakawa T. Induction of angiogenesis in chick yolk-sac membrane by polyamines and its inhibition by tissue inhibitors of metalloproteinases (TIMP and TIMP-2). *Biochem Biophys Res Commun* 1990; 171:1264–1271.
67. Albini A, Fontanini G, Masiello L, Tacchetti C, Bigini D, Luzzi P, Noonan DM, Stetler-Stevenson WG. Angiogenic potential in vivo by Kaposi's sarcoma cell-free supernatants and HIV-1 tat product: inhibition of KS-like lesions by tissue inhibitor of metalloproteinase-2. *Aids* 1994; 8:1237–1244.
68. Schnaper HW, Grant DS, Stetler-Stevenson WG, Fridman R, D'Orazi G, Murphy AN, Bird RE, Hoythya M, Fuerst TR, French DL, et al. Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis in vitro. *J Cell Physiol* 1993; 156:235–246.
69. Tsuboi R, Rifkin DB. Bimodal relationship between invasion of the amniotic membrane and plasminogen activator activity. *Int J Cncer* 1990; 46:56–60.
70. Moses MA, Sudhalter J, Langer R. Identification of an inhibitor of neovascularization from cartilage. *Science* 1990; 248:1408–1410.
71. Murphy AN, Unsworth EJ, Stetler-Stevenson WG. Tissue inhibitor of metalloproteinases-2 inhibits bFGF-induced human microvascular endothelial cell proliferation. *J Cell Physiol* 1993; 157:351–358.
72. Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol* 1992; 3:65–71.
73. Folkman J, Hanahan D. Switch to the angiogenic phenotype during tumorigenesis. *Princess Takamatsu Symp* 1991; 22:339–347.
74. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 1991; 324:1–8.
75. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, Gasparini G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma (*see* comments). *J Natl Cancer Inst* 1992; 84:1875–1887.
76. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993; 143:401–409.
77. Huynh HT, Tetenes E, Wallace L, Pollak M. In vivo inhibition of insulin-like growth factor I gene expression by tamoxifen. *Cancer Res* 1993; 53:1727–1730.
78. Falkson G, Gelman R, Glick J, Falkson CI, Harris J. Reinduction with the same cytostatic treatment in patients with metastatic breast cancer: an Eastern Cooperative Oncology Group study. *J Clin Oncol* 1994; 12:45–49.
79. Abrams WB, Davies RO, Ferguson RK. Overview: the role of angiotensin-converting enzyme inhibitors in cardiovascular therapy. *Fed Proc* 1984; 43:1314–1321.
80. Ashworth RW. Drugs affecting the renin-angiotensin system. *Prog Drug Res* 1982; 26:207–223.
81. Van Zwieten PA, De Jonge A, Timmermans PB. Inhibitors of the angiotensin I converting enzyme as antihypertensive drugs. *Pharm Weekbl (Sci)* 1983; 5:197–204.

82. Vincenti MP, Clark IM, Brinckerhoff CE. Using inhibitors of metalloproteinases to treat arthritis. Easier said than done? *Arthritis Rheum* 1994; 37:1115-1126.
83. Schwartz MA, Van Wart HE. Synthetic inhibitors of bacterial and mammalian interstitial collagenases. *Prog Med Chem* 1992; 29:271-334.
84. Beeley NRA, Ansell PRJ, Docherty AJP. Inhibitors of matrix metalloproteinases (MMPP's). *Curr Opinion Ther Patents* 1994; 4:7-16.
85. Brown PD. Clinical trials of a low molecular weight matrix metalloproteinase inhibitor in cancer. *Ann NY Acad Sci* 1994; 732:217-221.
86. Wang X, Fu X, Brown PD, Crimmin MJ, Hoffman RM. Matrix metalloproteinase inhibitor BB-94 (batimastat) inhibits human colon tumor growth and spread in a patient-like orthotopic model in nude mice. *Cancer Res* 1994; 54:4726-4728.
87. Naito K, Kanbayashi N, Nakajima S, Murai T, Arakawa K, Nishimura S, Okuyama A. Inhibition of growth of human tumor cells in nude mice by a metalloproteinase inhibitor. *Int J Cancer* 1994; 58:730-735.
88. Okuyama A, Naito K, Morishima H, Suda H, Nishimura S, Tanaka N. Inhibition of growth of human tumor cells in nude mice by a metalloproteinase inhibitor. *Ann NY Acad Sci* 1994; 732:408-410.
89. Tressler RJ, Wee J, Summers B, Galardy R. Galardin, a potent metalloproteinase inhibitor, prolongs survival time in a B16-F10 melanoma experimental metastasis model. *Clin Exp Metastasis* 1994; 12:28.
90. Taraboletti G, Garofalo A, Belotti D, Drudis T, Borsotti P, Scanziani E, Brown PD, Giavazzi R. Inhibition of angiogenesis and murine hemangioma growth by Batimastat, a synthetic inhibitor of matrix metalloproteinases. *J Nat Cancer Inst* 1995; 87:293-298.
91. Galardy RE, Grobelny D, Foellmer HG, Fernandez LA. Inhibition of angiogenesis by the matrix metalloprotease inhibitor N-[2R-2-(hydroxamidocarbonymethyl)-4-methylpentanoyl)]-L-tryptoph- an methylamide. *Cancer Res* 1994; 54:4715-4718.
92. Drummond AH, Beckett P, Boone EA, Brown PD, Davis M, Galloway WA, Taupin P, Wood LM, Davidson AH. BB-2516: An orally bioavailable matrix metalloproteinase inhibitor with efficacy in animal cancer models. *Proc Am Assoc Cancer Res* 1995; 36:100
93. Docherty AJP, Cockett MI, Birch ML, Chander S, Willmott N, O'Connell JP, Crabbe T, Mountain A, Morphy JR, Millican TA, Beeley NRA, Murphy G, Hart IR, Stamp G, Mahadevan V. Gelatinase inhibitors for the treatment of cancer. *Clin Exp Metastasis* 1994; 12:12.
94. Spurlino JC, Smallwood AM, Carlton DD, Banks TM, Vavra KJ, Johnson JS, Cook ER, Falvo J, Wahl RC, Pulvino TA, et al. 1.56 A structure of mature truncated human fibroblast collagenase. *Proteins* 1994; 19:98-109.
95. Lovejoy B, Hassell AM, Luther MA, Weigl D, Jordan SR. Crystal structures of recombinant 19-kDa human fibroblast collagenase complexed to itself. *Biochemistry* 1994; 33:8207-8217.
96. Lovejoy B, Cleasby A, Hassell AM, Luther MA, Weigl D, McGeehan G, Lambert MH, Jordan SR. Structural analysis of the catalytic domain of human fibroblast collagenase. *Ann NY Acad Sci* 1994; 732:375-378.
97. Hassell AM, Anderegg RJ, Weigl D, Milburn MV, Burkhart W, Smith GF, Graber P, Wells TN, Luther MA, Jordan SR. Preliminary X-ray diffraction studies of recombinant 19 kDa human fibroblast collagenase. *J Mol Biol* 1994; 236:1410-1412.
98. Bode W, Reinemer P, Huber R, Kleine T, Schnierer S, Tschesche H. The X-ray crystal structure of the catalytic domain of human neutrophil collagenase inhibited by a substrate analogue reveals the essentials for catalysis and specificity. *EMBO J* 1994; 13:1263-1269.
99. Reinemer P, Grams F, Huber R, Kleine T, Schnierer S, Piper M, Tschesche H, Bode W. Structural implications for the role of the N terminus in the "superactivation" of collagenases. A crystallographic study. *FEBS Lett* 1994; 338:227-233.
100. Stetler-Stevenson WG, Krutzsch HC, Wacher MP, Marguiles IMK, Liotta LA. The activation of human type IV Collagenase proenzyme. Sequence identification of the major conversion product following organomercurial activation. *J Biol Chem* 1989; 264:1353-1356.
101. Stetler-Stevenson WG, Talano JA, Gallagher ME, Krutzsch HC, Liotta LA. Inhibition of human type IV collagenase by a highly conserved peptide sequence derived from its prosegment. *Am J Med Sci* 1991; 302:163-170.

102. Van Wart H, Birkedal HH. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc Natl Acad Sci USA* 1990; 87:5578–5582.
103. Sanchez-Lopez R, Nicholson R, Gesnel M-C, Matrisian LM, Breathnach R. Structure–function relationships in the collagenase gene family member transin. *J Biol Chem* 1988; 263: 11,892–11,899.
104. Melchiori A, Albini A, Ray JM, Stetler-Stevenson WG. Inhibition of tumor cell invasion by a highly conserved peptide sequence from the matrix metalloproteinase enzyme prosegment. *Cancer Res* 1992; 52:2353–2356.
105. Benelli R, Adatia R, Ensoli B, Stetler-Stevenson WG, Santi L, Albini A. Inhibition of AIDS-Kaposi's sarcoma cell induced endothelial cell invasion by TIMP-2 and a synthetic peptide from the metalloproteinase propeptide: implications for an anti-angiogenic therapy. *Oncol Res* 1994; 6:251–257.
106. Muir D. Metalloproteinase-dependent neurite outgrowth within a synthetic extracellular matrix is induced by nerve growth factor. *Exp Cell Res* 1994; 210:243–452.
107. Hanglow AC, Lugo A, Walsky R, Finch-Arietta M, Lusch L, Visnick M, Fotouhi N. Peptides based on the conserved predomain sequence of matrix metalloproteinases inhibit human stromelysin and collagenase. *Agents Actions* 1993; 39 spec no:C148–50.
108. Fotouhi N, Lugo A, Visnick M, Lusch L, Walsky R, Coffey JW, Hanglow AC. Potent peptide inhibitors of stromelysin based on the prodomain region of matrix metalloproteinases. *J Biol Chem* 1994; 269:30,227–30,231.
109. Hanglow AC, Lugo A, Walsky R, Visnick M, Coffey JW, Fotouhi N. Inhibition of human stromelysin by peptides based on the N-terminal domain of tissue inhibitor of metalloproteinases-1. *Biochem Biophys Res Commun* 1994; 205:1156–1163.
110. Anderson TC, Shipp MA, Docherty AJP, Teicher BA. Combination therapy including a gelatinase inhibitor and cytotoxic agent reduces local invasion and metastasis of murine Lewis lung carcinoma. *Cancer Res* 1996; 56:715–718.

13 Interferons and Other Cytokines

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CONTENTS

1. INTRODUCTION

Cancer therapy with cytokines is a rapidly growing field owing to recent advances in immunology, molecular biology, and cell biology. In normal physiology, cytokines are proteins that regulate cell behavior in a paracrine or autocrine manner. When administered exogenously, these proteins often possess biological activities that make them attractive for cancer therapy. Results of clinical trials to date indicate that cytokines may be used along with other current cancer therapies and may eventually replace some of these.

Interferon alpha (IFN-α) was the first cytokine to be used in clinical trials, and the research experience that was obtained has formed the foundation for the investigation of other cytokines as cancer therapeutics. Preclinical studies with IFN-α have had a substantial impact on its clinical development. Similarly, experimental findings have been critical to the subsequent and more rapid development of another cytokine, interleukin-2 (IL-2). Recent preclinical studies demonstrating the potential of interleukin-12 (IL-12) as an anticancer agent have also led this cytokine to the clinic; however, the clinical data are not yet available. This chapter, not intended to be a comprehensive review of the literature, highlights the important steps in the development of IFN-α, IL-2, and IL-12, the cytokines that currently appear to have the most potential as cancer therapeutics.

2. IFN-α

2.1. Experimental Data

IFNs were discovered more than 30 years ago when Isaacs and Lindenmann demonstrated that cells infected with viruses produced proteins that could induce an antiviral state in uninfected cells (*1*). Extensive research conducted since that time has revealed

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

that there are several different types of IFN proteins and that they have many different effects on cellular functions in addition to their antiviral activity. With the molecular cloning and sequencing of the IFN genes, it has become clear that there are three major types of IFN, α, β, and γ. Although there is only one IFN-β gene and one IFN-γ gene, there are at least 23 different IFN-α genes clustered on chromosome 9, which code for at least 15 functional proteins (*2*). The recombinant IFN-α proteins have molecular weights of approx 17 kDa. The IFNs, present in small amounts and active under normal physiologic conditions, are produced in much greater amounts following stimulation by an IFN inducer (*13*). IFN-α is produced primarily by leukocytes, IFN-β is produced by fibroblasts and epithelial cells, and IFN-γ is produced by T-cells and NK cells. The majority of cells in the body possess IFN receptors. IFN-α and IFN-β share components of the same receptor and are known as Type I IFNs, whereas IFN-γ uses a separate receptor and is known as a Type II IFN (*2*). Several components of the IFN-α receptor have been cloned (*4,5*), but this receptor complex is not as well characterized as receptors for other cytokines.

Subsequent to its discovery as an antiviral agent, IFN-α has been shown to exert direct effects on cell growth and differentiation. Early studies demonstrating the ability of IFN to inhibit tumor cell growth (*6,7*) were controversial, because purified IFN was not yet available and impure preparations derived from leukocyte cell cultures, animal tissues, or serum were used. Since then, however, many tumor cell lines have been shown to be sensitive to the growth-inhibitory actions of purified IFN-α in vitro (*8*). Assays used to measure antiproliferative activity include cellular incorporation of ^{3}H-thymidine, clonogenic assays to measure colony growth in agar (*9*), and incubation of solid tumor specimens with IFN-α (*10*). Higher concentrations of IFN-α were often required to achieve the antiproliferative effect, as compared to the antiviral effect, and the result was usually a reversible cytostasis (*2*).

The in vitro antiproliferative effects of IFN-α in combination with either cytotoxic agents or other cytokines have also been examined. IFN-α has been used in combination with cytotoxic agents, such as doxorubicin, cisplatin, vinblastine, methotrexate, bleomycin, and 5-fluorouracil (5-FU), and synergistic effects on growth inhibition were often observed on a variety of human tumor cell lines or cells from primary tumors (*11*). The mechanism by which growth is inhibited may involve either modulation of the actions of IFN-α by the cytotoxic drugs or modulation of the action of the cytotoxic drugs by IFN-α (*11*). Synergistic activity in the inhibition of cell growth can also be obtained when IFN-α is combined with IFN-γ (*12,13*) or with tumor necrosis factor (TNF) (*14,15*).

The precise mechanism by which IFN-α inhibits cell growth has been the subject of more recent investigations. IFN-α slows the growth of tumor cells by increasing the length of their cell cycle, particularly at the G_0/G_1 transition phase (*2*). These cytokinetic effects may occur through the modulation of 2′5′-oligoadenylate synthetase (a protein translation inhibitor involved in the antiviral activity of IFN-α) or cellular oncogenes, such as c-*myc* and c-*fos* (*16–18*). In addition, IFN-α can also inhibit the gene for the enzyme ornithine decarboxylase, thus depleting the cell of essential metabolites (*19*). Basic studies of IFN signaling have identified some of the factors involved in this process (*20*). Thus, the ability of IFN-α to modulate gene expression may be an important component of its antitumor activity.

In addition to direct effects on tumor growth, IFN-α may also affect tumor growth by indirect mechanisms. Early experiments showed that a mouse L1210 leukemia cell line resistant to the antiproliferative effects of IFN in vitro could be inhibited from growing in vivo by IFN treatment (*21,22*). It is now known that IFN-α has numerous immunoregulatory activities. IFN-α can induce the differentiation of pre-NK cells (*2*) as well as stimulate NK activity (*23*). Furthermore, IFN-α has been shown to enhance antibody-dependent cellular cytotoxicity (ADCC) of both NK cells (*24*) and neutrophils (*25*). The cytotoxic function of macrophages is also stimulated by IFN-α (*26*). In addition, IFN-α can increase the expression of class I major histocompatibility complex (MHC) antigens (*27,28*) and tumor-specific antigens (*29,30*) on tumor cells. The increased expression of cell-surface antigens on tumor cells by IFN-α results in more efficient recognition and killing of tumor cells by cytotoxic T-cells and NK cells.

IFN-α also appears to have an indirect effect on tumors by inhibiting angiogenesis, the formation of a blood supply that is critical to the growth of solid tumors. Leukocyte IFN (α/β) was shown to inhibit capillary endothelial cell motility in vitro (*31*). In another report, the treatment of tumor-bearing mice with IFN-α/β resulted in the early degeneration of the endothelial cells in the vessel of the tumor (*32*). Studies such as these suggest that the antiangiogenic potential of IFN-α should be further investigated.

Many of the antitumor activities of IFN-α described above have been confirmed in murine tumor models. Using impure preparations of IFN, Gresser and others were the first to show that IFN could inhibit not only the growth of virally induced tumors (*33*), but also the growth of spontaneous (*34*) and transplantable (*35*) tumors. These observations, along with the in vitro data on the antiproliferative effects of IFN-α, led researchers to test the antitumor efficacy of IFN-α against a wide range of tumors, including established sc tumors, murine leukemias, and experimental and spontaneous metastases.

Human tumor xenograft models have been used to investigate the direct effects of human IFN-α on tumor growth, since these mice do not have a competent immune system, and human IFN-α is not active on mouse cells. Often, the response of a particular tumor to IFN-α in vivo is correlated with antiproliferative effects of IFN-α on the tumor cells in vitro. Many types of tumors have responded to IFN-α, including breast, colon, melanoma, and osteosarcoma (*36*). The results of these studies have shown that the antitumor effect is greater when therapy is initiated soon after tumor injection, repeated doses are more effective than single doses, and tumor stasis is more commonly achieved than tumor regression or cures (*36*).

Murine tumors growing in syngeneic hosts or transplantable tumor models have been used to examine the indirect antitumor effects of IFN-α. Since human IFN-α is not active on the murine tumors, either murine IFN-α or human IFN-α A/D (a hybrid human IFN-α that has activity on murine cells) has been administered to mice. As with the xenograft model, efficacy of treatment is associated with low tumor load and frequent dosing, and cures are rarely seen (*36*). In addition, indirect antitumor effects of IFN-α on the immune response have been confirmed. For example, IFN-α A/D-induced inhibition of experimentally induced pulmonary metastases was correlated with enhanced NK cytotoxicity (*37*). In another report, a variety of effector cells were shown to be involved in the IFN-α/β-induced suppression of Friend erythroleukemia cell (FLC) metastases (*38*). However, results of studies with animal models

have suggested that although indirect effects on the immune system may be involved in mediating the antitumor activity of IFN-α against some tumors, IFN-α appears to exert its major activity primarily through direct effects on tumor growth.

Murine tumor models have also been used to evaluate the antitumor efficacy of IFN-α in combination with cytotoxic drugs or other cytokines. Early observations that IFN could enhance the activity of chemotherapeutic agents in vivo (*39,40*) have been extended to human tumor xenografts, in which IFN-α has been found to increase the antitumor activity of cyclophosphamide, doxorubicin, cisplatin, and mitomycin C against various tumor types (*41–43*). Many studies have addressed the optimal timing for the administration of IFN and cytotoxic agents. Although it was initially thought that the cytotoxic drug should be given first to decrease a large tumor volume so that IFN-α could eradicate the minimum residual disease, recent studies have indicated that the interaction is complex and other types of scheduling may work just as well (*11*).

The synergy between IFN-α and other cytokines in antitumor efficacy was first demonstrated with the combination of IFN-α and IL-2 using the M5076 reticulum cell sarcoma (*44*). The synergistic or additive effects of IFN-α used in combination with IL-2 have since been confirmed in many experimental primary sc and metastatic murine tumor models (reviewed in ref. *45*). In addition to IL-2, additive or synergistic antitumor activity has also been observed when IFN-α is administered in combination with IFN-γ (*46–48*), IL-1α (*49*), or TNF (*50*). Recent studies have also shown therapeutic efficacy against solid tumors with IFN-α in combination with both a cytokine and a chemotherapeutic agent (*51*). It should be noted that the combination of cytotoxic drugs or cytokines with IFN-α does not always result in a synergistic or additive effect.

Gene therapy is currently being evaluated in mice as an alternative method to the systemic use of IFN-α. Fibroblasts transfected with the human IFN-α gene secreted human IFN-α and inhibited the growth of chronic myelocytic leukemia in nude mice (*52*). In addition, metastatic IFN-α/β-resistant FLC (*53*) or metastatic murine adenocarcinoma cells (*54*) transfected with a murine IFN-α gene failed to grow in mice and immunized mice to rechallenge with the parental non-IFN-α-secreting tumor cells. Importantly, the IFN-α-producing FLC were also shown to inhibit tumor growth in mice with established metastatic tumors (*53*). Thus, these results provide a rationale for the use of gene therapy in the clinic.

2.2. Clinical Trials

Information obtained from studying the antitumor efficacy of IFN-α in murine tumor models has been useful in designing clinical trials. In the early clinical trials, natural leukocyte IFN (a mixture of IFNs with only 1% purity) was shown to have antitumor activity against several types of malignancies, including osteosarcoma, lymphoma, and breast cancer (*55*), and it was believed that IFNs would act on a wide range of tumor types. We now know, using both recombinant and purified natural material, that IFN-α has a more restricted activity, being effective mainly against hematological malignancies. However, the use of IFN-α in the treatment of solid tumors is likely to expand, since it has shown some efficacy and is less toxic than most existing forms of therapy. The effective doses of IFN-α range from 1 million units (MU) to 36 MU/injection and vary with the particular malignancy being treated (*56,57*). Side effects typically consist of "flu-like" symptoms, such as fever, chills,

myalgia, and headache, but may also include gastrointestinal and CNS effects at doses >18 MU (*16*).

IFN-α has been approved by many health authorities for use in hairy cell leukemia (HCL), a rare B-lymphocyte malignancy, characterized by peripheral blood mononuclear cells that display hair-like protrusions from their cytoplasm. A summary of the largest trials of IFN-α in HCL has shown that long-term treatment with frequent low doses of IFN-α resulted in an overall response rate of 80–90%, with 5% complete remissions (*56*). Responders could be held in remission using prolonged less frequent maintenance doses, and in one study, overall survival rate was 82% (*58*). The antitumor mechanism of IFN-α is mainly owing to direct antiproliferative effects (differentiation, cell-cycle arrest, and/or apoptosis) (*59,60*), but immunomodulatory effects, such as stimulation of NK activity and enhancement of HLA-DR antigens on HCL cells, may add to the activity of IFN-α (*16*).

IFN-α has also shown biological activity in chronic myelogenous leukemia (CML), a progressive myeloproliferative disorder arising from the neoplastic transformation of pluripotent stem cells (*16*). CML is associated with the activation of a cellular oncogene by chromosomal translocation, and a shortened chromosome 22 (the Philadelphia chromosome) is present in hemopoietic cells. Survival prognosis is correlated with a cytogenetic (karyotypic) response. In the largest reported clinical trial, IFN-α treatment resulted in 73% complete hematologic remissions (normalization of peripheral blood counts and disappearance of symptoms) and 19% complete cytogenetic remissions (*61*). It is likely that this antitumor activity was mediated by direct effects of IFN-α on cell growth (*16*). In a recent study, patients were treated first with hydroxyurea and then with IFN-α in combination with ara-C, resulting in a significant increase in the rate of complete cytogenetic remission (*62*). IFN-α may also be effective for the treatment of minimal residual disease following bone marrow transplant (*63*). In addition to CML and HCL, IFN-α has shown some activity against other hematological malignancies, such as multiple myeloma, non-Hodgkin's lymphoma, and cutaneous T-cell lymphoma (*16*).

Although IFN-α activity against solid tumors has been generally disappointing, significant responses have been observed in certain tumors that have been difficult to treat, but are sensitive to immunomodulation, such as malignant melanoma, renal cell carcinoma, and Kaposi's sarcoma. Individuals with malignant melanoma have been treated with IFN-α alone (average response rate 20%) or in combination with cytotoxic drugs, such as cimetidine, decarbazine, cisplatin, and cyclophosphamide (*16*). Thus far, combinations with cytotoxic drugs have not shown increased benefit over IFN-α monotherapy (*16*). Similarly, the combination of IFN-α and IL-2 in Phase I and II trials has been disappointing, with an overall response rate between 0 and 33% (*64*). Recent trials have indicated that IFN-α in combination with both IL-2 and cisplatin may be effective (*65*), but these results must be confirmed. IFN-α therapy is most effective in patients with nonbulky disease limited to cutaneous, soft tissue, or pulmonary sites, or in an adjuvant setting after surgery (*16*).

A review of seven recent studies indicated an overall response rate of 19% for the treatment of renal cell carcinoma with IFN-α (*66*). Combinations of IFN-α with cytotoxic drugs generally did not improve antitumor efficacy; however, a response rate of up to 43% has been observed with IFN-α in combination with vinblastine (*67*). Other drugs that have shown some efficacy in combination with IFN-α are pre-

dnisone (*68*) and 5-FU (*69*). IFN-α in combination with IL-2 has given response rates of 0–50%, with a median of 25% (*57*), and has often resulted in severe side effects (*70*). Some recent studies, however, suggest that the combination of IFN-α, IL-2, and 5-FU may prove to be effective (*71*).

IFN-α has demonstrated sufficient efficacy against Kaposi's sarcoma to be approved by the US FDA and other health authorities. Kaposi's sarcoma, a disease of endothelial or spindle-cell origin, has been observed in AIDS patients and other immunosuppressed individuals. Treatment with relatively high doses of IFN-α results in an overall response rate of 30–40%, with better responses in those with higher $CD4^+$ T-cell counts (*72*). The antitumor mechanism of IFN-α is not known, but the activity may be mediated in part by an inhibition of viral replication, with a corresponding increase in $CD4^+$ T-cell numbers (*73*). Also, since Kaposi's sarcoma is an angiogenic disease of endothelial cell origin, IFN-α may exert anti-angiogenic effects. In support of this concept, IFN-α has recently been shown to induce regressions in children with pulmonary hemangioma (*74*). Beneficial effects of IFN-α in combination with AZT has been observed, but were associated with significant toxicity (*75*). In addition to Kaposi's sarcoma and the other solid tumors described above, IFN-α may be effective for localized therapy in bladder and ovarian cancer (*16*).

2.3. Experimental vs Clinical Data

Although there have been limitations, the experimental data on the antitumor effects of IFN-α have been quite useful for the clinical setting. Although IFN-α has clearly demonstrated antitumor efficacy in clinical trials, it is active over a more restricted range of tumor types than what was predicted based on experimental data. This difference cannot be attributed to the use of impure IFN preparations in many experimental studies, since subsequent studies with purified recombinant or natural IFN-α generally confirmed the earlier results. Instead, it may be that antiproliferative effects on tumor cells in vitro were often achieved with higher concentrations of IFN-α than could be used clinically. Owing to the species-restricted activity of IFN-α, animal tumor models failed to predict the toxicity that has been observed in patients given higher doses of IFN-α. Also, some of the antitumor effects of IFN-α observed in animals may have been associated with the use of murine rather than human IFN-α or may have been owing to the increased immunogenicity of some syngeneic tumors.

Some important information has been extrapolated from these experimental data, however. Studies in animal tumor models have indicated that IFN-α may be most effective when it is administered frequently and tumor burden is low. In the clinic, IFN-α is also most effective with frequent dosing, and its usefulness as maintenance therapy for minimal residual disease or as an adjuvant is becoming apparent. Regressions or cures were rarely seen in animals, and the same has been true for humans. The experimental data have also been very useful in elucidating the complex mechanism of the antitumor activity of IFN-α. As in preclinical studies, the clinical data suggest that IFN-α mainly exerts direct effects on tumor cells, but can indirectly affect the immune system as well. Mechanistic studies are important for the identification of novel treatment strategies, and experimental data have already provided some new directions for IFN-α therapy. Clinical studies in which IFN-α is used as an adjuvant, in combination with cytotoxic drugs and/or cytokines, or in gene therapy may result in some very effective treatments for some malignancies.

3. IL-2

3.1. Experimental Data

IL-2 was originally described as a soluble factor, derived from phytohemagglutinin-stimulated human lymphocytes, that could induce the outgrowth of T-cells from bone marrow (*76*). Subsequent studies have demonstrated that IL-2 is a single-chain glycoprotein of approx 15 kDA (*77*). Based on its ability to stimulate T-cell proliferation, assays were developed to quantitate its activity (*78*), and the gene for this cytokine was cloned (*79*). Although the recombinant protein is nonglycosylated, it retains all of the evaluated biological properties of IL-2.

IL-2 mediates its effect on the immune system by interacting with receptors present on various cell types. The IL-2 receptor (IL-2R) is comprised of three chains, α, β, and γ (*80–83*). The α chain is a low-affinity receptor for IL-2, the β and γ chains form an intermediate-affinity receptor, but all three chains are necessary for the high-affinity IL-2R (*84,85*). Signaling results following interaction of IL-2 with either the intermediate- or high-affinity IL-2R (*84,85*). Ligand–receptor interaction triggers a series of complex biochemical events within cells, which are the focus of much experimental study (*84,85*), and eventually results in the biological activities associated with IL-2.

In more recent years, it has been demonstrated that IL-2 is produced by the Th1 helper T-cells, a subset of cells that has been implicated in cell-mediated immune responses (*86*). Consistent with this observation, IL-2 has been shown to be a critical regulator of a variety of biological functions, which have been reviewed in more detail (*87,88*). With respect to its antitumor potential, one of the most important biological properties of IL-2 is its ability to induce the activation/differentiation of various cell populations that can lyse tumor cells. IL-2 can enhance the generation of murine or human cytotoxic T-lymphocyte (CTL) responses in vitro (*89,90*), as well as augment responses in animals in vivo (*91,92*). Likewise, brief exposure of murine or human NK cells in vitro to IL-2 can augment their cytolytic responses (*93,94*), and increased NK activity has also been demonstrated in animals injected with IL-2 (*91,95*). Longer-term culture of lymphocytes with IL-2 results in the development of lymphokine-activated killer (LAK) cells (*96*); LAK cells can kill NK cell-resistant tumor targets and are derived primarily from NK cells (*97*). Highly specific CTL derived from tumors, tumor-infiltrating lymphocytes (TILs) are also generated by culture of these cells with IL-2 (*98*). In addition to its effects on lymphocytes, under some conditions, macrophages can also be activated by IL-2, resulting in cells capable of lysing tumor target cells (*99*). Thus, the cytolytic potential of a diverse set of immune effector cells can be upregulated by IL-2.

IL-2 has also been shown to induce other cytokines, especially IFN-γ (*100*). In addition to IFN-γ, mRNA, or protein for tumor growth factor-β, TNF-α, lymphotoxin, IL-1, IL-6, IL-5, granulocyte macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF) have been demonstrated in IL-2-incubated human peripheral blood mononuclear cells (*101*). These cytokines may be important in mediating the efficacy of IL-2.

Based on the in vitro and in vivo biological effects of IL-2, substantial interest arose for use of this protein as an antitumor effector molecule. It should be noted that human IL-2, in contrast to IFN-α, is active on both human and mouse cells, thus

permitting the use of human IL-2 for studies in rodent animal models. Studies were performed to evaluate the antitumor efficacy of IL-2 as monotherapy in tumor models (*102–106*); positive effects have been observed against experimental and spontaneous pulmonary and hepatic metastases (*102,103,105,106*), in murine leukemia models (*104*), and against established sc tumors (*102,106*). In these models, reduction in the number of metastases (*102,103,105,106*), inhibition of tumor growth (*102,106*), and prolongation of survival were reported (*102,104*). However, in most of these models, treatment with IL-2 was initiated soon after tumor implantation and rarely resulted in long-term cures.

Since many tumor models and experimental conditions have been evaluated, it is difficult to generalize about the optimal utility of IL-2 and its mechanism of action, but a few observations can be made. Although there have been some studies suggesting efficacy at low doses of IL-2 (*105*), the most success has been observed utilizing high doses of IL-2, at or near the maximum tolerated dose of this cytokine (*102,103,106*). At higher doses, IL-2 is quite toxic, and the toxicities in mice (*107*) seem to be similar to those observed in patients. The timing of the initiation of IL-2 therapy seems to play some role in the ability of IL-2 to mediate its antitumor effects. It is more difficult to treat larger, more established sc tumors with IL-2 (G. Truitt, personal communication; M. Brunda, unpublished observations), but this is not necessarily the case with metastases (*102*) or in leukemia (*104*) models.

It is clear that IL-2 does not have a direct antiproliferative effect on tumor cells, but rather mediates its effects through some component of the immune system; however, some of the cytokines induced by IL-2 treatment, for example, IFN-γ or TNF, may directly affect tumor cell proliferation. In various tumor models, the antitumor efficacy of IL-2 is greatly diminished in irradiated mice (*102*) or mice treated with antibodies directed to various immune cell populations (*108*), or in mice concomitantly receiving corticosteroids (*109*). IL-2 treatment induced cytotoxic LAK cells in tumor-bearing mice, and a correlation between the induction of LAK activity and efficacy was observed (*108*). The best effects of IL-2 on metastases were found in the therapy of weakly immunogenic tumors as well as some "nonimmunogenic" tumors, if treatment with IL-2 was initiated on day 3 (*110*). However, initiation of treatment of metastases from nonimmunogenic tumors with IL-2 on day 10 resulted in no therapeutic benefit (*108*). The antitumor effect of IL-2 against poorly responsive tumors, which lack MHC class I, is enhanced following transfection of an MHC class I gene into these tumor cells (*111*), suggesting a role for $CD8^+$ T-cells. Furthermore, an increased lymphocytic infiltrate was evident following histological evaluation of tumors undergoing IL-2-induced regression (*102*), suggesting a possible involvement in the observed tumor inhibition. Whether the immune cells demonstrated in the models inhibit tumor growth through cytolytic mechanisms, cytokine induction, or other mechanisms is not firmly established.

Since IL-2 can induce cytotoxic lymphocyte activity in vitro, a large number of preclinical studies have focused on the use of the combination of IL-2-activated cells and lower levels of systemically administered IL-2 for the therapy of murine metastases. The initial work in this area demonstrated that in several murine pulmonary metastasis models, a substantially greater inhibition of metastases was observed in mice receiving both LAK cells and IL-2 than in mice receiving either component alone (*112–114*). In contrast to the reduced efficacy of IL-2 in irradiated mice, no reduction of the antimetastatic effect of IL-2 plus LAK was seen, most probably reflecting the

ability of the transferred LAK cells to mediate the efficacy (*113,115*). One of the main questions associated with this form of therapy is whether the injected LAK cells can selectively migrate to the tumor; accumulation of only small numbers of the injected LAK cells have been found within tumors (*116,117*) and, of possible importance, injected cells were not seen in all metastatic foci (*116*).

Another form of IL-2-adoptive cellular therapy is the use of IL-2 plus TILs (*118*). Therapy with IL-2 plus TILs was found to be up to 100 times more efficacious than IL-2 plus LAK cells in metastatic tumor models; the best synergy was obtained in mice receiving sublethal irradiation or cyclophosphamide treatment, in addition to IL-2 plus TILs (*118–120*). TILs can persist for months in animals, even in the absence of IL-2 therapy (*119,120*).

In animal models, significant effort has also been placed on the therapeutic benefit of IL-2 combined with other antitumor drugs. As discussed earlier, a large amount of data demonstrated substantially increased activity of IL-2 combined with IFN-α (*44,106*); at higher doses of these two cytokines, increased toxicity was also observed (*106*). Combinations with other cytokines, including IFN-γ, TNF, and IL-1, have been evaluated and resulted in modest levels of increased efficacy (*45*). Likewise, combination of IL-2 and cytotoxic drugs has been evaluated in animal models with some improved results (*121,122*).

In addition to the systemic use of IL-2, experimental systems utilizing transfection of tumor cells with the IL-2 gene, resulting in cells secreting IL-2, have also been studied (*123,124*). IL-2-transfected tumor cells do not form tumors and can immunize mice to rechallenge with parental, non-IL-2-secreting tumor cells (*123,124*); variations on this line of research include immunization with tumor cells or tumor antigen and IL-2-secreting fibroblasts (*125*). Antitumor efficacy is dependent on $CD8^+$ T-cells and has been correlated with the induction of CTL (*123–125*). In contrast to the marked effect with IL-2-secreting tumor cells in preimmunizing mice to subsequent tumor challenge, there are few data to support the therapeutic utility of such cells in mice with established tumors.

3.2. Clinical Trials

IL-2 has been clearly demonstrated to have antitumor efficacy in patients with renal cell carcinoma (*126,127*). In the trials used for registration of the drug, 600,000 IU/kg of IL-2 were administered iv every 8 h for 5 d, followed by a second course after 2 wk of rest; dose reduction occurred frequently owing to toxicities (*128*). An objective response rate of 15% (4% complete response, 11% partial response), was observed (*128*); the median duration of response was 23.2 mo (*128*). In other trials utilizing various regimens and high doses of IL-2, the overall response rate in renal cell carcinoma ranged from 0 to 40% with a median response of 20% (*126,127*). Toxicities are quite severe with capillary leak syndrome and hypotension among the major problems (*126,127*). Because of high-dose IL-2 toxicities, a variety of schedules/regimens and combinations have been evaluated in an attempt to maintain efficacy and reduce toxicity (*127*). It does not appear that combinations of IL-2 plus IFN-α (*see* Section 2) or other drugs result in a better response overall (*126,127*), although enhanced efficacy has been reported in some trials. One of the more potentially promising approaches is the use of sc administered IL-2, which in early studies appears to give comparable response rates, but less toxicity (*127*).

IL-2 has been shown to have some activity in several other malignancies. In patients with malignant melanoma, using various regimens/doses, an average response rate of approx 13% (range of 3–60%) was achieved, but at doses often resulting in marked toxicity (*126,127*). Studies evaluating multiple-drug combinations of IL-2, IFN-α, and other chemotherapeutic drugs are in progress and may improve response (*126, 127*). IL-2 has also been tested in a limited number of patients with colorectal, ovarian, bladder, neuroblastoma, non-Hodgkin's lymphoma, and acute myeloid leukemia (*126,127*). Although some responses have been observed, the utility of IL-2 in these malignanies has not been firmly established.

With respect to IL-2 combined with LAK cells, early clinical studies suggested that some efficacy was achievable with this combination (*129*). However, more extensive testing has demonstrated a response rate similar to that obtained with IL-2 alone and with similar toxicities (*126,127*). For example, in a summary of European studies, an overall response rate of 16% (5% CR, 11% PR) was observed in a total of approx 300 renal cell carcinoma patients treated with IL-2 alone or IL-2 plus LAK (*130*). In melanoma patients, although there is no increase in response rate, there is a trend toward longer survival in patients receiving IL-2 plus LAK cells compared to those treated with IL-2 alone (*131*), but additional studies will be needed to confirm this finding. Thus, overall, it appears that there is no significant benefit of IL-2 plus LAK cell therapy compared to treatment with IL-2 alone.

Some of the other experimental approaches developed in animal models are now beginning to be investigated in clinical trials. Early results with IL-2 combined with TILs have also demonstrated some responses, but further testing is required (*132*). Experimental tumor vaccine protocols utilizing IL-2-transfected tumor cells are also in progress.

3.3. Experimental vs Clinical Data

Based on its biological properties and animal experimental data, IL-2 was evaluated in the clinic and shown to have efficacy in some malignancies, in particular renal cell carcinoma. Thus, the experimental data clearly demonstrated the utility of this cytokine for cancer therapy. However, it is also obvious that IL-2 was much more active in murine models than in people. This may reflect the relative immunogenicities of murine vs human tumors, with the murine tumors being more immunogenic. It should be noted that, even in the case of chemotherapeutic drugs, it has been difficult to predict which histologic type of malignancy will respond to therapy by use of animal models. In addition to its potential efficacy, the experimental data have provided a framework for understanding the mechanism(s) mediating the antitumor efficacy observed with IL-2. Thus, IL-2 does not exert its effects on the tumor directly, but rather stimulates the host to mount an immune response through interaction of IL-2 with its receptor on various cell populations. Some useful, but limited information was obtained in animal models for dosing regimen, which began as a starting point for clinical trials.

The marked efficacy of IL-2 combined with cellular therapy, in particular LAK cells, appears not be supported by clinical results to date. However, on closer inspection of these experimental data, cures were rarely obtained in these acute animal models. Furthermore, the large majority of studies utilized metastatic models, which may be "easier" to treat than more established sc tumors. The rather substantial effects seen in animal models may, therefore, translate into marginal effects observed in

patients with more chronic disease. Likewise, the success of combination therapy in animal models may translate into only a small incremental effect in patients. However, it should be emphasized that these experimental models have given new directions for therapeutic intervention, which as our knowledge on the use of IL-2 increases, may eventually result in greater benefit to patients. In the future, the use of TILs or IL-2-transfected tumor cells, for example, may result in greater efficacy in some human malignancies.

4. IL-12

In contrast to the relatively long histories of IFNs and IL-2, IL-12 is a more recently discovered cytokine. It has a unique structure, since it is the only characterized interleukin that is composed of two disulfide-bridged subunits, designated p35 and p40 (*133–136*). Full biological activity of this protein is only obtained with the heterodimer and not with the individual subunits alone (*135,136*). Although not fully characterized, the receptor for IL-12 is distinct from the receptor for IL-2 or other cytokines (*137–139*). As with other cytokines, initial studies with recombinant human IL-12 were conducted in various in vitro immunologic assays. A number of the biologic activities of IL-12 (reviewed in *140–143*) are similar to those of IL-2, including enhancement of specific CTL responses, activation of NK/LAK cells, stimulation of T- and NK cell proliferation, and induction of IFN-γ production from both T- and NK cells. However, in addition to activities it shares with IL-2, IL-12 has the unique property of inducing the maturation of the Th1 helper cell population; Th1 cells secrete IL-2 and IFN-γ and promote cell-mediated immune responses. With the availability of recombinant murine IL-12 (rMuIL-12) (*144*), many of these biological effects previously observed in vitro have been confirmed following administration of rMuIL-12 to mice (*145*).

Based on these potent immunomodulatory effects of IL-12, experiments have been performed to evaluate the potential antitumor and antimetastatic effects of IL-12 in murine models. To date, experiments with IL-12 have focused in three areas:

1. Therapeutic effects on sc tumors;
2. Inhibition of experimental and spontaneous metastases; and
3. Tumor cells transfected with the genes for IL-12.

With respect to sc tumors, treatment of tumor-bearing mice with rMuIL-12 can result in tumor growth inhibition, tumor stasis, prolongation of survival, and in some tumor models, regression of established tumors (*146–155*). For example, tumor regression occurs in approx 75% of mice bearing 14-d old Renca renal cell carcinoma tumors that are 1 cm or greater in diameter (*142,146,149,155*); these mice die at 5–7 wk if untreated. Mice whose tumors regressed following treatment with rMuIL-12 rejected subsequent challenge with the same, but not other syngeneic tumors, indicating that tumor-specific immunity had been induced in these animals (*143*). At the effective dose/treatment regiment of IL-12, mild to moderate toxicities (*141;* M. Brunda and T. Anderson, unpublished), primarily leucopenia and elevated hepatic enzymes, were found, demonstrating antitumor efficacy was achievable under conditions that were well tolerated. The antitumor efficacy obtained with IL-12 was superior to treatment with either IFN-α or IL-2 in some models (*153,155;* Brunda et al., unpublished). The mechanism through which IL-12 mediates its antitumor efficacy has not been fully characterized, although IL-12 has no direct antiproliferative effects on tumor

cells in vitro (*151*) and is probably immune-mediated, since both T-cells and the induction of IFN-γ are necessary for optimal effects to be seen (*146,151,155*).

Treatment with IL-12 also results in marked antimetastatic effects (*141,146,147, 154*). For example, following iv injection of B16F10 tumor cells, treatment with IL-12 initiated on day 7 resulted in a 75% reduction in the number of pulmonary metastases (*146*). In one experimental metastasis model, treatment with IL-12 gave comparable efficacy, but substantially less toxicity than IL-2 (*147*). Experimental hepatic metastases were also inhibited following treatment with IL-12 (*146*). Similar to its effects in experimental metastasis models, treatment with IL-12 likewise substantially reduced either pulmonary (*154*) or hepatic spontaneous metastases (*141*).

The potential use of IL-12 in tumor vaccines has been demonstrated in a murine model (*150*). When murine fibroblasts secreting biologically active murine IL-12 were mixed with viable BL-6 melanoma cells, a significant delay in tumor growth was observed. Histological evaluation of injection sites demonstrated an increased accumulation of macrophages and a decrease in $CD4^+$ T-cells at sites of IL-12-secreting fibroblasts and tumor cells compared to sites injected with tumor cells alone. Therefore, it appears that IL-12 has the ability to modulate immune responses locally, resulting in increased antitumor efficacy.

Thus, from these preclinical experiments, IL-12 appears to hold promise for the therapy of malignancies in patients. Clinical trials with this cytokine have been initiated, and future studies will determine if the success of IL-12 in animal models will translate into clinical efficacy.

5. CONCLUSIONS

This chapter demonstrates the important role of preclinical studies in the clinical development of cytokines for cancer therapy. Biological activities of a cytokine that are relevant to cancer therapy must first be demonstrated using both in vitro and in vivo assays. Once these effects have been established, the cytokine can be tested in the appropriate animal tumor models. The tumor models are useful in providing the rationale for clinical testing of the cytokine, as well as an initial dose range and treatment schedule to begin clinical trials. Furthermore, the data obtained from these experiments can be used to identify factors that determine a positive response so patient subgroups likely to respond will be identified. However, limitations to these models include the species-specific effects of some cytokines, the immunogenicity of some syngeneic tumors, and the frequent inability of commonly used end points, such as tumor growth inhibition and reduction in metastases, to translate into positive clinical responses. There is a need to refine the animal models used and to evaluate antitumor efficacy in the context of the toxicities induced by the treatment. Nevertheless, the results of these studies provide a starting point for use in the clinic and then continue to influence the direction of clinical testing. Thus, a constant interaction between preclinical and clinical research is essential to realize fully the potential of cytokines in cancer therapy.

In addition, the experience gained from the clinical development of one cytokine can provide some of the directions needed for the development of other cytokines. Almost 30 years passed from the discovery of IFN-α to its approval for clinical use, whereas the time for IL-2 was considerably shorter. Hopefully, the principles learned from the development of these cytokines will lead to the rapid development of IL-12, as well as other cytokines, for the treatment of human malignancies.

REFERENCES

1. Isaacs A, Lindenmann J. Virus interference I. The interferon. *Proc R Soc Lond Ser B* 1957; 147:258-267.
2. Balkwill FR. *Cytokines in Cancer Therapy.* New York: Oxford University Press, 1989.
3. Jahiel RI, Krim M. Interferons: biology, clinical trials, and effects on hematologic neoplasms. In: Chiao JW, ed. *Biological Response Modifiers and Cancer Therapy.* New York: Marcel Dekker. 1988:197-266.
4. Uze G, Lutfalla, Gresser I. Genetic transfer of a functional human interferon α receptor into mouse cells: cloning and expression of its cDNA. *Cell* 1990; 60:225-234.
5. Novick D, Cohen B, Rubinstein M. The human interferon α/β receptor: characterization and molecular cloning. *Cell* 1994; 77:391-400.
6. Paucker K, Cantell K. Henle W. Quantitative studies on viral interference in suspended L cells. III. Effect of interfering viruses and interferon on the growth rate of cells. *Virology* 1962; 17: 324-334.
7. Gresser I. Antitumor effects of interferon. *Adv Cancer Res* 1972; 16:97-140.
8. Taylor-Papadimitriou J, Rozengurt E. Interferons as regulators of cell growth and differentiation. In: Taylor-Papadimitriou J, ed. *Interferons, Their Impact in Biology and Medicine.* Oxford: Oxford Medical Publications. 1985:81-98.
9. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977; 197: 461-463.
10. Salmon SE, Durie BG, Young L, Liu RM, Trown PW, Stebbing N. Effects of cloned human leukocyte interferons in the human tumor stem cell assay. *J Clin Oncol* 1983; 1:217-225.
11. Wadler S, Schwarz EL. Antineoplastic activity of the combination of interferon and cytotoxic agents against experimental and human malignancies: a review. *Cancer Res* 1990; 50:3473-3486.
12. Denz H. Lechleitner M, Marth Ch, Daxenbichler G, Gastl G, Braunsteiner H. Effect of human recombinant alpha-2 and gamma-interferon on the growth of human cell lines from solid tumors and hematologic malignancies. *J Interferon Res* 1985; 5:147-157.
13. Brunda MJ, Wright RB. Differential antiproliferative effects of combinations of recombinant interferons alpha and gamma on two murine tumor cell lines. *Int J Cancer* 1986; 37:287-291.
14. Kikuchi A, Holan V, Minowada J. Effects of tumor necrosis factor alpha, interferon alpha and interferon gamma on non-lymphoid leukemia cell lines: growth inhibition, differentiation induction and drug sensitivity modulation. *Cancer Immunol Immunother* 1992; 35(4):257-263.
15. Matsubara N, Fuchimoto S, Orita K. Antiproliferative effects of natural human tumor necrosis factor-alpha, interferon-alpha, and interferon-gamma on human pancreatic carcinoma cell lines. *Int J Pancreatol* 1991; 8(3):235-243.
16. Dorr RT. Interferon-α in malignant and viral diseases. *Drugs* 1993; 45(2):177-211.
17. Einhorn S, Showe L, Ostlund L, Juliusson G, Robert KH, Gahrton G, Croce C. Influence of interferon-alpha on the expression of cellular oncogenes in primary chronic lymphocytic leukemia cells. *Oncogene Res* 1988; 3(1):39-49.
18. Hannigan GE, Williams BR. Interferon-alpha activates binding of nuclear factors to a sequence element in the c-fos proto-oncogene 5'-flanking region. *J Interferon Res* 1992; 12(5): 355-361.
19. Baron S. Tyring SK, Fleischmann WR, Coppenhaver DH, Niesel DW, Klimpel GR, Stanton GJ, Hughes TK. The interferons: mechanisms of action and clinical applications. *J Am Med Assoc* 1991; 266:1375-1383.
20. Pelligrini S, Schindler C. Early events in signalling by interferons. *Trends Biochem Sci* 1993; 18:338-342.
21. Gresser I, Brouty-Boye D, Thomas MT, Macieira A. Interferon and cell division. I. Inhibition of the multiplication of mouse leukemia L1210 cells in vitro by interferon preparations. *Proc Natl Acad Sci USA* 1970; 66:1052-1058.
22. Gresser I, Maury C, Brouty-Boye D. Mechanisms of the antitumor effect of interferon in mice. *Nature (Lond)* 1972; 239:167,168.
23. Lee SH, Kelley S, Chiu H, Stebbing N. Stimulation of natural killer cell activity and inhibition of proliferation of various leukemic cells by purified human leukocyte interferon subtypes. *Cancer Res* 1982; 42:1312-1315.
24. Vuist WM, Visseren MJ, Otsen M, Bos K, Vyth-Dreese FA, Figdor CG, Melief CJ, Hekman

A. Enhancement of the antibody-dependent cellular cytotoxicity of human peripheral blood lymphocytes with interleukin-2 and interferon alpha. *Cancer Immunol Immunother* 1993; 36(3):163–170.
25. Hokland P, Berg K. Interferon enhances the antibody-dependent cellular cytotoxicity (ADCC) of human polymorphonuclear lymphocytes. *J Immunol* 1981; 127:1585–1588.
26. Webb DSA, Zur Nedden D, Miller DM, Zoon KC, Gerrard TL. Enhancement of monocyte-mediated tumoricidal activity by multiple interferon-α species. *Cell Immunol* 1989; 124: 158–167.
27. Oberg K. The action of interferon alpha on human carcinoid tumours. *Semin Cancer Biol* 1992; 3(1):35–41.
28. Angus R, Collins CM, Symes MO. The effect of alpha and gamma interferon on cell growth and histocompatibility antigen expression by human renal carcinoma cell in vitro. *Eur J Cancer* 1993; 29A(13):1879–1885.
29. Gaicomini P, Aguzzi A, Pestha S. Modulation by recombinant DNA leukocyte (α) and fibroblast (β) interferons of the expression and shedding of HLA- and tumor-associated antigens by human melanoma cells. *J Immunol* 1984; 133:1649–1655.
30. Greiner JW, Hand PH, Noguchi P, Fisher PB, Pestka S, Schlom J. Enhanced expression of surface tumor associated Ag on human breast and colon tumor cells after recombinant human leukocyte α-interferon treatment. *Cancer Res* 1984; 44:3208–3211.
31. Brouty-Boye D, Zetter BR. Inhibition of cell motility by interferon. *Science* 1980; 208:516–518.
32. Dvorak H, Gresser I. Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumors in mice. *J Natl Cancer Inst* 1989; 81:497–502.
33. Gresser I, Coppey J, Falcoff E, Fontaine D. Interferon and murine leukemia. I. Inhibitory effects of interferon preparations on development of Friend leukemia in mice. *Proc Soc Exp Biol Med* 1967; 124:84–91.
34. Gresser I, Coppey J, Bourali C. Interferon and murine leukemia. 6. Effect of interferon preparations on lymphoid leukemia of AKR mice. *J Natl Cancer Inst* 1969; 43:1083–1089.
35. Gresser I, Bourali C. Exogenous interferon and inducers of interferon in the treatment of Balb/c mice inoculated with RC_{19} tumor cells. *Nature* 1969; 223:844–845.
36. Thomas H, Balkwill FR. Effects of interferons and other cytokines on tumors in animals: a review. *Pharmacol Ther* 1991; 52:307–330.
37. Brunda MJ, Rosenbaum D, Stern L. Inhibition of experimentally-induced murine metastases by recombinant alpha interferon: correlation between the modulatory effect of interferon treatment on natural killer cell activity and inhibition of metastases. *Int J Cancer* 1984; 34: 421–426.
38. Gresser I, Maury C, Carnaud C, De Maeyer E, Maunroy MT, Belardelli F. Antitumor effects of IFN in mice injected with IFN sensitive and IFN-resistant Friend erythroleukaemia cells. VIII. Role of the immune system in the inhibition of visceral metastases. *Int J Cancer* 1990; 46:468–474.
39. Chirigos MA, Pearson JW. Brief communication: cure of murine leukemia with drug and interferon treatment. *J Natl Cancer Inst* 1973; 51:1367,1368.
40. Gresser I, Maury C, Tovey M. Efficacy of combined interferon cyclophosphamide therapy after diagnosis of lymphoma in AKR mice. *Eur J Cancer* 1978; 14:97–99.
41. Balkwill FR, Moodie EM. Positive interactions between human interferon and cyclophosphamide or Adriamycin in a human tumor model system *Cancer Res* 1984; 44:904–907.
42. Carmichael J, Fergusson RJ, Wolf CR, Balkwill FR, Smyth JF. Augmentation of cytotoxicity of chemotherapy by human α-interferons in human non-small cell lung cancer xenografts. *Cancer Res* 1986; 46:4916–4920.
43. Sklarin NT, Chahinian AP, Feuer EJ, Lahman LA, Szrajer L, Holland JR. Augmentation of activity of cis-diamminedichloroplatinum (II) and mitomycin C by interferon in human malignant mesothelioma xenografts in nude mice. *Cancer Res* 1988; 48:64–67.
44. Brunda MJ, Bellantoni D, Sulich V. In vivo anti-tumor activity of combinations of interferon alpha and interleukin-2 in a murine model. Correlation of efficacy with the induction of cytotoxic cells resembling natural killer cells. *Int J Cancer* 1987; 40:365–371.
45. Brunda MJ. Antitumour activity of interleukin-2 combined with other cytokines. In: Waxman J, Balkwill F, eds. *Interleukin-2.* Oxford: Blackwell Scientific Publications. 1992:106–121.

46. Koren S, Fleischmann WR. Quantitation of *in vivo* potentiation resulting from combined interferon therapy: antitumor effect against B-16 melanoma in mice. *J Interferon Res* 1986; 6: 473–482.
47. Truitt GA, Bontempo JM, Stern LL, Sulich V, Bellantoni D, Trown PW, Brunda MJ. Efficacy and toxicity elicited by recombinant interferons alpha and gamma when administered in combination to tumor-bearing mice. *Biotech Ther* 1989-90; 1(1):1–16.
48. Sayers TJ, Wiltrout TA, McCormick K, Husted C, Wiltrout RH. Antitumor effects of α-interferon and γ-interferon on a murine renal cancer (Renca) *in vitro* and *in vivo*. *Cancer Res* 1990; 50:5414–5420.
49. Brunda MJ, Wright RB, Luistro L, Harbison ML, Anderson TD, McIntyre KW. Enhanced antitumor efficacy in mice by combination treatment with interleukin-1α and interferon-α. *J Immunother* 1994; 15:233–241.
50. Sanada E, Fuchimoto S, Orita, K. Synergistic antiproliferative effect of the combination of natural human tumor necrosis factor-alpha and natural murine interferon-alpha/beta against colon-26 adenocarcinoma hepatic metastases in a murine model. *Acta Med Okayama* 1990; 44(4):217–222.
51. Kedar E, Rutkowski Y, Leshem B. Chemo-immunotherapy of murine solid tumors: enhanced therapeutic effects by interleukin-2 combined with interferon alpha and the role of specific T cells. *Cancer Immunol Immuother* 1992; 35(1):63–68.
52. Ogura H, Tani K, Ozawa K, Nagata S, Asano S, Takaku F. Implantation of genetically manipulated fibroblasts into mice as antitumour alpha-IFN therapy. *Cancer Res* 1990; 50:5102–5106.
53. Ferrantini M, Proietti E, Santodonato L, Gabriele L, Peretti M, Plavec I, Meyer F, Kaido T, Gresser I, Belardelli F. α_1-Interferon gene transfer into metastatic Friend leukemia cells abrogated tumorigenicity in immunocompetent mice: antitumor therapy by means of interferon-producing cells. *Cancer Res* 1993; 53:1107–1112.
54. Ferrantini M, Giovarelli M, Modesti A, Musiani P, Modica A, Venditti M, Peretti E, Lollini P, Nanni P, Forni G, Belardelli F. IFN-α1 gene expression into a metastatic murine adenocarcinoma (TS/A) result in $CD8^+$ T cell-mediated tumor rejection and development of antitumor immunity. *J Immunol* 1994; 153:4604–4615.
55. Came PE. Interferon: its application and future as an antineoplastic agent. In: Ottenbrite RM, Butler GB, eds. *Anticancer and Interferon Agents. Synthesis and Properties.* New York: Marcel Dekker. 1984:301–319.
56. Platanias LC, Golomb HM. Clinical use of interferons: hairy cell, chronic myelogenous and other leukemias. In: Baron S. Coppenhaver DH, Dianzani F, Fleischmann WR, Hughes TK, Klimpel GR, Niesel DW, Stanton GJ, Tyring SK, eds. *Interferon: Principles and Medical Applications.* Galveston: The University of Texas Medical Branch. 1992:487–499.
57. Strander H, Oberg K. Clinical use of interferons: solid tumors. In: Baron S. Coppenhaver DH, Dianzani F, Fleischmann WR, Hughes TK, Klimpel GR, Niesel DW, Stanton GJ, Tyring SK, eds. *Interferon: Principles and Medical Applications.* Galveston: The University of Texas Medical Branch. 1992:533–561.
58. Smith JW, Longo DL, Urba WJ, Clark JW, Watson T, Beveridge J, Conlon K, Sznol M, Creekmore SP, Alvord WG, Lawrence JB, Steis RG. Prolonged, continuous treatment of hairy cell leukemia patients with recombinant interferon-α2a. Blood 1991; 78:1664–1671.
59. Vedantham S, Gamliel H, Golomb HM. Mechanism of interferon action in hairy cell leukemia: a model of effective cancer biotherapy. *Cancer Res* 1992; 52:1056–1066.
60. Gutterman JU. Cytokine therapeutics: lessons from interferon α. *Proc Natl Acad Sci USA* 1994; 91:1198–1205.
61. Talpaz M, Kantarjian H, Kurzrock R, Trujillo JM, Gutterman JU. Interferon-alpha produces sustained cytogenetic responses in chronic myelogenous leukemia. *Ann Intern Med* 1991; 114: 532–538.
62. Arthur CK, Ma DDF. Combined interferon alpha-2a and cytosine arabinoside as first-line treatment for chronic myeloid leukemia. *Acta Haematol* 1992; 89(suppl 1):15–21.
63. Higano CS, Raskind WH, Singer JW. Use of α interferon for the treatment of relapse of chronic myelogenous leukemia in chronic phase after allogeneic bone marrow transplantation. *Blood* 1992; 80:1437–1442.
64. Keilholz U, Scheibenbogen C, MacLachlan D, Jochim A, Bergmann L, Weidmann E, Mitrou

PS, Tilgen W, Hunstein W. Treatment of metastatic melanoma with interferon-α and high dose interleukin-2. In: Bergmann L, Mitrou PS, eds. *Cytokines in Cancer Therapy. Contributions to Oncology.* Basel: Karger. 1994:191–200.

65. Khayat D, Antoine E, Rixe O, Tourani JM, Vuillemin E, Borel C, Benhammouda A, Thill L, Franks C, Auclerc G, et al. Chemoimmunotherapy of metastatic malignant melanoma. The Salpetriere Hospital (SOMPS) experience. *Eur J Cancer* 1993; 29A(S5):S2–S5.
66. Fossa SD. Proc Symp, Progress in the treatment of renal cell carcinoma. Cambridge: Queens College, 1989:12–21.
67. Bergerat JP, Herbrecht R, Dufour P, Jacqmin D, Bollack C, Prevot G, Bailly G, De Garis S, Juraschek F, Oberling F. Combination of recombinant interferon alpha-2a and vinblastine in advanced renal cell cancer. *Cancer* 1988; 62:2320–2324.
68. Fossa SD, Gunderson R, Moe B. Recombinant interferon-alpha combined with prednisone in metastatic renal cell carcinoma. *Cancer* 1990; 65:2451–2454.
69. Sella A, Logothetis J, Fitz K, Dexeus FH, Amato R, Kilbourn R, Wallace S. Phase II study of interferon-α and chemotherapy (5-fluorouracil and mitomycin C) in metastatic renal cell cancer. *J Urol* 1992; 147:573–577.
70. Rosenberg SA, Lotze MT, Yang JC, Linehan WM, Seipp C, Calabro S, Karp SE, Sherry RM, Steinberg S, White DE. Combination therapy with interleukin-2 and alpha-interferon for the treatment of patients with advanced cancer. *J Clin Oncol* 1989; 7:1863,1874.
71. Atzpodien J, Kirchner H, Poliwoda H. Treatment strategies employing chemoimmunotherapy in patients with metastatic renal carcinoma. In: Bergmann L, Mitrou PS, eds. *Cytokines in Cancer Therapy. Contributions to Oncology.* Basel: Karger. 1994:211–217.
72. Evans LM, Itri LM, Campion M, Wyler-Plaut R, Krown SE, Groopman JE, Goldsweig H, Volberding PA, West SB, Mitsuyasu RT, et al. Interferon-alpha-2a in the treatment of acquired immunodeficiency syndrome-related Kaposi's sarcoma. *J Immunother* 1991; 10:39–50.
73. De Wit R, Schattenkerk JKME, Boucher CAB, Bakker PJM, Veenhof KHN, Danner SA. Clinical and virological effects of high-dose recombinant interferon-alpha in disseminated AIDS-related Kaposi's sarcoma. *Lancet* 1988; 2:1214–1217.
74. Folkman J, Shing Y. Minireview: Angiogenesis. *J Biol Chem* 1992; 267:10,931–10,934.
75. Fischl MA. Antiretroviral therapy in combination with interferon for AIDS-related Kaposi's sarcoma. *Am J Med* 1991; 90(suppl 4A):2S–7S.
76. Morgan D, Ruscetti FW, Gallo R. Selective *in vitro* growth of T-lymphocytes from normal bone marrow. *Science* 1976; 193:1007,1008.
77. Welte K, Wang CY, Mertelsmann R, Venuta S, Felman SP, Moore MA. Purification of human interleukin-2 to apparent homogeneity and its molecular heterogeneity. *J Exp Med* 1982; 156: 454–464.
78. Gillis S, Ferm MM, Ou W, Smith KA. T-cell growth factor: Parameters of production and a quantitative microassay for activity. *J Immunol* 1978; 120:2027–2032.
79. Taniguchi T, Matsui H, Fujita T, Fakaoka C, Kashima N, Yoshimoto R, Hamuro J. Structure and expression of a cloned cDNA for human interleukin-2. *Nature* 1983; 302:305–310.
80. Leonard WJ, Depper JM, Crabtree GR, Rudikoff S, Pumphrey J, Robb RJ, Kronke M, Svetlik, PB, Peffer NJ, Waldmann TA, Greene WC. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. *Nature* 1984; 311:626–631.
81. Nikaido T, Shimizu A, Ishida N, Sabe H, Teshigawara K, Maeda M, Uchiyama T, Yodoi J, Honjo T. Molecular cloning of cDNA encoding human interleukin-2 receptor. *Nature* 1984; 311:631–635.
82. Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyasaka M, Taniguchi T. Interleukin-2 receptor β chain gene: Generation of three receptor forms by cloned human α and β chain cDNAs. *Science* 1989; 244:551–556.
83. Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, Munakata H, Natamura M, Sugamura K. Cloning of the γ chain of the human IL-2 receptor. *Science* 1992; 257:379–382.
84. Smith KA. Lowest dose interleukin-2 immunotherapy. *Blood* 1993; 81:1414–1423.
85. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: A current overview. *Cell* 1993; 73:5–8.
86. Mosmann TR, Coffman RL. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; 7:145–173.
87. Rees RC. The biological response to interleukin-2. In: Waxman J, Balkwill F, eds. *Interleukin-2.* Oxford: Blackwell Scientific Publications. 1992:47–68.

88. Kintzel PE, Calis KA. Recombinant interleukin-2: a biological response modifier. *Clin Pharm* 1991; 10:110–128.
89. Takai Y, Herrmann S, Greenstein JL, Spitalny GL, Burakoff SJ. Requirement for three distinct lymphocytes for the induction of cytotoxic T lymphocytes from thymocytes. *J Immunol* 1986; 137:3494–3500.
90. Gately MK, Wilson DE, Wong HL. Synergy between recombinant interleukin 2 (rIL 2) and IL 2-depleted lymphokine-containing supernatants in facilitating allogeneic human cytolytic T lymphocyte responses *in vitro*. *J Immunol* 1986; 136:1274–1282.
91. Hefeneider SH, Conlon PJ, Henney CS. Gillis S. *In vivo* interleukin 2 administration augments the generation of alloreactive cytolytic T lymphocytes and resident natural killer cell activity. *J Immunol* 1983; 130:222–227.
92. Rosenberg SA, Grimm EA, McGrogan M, Doyle M, Kawasaki E, Koths K, Mark DF. Biological activity of recombinant human interleukin-2 produced in *Escherichia coli*. *Science* 1984; 223:1412–1415.
93. Henney CS, Kuribayashi K, Kern DE, Gillis S. Interleukin-2 augments natural killer cell activity. *Nature* (*Lond*) 1981; 291:335–338.
94. Brunda MJ, Tarnowski D, Davatelis V. Interaction of recombinant interferons with recombinant interleukin-2: differential effects on natural killer cell activity and interleukin-2 activated killer cells. *Int J Cancer* 1986; 37:787–793.
95. Talmadge JE, Herberman RB, Chirigos MA, Maluish AE, Schneider MA, Adams JS, Philips H, Thurman GB, Varesio L, Long C, Oldham RK, Wiltrout RH. Hyporesponsiveness to augmentation of murine natural killer cell activity in different anatomical compartments by multiple injections of various immunomodulators including recombinant interferons and interleukin-2. *J Immunol* 1985; 135:2483–2491.
96. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokine-activated killer cell phenomenon: lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J Exp Med* 1982; 155:1823–1841.
97. Ortaldo JR, Mason AA, Overton R. Lymphokine-activated killer cells. Analysis of progenitors and effectors. *J Exp Med* 1986; 164:1193–1205.
98. Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science* 1986; 233:1318–1321.
99. Espinoza-Delgado I, Bosco MC, Musso T, Gusella GL, Longo DL, Varesio L. Interleukin-2 and human monocyte activation. *J Leuk Biol* 1995; 57:13–19.
100. Farrar WL, Johnson HM, Farrar JJ. Regulation of the production of immune interferon and cytotoxic T lymphocytes by interleukin 2. *J Immunol* 1981; 124:1120–1125.
101. Saraya KA. Interleukin-2 and its place in the cytokine network. In: Waxman J, Balkwill F, eds. *Interleukin-2*. Oxford: Blackwell Scientific Publications. 1992:69–77.
102. Rosenberg SA, Mule JJ, Spiess PJ, Reichert CM, Schwarz SL. Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. *J Exp Med* 1985; 161:1169–1188.
103. Lafreniere R, Rosenberg SA. Successful immunotherapy of murine experimental hepatic metastases with lymphokine-activated killer cells and recombinant interleukin 2. *Cancer Res* 1985; 45:3735–3741.
104. Thompson JA, Peace DJ, Klarnet JP, Kern DE, Greenberg PD, Cheever MA. Eradication of disseminated murine leukemia by treatment with high-dose interleukin 2. *J Immunol* 1986; 137:3675–3680.
105. Talmadge JE, Philips H, Schindler J. Tribble H, Pennington R. Systematic preclinical study on the therapeutic properties of recombinant human interleukin 2 for the treatment of metastatic disease. *Cancer Res* 1987; 47:5752.
106. Truitt GA, Brunda MJ, Levitt D, Anderson TD, Sherman MI. The therapeutic activity in cancer of IL-2 in combination with other cytokines. *Cancer Surveys* 1989; 8:875–889.
107. Gately MK, Anderson TK, Hayes TJ. Role of asialo-GM_1-positive lymphoid cells in mediating the toxic effects of recombinant IL-2 in mice. *J Immunol* 1988; 141:189–200.
108. Mule JJ, Yang JC, Lafreniere RL, Shu S, Rosenberg SA. Identification of cellular mechanisms operational *in vivo* during the regression of established pulmonary metastases by the systemic administration of high-dose recombinant interleukin 2. *J Immunol* 1987; 139:285–294.
109. Papa MZ, Vetto JT, Ettinghausen SE, Mule JJ, Rosenberg SA. Effect of corticosteroid on the

antitumor activity of lymphokine-activated killer cells and interleukin 2 in mice. *Cancer Res* 1986; 46:5618-5623.

110. Papa MZ, Mule JJ, Rosenberg SA. Antitumor efficacy of lymphokine-activated killer cells and recombinant interleukin 2 *in vivo*: Successful immunotherapy of established pulmonary metastases from weakly immunogenic and nonimmunogenic murine tumors of three distinct histological types. *Cancer Res* 1986; 46:4973-4978.
111. Weber JS, Jay G, Tanaka K, Rosenberg SA. Immunotherapy of a murine tumor with interleukin 2. Increased sensitivity after MHC class I gene transfer. *J Exp Med* 1987; 166:1716-1733.
112. Mule JJ, Shu S, Schwarz SL, Rosenberg SA. Adoptive immunotherapy of established pulmonary metastases with LAK cells and recombinant interleukin-2. *Science* 1984; 225:1487-1489.
113. Mule JJ, Shu S, Rosenberg SA. The anti-tumor efficacy of lymphokine-activated killer cells and recombinant interleukin 2 *in vivo*. *J Immunol* 1985; 135:646-652.
114. Lafreniere R, Rosenberg SA. Successful immunotherapy of murine experimental hepatic metastases with lymphokine-activated killer cells and recombinant interleukin 2. *Cancer Res* 1985; 45:3735-3741.
115. Mule JJ, Yan J, Shu S, Rosenberg SA. The anti-tumor efficacy of lymphokine-activated killer cells and recombinant interleukin 2 *in vivo*: Direct correlation between reduction of established metastases and cytolytic activity of lymphokine-activated killer cells. *J Immunol* 1986; 136: 3899-3909.
116. Basse P, Herberman RB, Nannmark U. Johansson BR, Hokland M, Wasserman K, Goldfarb RH. Accumulation of adoptively transferred adherent, lymphokine-activated killer cells in murine metastases. *J Exp Med* 1991; 174:479-488.
117. Futami H, Pilar o AM, Gruys ME, Back TC, Young HA, Wiltrout RH. *In vivo* distribution and cytokine expression by enriched mouse LAK effector cells. *Biotherapy* 1991; 3:219-232.
118. Spiess PJ, Yang JC, Rosenberg SA. In vivo antitumor activity of tumor-infiltrating lymphocytes expanded in recombinant interleukin-2. *J Natl Cancer Inst* 1987; 79:1067-1075.
119. Alexander R, Rosenberg SA. Long term survival of adoptively transferred tumor-infiltrating lymphocytes in mice. *J Immunol* 1990; 145:1615-1620.
120. Alexander RB, Rosenberg SA. Adoptively transferred tumor-infiltrating lymphocytes can cure established metastatic tumor in mice and persist long-term *in vivo* as functional memory T lymphocytes. *J Immunother* 1991; 10:389-397.
121. Salup RR, Back TC, Wiltrout RH. Successful treatment of advanced murine renal cell cancer by bicompartmental adoptive chemoimmunotherapy. *J Immunol* 1987; 138:641-647.
122. Papa MZ, Yang JC, Vetto J, Shiloni E, Eisenthal A, Rosenberg SA. Combined effects of chemotherapy and interleukin 2 in the therapy of mice with advanced pulmonary tumors. *Cancer Res* 1988; 48:122-129.
123. Fearon ER, Pardoll DM, Itaya T, Golumbek P, Levitsky HI, Simons JW, Karasuyama H, Vogelstein B, Frost P. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell* 1990; 60:397-403.
124. Gansbacher B, Zier K, Daniels B, Cronin K, Bannerji R, Gilboa E. Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *J Exp Med* 1990; 172:1217-1224.
125. Kim TS, Cohen EP. Interleukin-2-secreting mouse fibroblasts transfected with genomic DNA from murine melanoma cells prolong the survival of mice with melanoma. *Cancer Res* 1994; 54:2531-2535.
126. Whittington R, Faulds D. Interleukin-2. A review of its pharmacological properties and therapeutic use in patients with cancer. *Drugs* 1993; 46:446-514.
127. Bruton JK, Koeller JM. Recombinant interleukin-2. *Pharmacotherapy* 1994; 635-656.
128. Physicians Desk Reference. Proleukin. Medical Economics, Montvale, NJ. 1994:801-804.
129. Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber J, Shiloni E, Vetto JT, Seipp CA, Simpson C, Reichert CM. Observations on the systemic administration of autologous lymphokine activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Eng J Med* 1985; 313:1485-1492.
130. Palmer PA, Vinke J, Evers P, Porreaux C, Oskam R, Roest G, Viems F, Becker L, Loriaux E, Franks GP. Continuous infusion of recombinant interleukin-2 with or without autologous lymphokine activated killer cells for the treatment of advanced renal cell carcinoma. *Eur J Cancer* 1992; 28A:1038-1044.

131. Rosenberg SA, Lotze MT, Yand JC, Topalian SL, Chang AE, Schwartzentruber DJ, Aebersold P, Leitman S, Linehan WM, Seipp CA, White DE, Steinberg SM. Prospective randomized trial of high-dose interleukin-2 alone or in combination with lymphokine-activated killer cells for the treatment of patients with advanced cancer. *J Natl Cancer Inst* 1993; 85:622–632.
132. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Lotze MT, Yang JC, Seipp CA, Simpson C, Carter C, Bock S, Schwartzentruber D, Wei JP, White DE. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. *N Eng J Med* 1988; 319:1676–1680.
133. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biological effects on human lymphocytes. *J Exp Med* 1989; 170:827–845.
134. Stern AS, Podlaski FJ, Hulmes JD, Pan Y-CE, Quinn PM, Wolitzky AG, Familletti PC, Stremio DL, Truitt T, Chizzonite R, Gately MK. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc Natl Acad Sci USA* 1990; 87:6808–6812.
135. Gubler U, Chua AO, Schoenbaut DS, Dwyer CM, McComas W, Motyka R, Nabavi N, Wolitzky AG, Quinn PM, Familletti PC, Gately MK. Coexpression of two distinct genes is required to generate secreted, bioactive cytoxic lymphocyte maturation factor. *Proc Natl Acad Sci USA* 1991; 88:4143–4147.
136. Wolf SF, Temple PA, Kobayashi M, Young D, Dicig M, Lowe L, Dzialo R, Fitz L, Ferenz C, Hewick RM, Kelleher K, Herrmann SH, Clark SC, Azzoni L, Chan SH, Trinchieri G, Perussia B. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J Immunol* 1991; 146:3074–3081.
137. Chizzonite R, Truitt T, Desai BB, Nunes P, Podlaski FJ, Stern AS, Gately MK. IL-12 receptor. I. Characterization of the receptor on phytohemagglutinin-activated human lymphocytes. *J Immunol* 1992; 148:3117–3124.
138. Desai BB, Quinn PM, Wolitzky AG, Mongini PKA, Chizzonite R, Gately MK. IL-12 receptor. II. Distribution and regulation of receptor expression. *J Immunol* 1992; 148:3125–2132.
139. Chua AO, Chizzonite R, Desai BB, Truitt T, Nunes P, Minetti LJ, Warrier RR, Presky DH, Levine JF, Gately MK, Gubler U. Expression cloning of a human IL-12 receptor component: a new member of the cytokine receptor superfamily with strong homology to gp130. *J Immunol* 1994; 153:128–136.
140. Brunda MJ. Interleukin-12. *J Leukocyte Biol* 1994; 55:280–288.
141. Gately MK, Gubler U, Brunda MJ, Nadeau RR, Anderson TD, Lipman JM, Sarmiento U. Interleukin-12: a cytokine with therapeutic potential in oncology and infectious diseases. *Therapeutic Immunol* 1994; 1:187–196.
142. Brunda MJ, Gately MK. Interleukin-12: potential role in cancer therapy. In: DeVita V, Hellman S, Rosenberg SA, eds. *Important Advances in Oncology 1995*. Philadelphia: JB Lippincott Company. 1995:3–18.
143. Gately MK, Brunda MJ. Interleukin-12: a pivotal regulator of cell-mediated immunity. In: Kurzrock R, Talpaz M. eds. *Cytokines: Interleukins and Their Receptors*. Norwell, MA: Kluwer Academic Publishers. 1995:341–366.
144. Schoenhaut DA, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, Familletti PC, Gately MK, Gubler U. Cloning and expression of murine IL-12. *J Immunol* 1992; 148: 3433–3440.
145. Gately MK, Warrier RR, Honasoge S, Carvajal DM, Faherty DA, Connaughton SE, Anderson TD, Sarmiento U, Hubbard BR, Murphy M. Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-γ in vivo. *Intl Immunol* 1994; 6:157–167.
146. Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, Wolf SF, Gately MF. Antitumor and antimetastatic activity of interleukin-12 against murine tumors. *J Exp Med* 1993; 178:1223–1230.
147. Nastala CL, Edington HD, McKinney TG, Tahara H , Nalesnik M, Brunda MJ, Gately MK, Wolf SF, Schreiber RD, Stewart T, Storkus WJ, Lotze MJ. Recombinant interleukin-12 (IL-12) administration induces tumor regression in association with interferon-gamma production. *J Immunol* 1994; 153:1697–1706.

148. Brunda MJ, Gately MK. Antitumor activity of interleukin-12. *Clin Immunol Immunopath* 1994; 71:253–255.
149. Brunda MJ, Luistro L, Hendrzak JA, Fountoulakis M, Garotta G, Gately MK. Interleukin-12: biology and preclinical studies of a new anti-tumor cytokine. In: Bukowski RM, Finke JH, Klein EA, eds. *The Biology of Renal Cell Carcinoma.* New York: Springer-Verlag, pp. 177–188.
150. Tahara J, Zeh HJ, Storkus WJ III, Pappo I, Watkins SC, Gubler U, Wolf SF, Robbins PD, Lotze MT. Fibroblasts genetically engineered to secrete IL-12 can suppress tumor growth and induce antitumor immunity to murine melanoma in vivo. *Cancer Res* 1994; 54:182–189.
151. Brunda MJ, Luistro L, Hendrzak JA, Fountoulakis M, Garotta G, Gately MK. Role of interferon gamma in mediating the antitumor efficacy of interleukin-12. *J Immunother* 1995; 17:71–77.
152. O'Toole M, Wolf SF, O'Brien C, Hubbard B, Herrmann S. Effect of in vivo IL-12 administration on murine tumor cell growth. *J Immunol* 1993; 150:294A.
153. Stern LL, Tarby CM, Tamborini B, Truitt GA. Preclinical development of IL-12 as an anticancer drug: comparison to IL-2. *Proc Am Assoc Cancer Res* 1994; 35:520.
154. Mayor SE, O'Donnell MA, Clinton SK. Interleukin-12 (IL-12) immunotherapy of experimental bladder cancer. *Proc Am Assoc Cancer Res* 1994; 35:474.
155. Hendrzak JA, Luistro L, Gately MK, Garotta G, Brunda MJ. Role of interferon gamma in mediating the antitumor effects of interleukin-12. *Proc Am Assoc Cancer Res* 1994; 35:524.

14 Discovery of TNP-470 and Other Angiogenesis Inhibitors

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Contents

1. INTRODUCTION

Most types of cancer chemotherapy, either currently available or in development, are cytotoxic in that they are designed to kill rapidly growing tumor cells. Because many normal tissues contain stem cells that also proliferate rapidly (e.g., bone marrow, hair follicles, intestines), these types of agents often cause many side effects, and thus, their clinical effectiveness is limited. For this reason, many in the cancer field have begun to pursue different approaches.

One of the most novel and promising approaches to anticancer therapy does not target the tumor cells directly. Instead, the objective is to inhibit the growth of new capillary blood vessels that feed the growing tumor, a process that is known as tumor "angiogenesis." This chapter will explain the concept behind this approach and review the process of discovery in the field of angiogenesis inhibition. In particular, it will focus on a serendipitous discovery I made 10 years ago while working as a postdoctoral fellow in the laboratory of Judah Folkman. That initial observation led to the development of TNP-470, a potent angiogenesis inhibitor that is now entering Phase II clinical trials. In the process of reviewing these discoveries, preclinical data will be described that, I believe, provide the "proof of principle" for a cancer therapy to be based on angiogenesis inhibition.

Importantly, the chapter that follows is not designed to serve as a thorough introduction to the field or a review of all angiogenesis inhibitors that have been identified. A number of excellent reviews of this type have been published recently (*1–3*). Rather, here I hope to convey the importance of serendipity in the process of cancer drug discovery and the equally important need for a receptive research environment.

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

2. IN SEARCH OF ANGIOGENESIS INHIBITORS

The search for angiogenesis inhibitors began almost 25 years ago when J. Folkman first suggested that tumors are angiogenesis-dependent (*4*). This hypothesis was based on results of animal studies and anecdotal observations in human cancer patients, which showed that for tumors to grow larger than approx 1–2 mm^3 in size, they must gain the ability to stimulate ingrowth of new capillaries (*5–8*). The reason for this is that tumor cells, like all living cells, require a continous supply of oxygen and nutrients in order to grow. In addition, effective blood flow may remove factors that suppress tumor cell death (i.e., apoptosis) (*9*).

The net result is that tumors generally will not grow significantly in size, and thus, will not be malignant or often clinically detectable if new capillary growth is prevented (*5,9–13*). A simple example is cutaneous melanoma. Extensive morphometric analysis of histological sections of human melanoma specimens shows that tumors that do not promote angiogenesis tend to remain < 0.76 mm in thickness (these are planar lesions) and exhibit a low incidence of metastasis (*14*). In contrast, once capillary ingrowth is stimulated, this size restriction is circumvented, and malignancy results. Quantitation of capillary densities in pathological specimens also have been found to be an excellent prognostic indicator in human patients with breast cancer (*15*).

The proposal that tumors depend on continual neovascularization for their own growth and expansion immediately stimulated a search for a tumor "angiogenesis factor," that is, the soluble mitogen that was thought to be responsible for induction of capillary ingrowth (*4,6,16*). The concept was simple from a therapeutic perspective: If one could identify this molecule, then specific inhibitors, such as blocking antibodies, could be developed to prevent tumor expansion. Combination of these growth-inhibitory agents with conventional drugs, radiotherapy, and surgical intervention could therefore lead, in theory, to rational management or even cure of this disease.

Great advances have been made in the search for angiogenic factors. Many angiogenic proteins have now been purified, sequenced, and cloned (*2,17,18*). Some angiogenic factors (e.g., acidic and basic FGFs) are mitogentic for a variety of cell types in addition to capillary cells. In contrast, others appear to be highly specific for endothelium (e.g., VEGF). Other factors (e.g., TGF-B, TNF-α) are not directly mitogenic for endothelial cells, and thus may act indirectly by activating or recruiting other cells, such as macrophages, that in turn release angiogenic mitogens. Alternatively, some angiogenic factors appear to promote angiogenesis directly by stimulating endothelial cell migration and/or capillary tube formation, rather than cell growth *per se* (*19*).

Importantly, as time went on, it became clear that there was enormous redundancy among angiogenic factors. Many different types of molecules can stimulate capillary growth, and a single tumor can produce more than one angiogenic mitogen (*3,17,18, 20*). Thus, more recently, emphasis has shifted away from growth factor identification to understanding the molecular basis of capillary growth control.

2.1. Rational Drug Discovery

In the angiogenesis field, as in many other areas of cancer research, the current concept is that a more in depth understanding of the molecular basis of cell and tissue regulation will lead directly to development of new therapeutic agents. In fact, there are many cases where this has been shown to be true. Examples of angiogenesis in-

hibitors that have been discovered based on a rational approach include: cartilage-derived inhibitor, protamine, platelet factor 4, medroxyprogesterone, proline analogues, retinoids, integrin receptor antagonists, thalidomide, and most recently, angiostatin.

Cartilage-derived inhibitor was first sought after (*21,22*) and later purified (*23*) based on the observation that cartilage is a relatively avascular tissue. Interestingly, sharks, which are highly cartilagenous creatures, both contain angiogenesis inhibitory activity in their cartilage and rarely develop solid tumors (*24*).

Protamine and platelet factor 4 were tested and found to inhibit new capillary growth based on their known high affinity for heparin (*25,26*). This work followed the discovery that heparin can enhance angiogenic factor activity in vitro (*27*). At about the same time, Shing and Klagsbrun (again working in Folkman's department) also found that many angiogenic factors can be purified based on their ability to bind heparin (*28,29*).

The finding that extracellular matrix proteolysis mediates initiation of angiogenesis in vivo (*30,31*) and endothelial cell migration in vitro (*32*) led to the testing of known collagenase inhibitors in angiogenesis assays. This approach resulted in the discovery of the antiangiogenic effects of medroxy-progesterone (*33*). More recently, cartilage-derived inhibitor was found to exhibit anticollagenase activities as well (*23*). In addition, BB94, a synthetic collagenase antagonist that was initially developed to inhibit tumor growth and metastasis, also has been found to inhibit neovascularization (*34*). BB94 is currently in Phase I clinical trials.

Proline analogs, retinoids, and other inhibitors of extracellular matrix deposition and processing (e.g., α,α-dipyridyl, β-aminopriopionitrile) were examined because other angiogenesis inhibitors (heparin-angiostatic steroid combinations) were found to induce capillary basement membrane dissolution as part of their action (*35*). All of these compounds inhibited angiogenesis in the CAM, and their inhibitory effects correlated directly with their ability to suppress collagen deposition in that assay (*36*).

Soluble antagonists of integrin $\alpha_V\beta_3$ receptors (e.g., blocking antibodies, synthetic RGD peptides) were tested and found to exhibit antiangiogenic activity based on the observation that growing endothelial cells appear to express preferentially this particular extracellular matrix receptor subtype on their surfaces (*37,38*). Work in my laboratory also had previously shown that extracellular matrix and integrins play a key role in capillary growth control in vitro (*39–43*) as well as in vivo (*35,36*).

The discovery of thalidomide's antiangiogenic activity is quite interesting. R. J. D'Amato, an opthalmologist working as a research fellow in Folkman's laboratory, decided that a rational way to identify new angiogenesis inhibitors would be to search the medical literature (using a computer) for existing drugs that exhibit "side effects" that might be expected to be associated with angiogenesis inhibition (e.g., increased rates of spontanteous abortion, large-scale development defects). The discovery of thalidomide's antiangiogenic capabilities resulted (*44*). Thalidomide has recently entered Phase I clinical trials for treatment of angiogenesis-dependent disease in the eye.

Most recently, angiostatin was discovered based on a search for an endogenous inhibitor in serum that might be responsible for the observed inhibitory influence exerted by large primary tumors on the growth of secondary metastatic lesions (a process that was sometimes referred to as "concomitant immunity" in the past). Although little was known about the mechanism behind this inhibition, Folkman had

suspected for many years that it could be due to production of systemic angiogenesis inhibitors that would effectively suppress growth of small tumors at a distance. In this model, tumor expansion would continue at the primary site, because short-lived angiogenic stimulators overcame the inhibitor locally and shifted the balance toward capillary growth at the primary site. However, if clearance of the inhibitors was much slower than that of the angiogenic mitogen, then capillary growth suppression might be expected to dominate systemically. M. O'Reilly, another surgical resident working as a research fellow in Folkman's laboratory, purified a fragment of plaminogen from serum and urine that has been named "angiostatin" (*13*). Angiostatin, which appears to be generated or released by primary tumors that suppress the growth of their metastases, effectively prevents the growth of both primary and metastatic lesions when administered to mice and, thus, exhibits all of the properties that Folkman envisioned.

2.2. Discovery by Chance: Angiostatic Steroids

Although the rational approach will likely pay off in the long term, it turns out that other angiogenesis inhibitors were discovered based entirely on chance. A classic example was when S. Taylor, a medical student working in Folkman's laboratory, discovered that heparin and cortisone inhibit angiogenesis (*10*). She actually made this discovery in the course of trying to optimize a screening assay for angiogenic modulators.

To study angiogenesis, Taylor had been placing tumor extracts on the surface of the chick chorioallantoic membrance (CAM). This embryonic membrane is used as an angiogenesis model, because it is underlined by a continously extending capillary network that grows rapidly from day 6 to day 10 of development (*35*). In earlier studies, it was shown that application of living tumor cells, tumor extracts, or partially purified angiogenic factor preparations to the surface of the CAM resulted in enhanced angiogenesis, as visualized by increased growth of vessels in a "spoke-wheel" pattern that converged toward the growth stimulus (*8,25*).

When using this CAM assay, Taylor first added heparin in an attempt to enhance the activity of the tumor-derived angiogenic factors and, thus, increase the sensitivity of the assay (*25*). Heparin had been previously shown to enhance angiogenic factor-induced capillary cell migration in vitro (*27*). However, in the assay Taylor used, dust often would fall onto the CAM from the surrounding egg shell causing nonspecific "inflammatory" angiogenesis that complicated the interpretation of her results. In an attempt to suppress this inflammatory activity and increase the signal-to-noise, Taylor and Folkman decided to add cortisone (a potent immune suppressant) to the assay. Surprisingly, they found that not only was this nonspecific background angiogenesis-inhibited, but also both normal embryonic capillary growth and tumor extract-induced angiogenesis were completely prevented as well. In addition, regression of pre-existing capillaries, but not neighboring epithelium, was observed over large regions of the CAM. These studies were extended by Folkman and coworkers to show that combinations of cortisone and heparin or heparin fragments can inhibit angiogenesis and suppress tumor growth in a variety of animal models (*10,45*).

Taylor and Folkman's discovery eventually led to the identification and characterization of an entirely new class of antiangiogenic or "angiostatic" steroids (*35, 46–48*). These steroids are potent inhibitors of neovascularization when combined with hep-

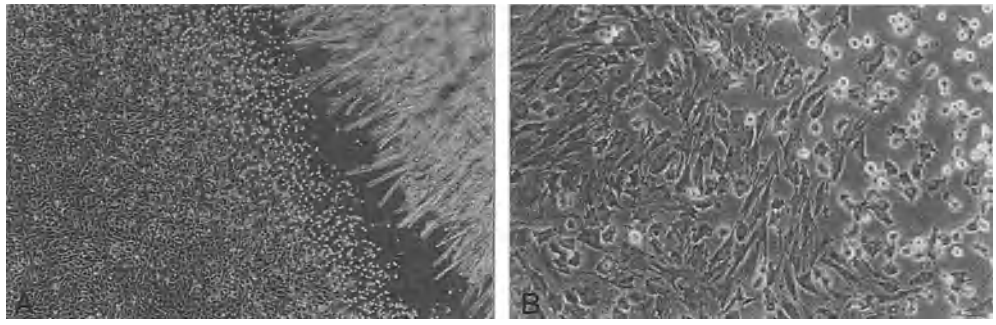


Fig. 1. The fungal contaminant that led to the development of TNP-470. (**A**) A low-magnification phase-contrast view showing the hyphae of the fungal contaminant at the top right of the culture well with the retracting endothelial monolayer below. (**B**) A higher magnification view of the endothelial monolayer near the edge of the fungal colony. A gradient of increased cell rounding can be observed, as one moves from the bottom left to the top right of the view.

arin, yet they have no known glucocorticoid, mineralocorticoid, or sex steroid activities. Importantly, some of these angiostatic steroids occur naturally as "inactive" steroid metabolites in urine (e.g., tetrahydrocortisol). The presence of these endogenous inhibitors may play an important role in maintaining the low cell turnover rate that is characteristic of the normal vascular endothelium (*49*). This work also indirectly led to the search for other angiogenesis inhibitors in urine; the discovery of genistein (*50*) and 2-methoxyestradiol (*51*) soon followed.

3. TNP-470: SERENDIPITY LEADS TO A NEW ANTICANCER DRUG

3.1. Initial Observation

Perhaps one of the best examples of the importance of serendipity in drug discovery in the angiogenesis field is the case of TNP-470 (originally called AGM-1470; *11*). This story is a very personal one since it began with an observation I made while working as a postdoctoral fellow in Folkman's laboratory. In the course of passing standard capillary endothelial cell cultures on November 1, 1985, I discovered fungal contaminants growing in three different wells of the same multiwell culture dish. One of these contaminants I had unfortunately seen many times in the past. Even when small, this type of fungus induced complete cell detachment and death in our endothelial cell cultures. It was a known scourge that would emerge from time to time. My immediate reflex response on seeing this fungus, based on past training and experience, was to immerse the entire culture dish immediately in disinfectant and to place it in line for autoclaving and disposal (the standard approach to contamination in our lab at that time).

However, the fungus in the second well immediately attracted my attention. In contrast to all other fungi I had seen in the past and to the contaminants in the neighboring wells, this fungus did not induce complete cell detachment even though it covered about one-third of a 35-mm well. Instead, this fungus appeared to induce a gradient of cell retraction and rounding in the adjacent endothelial monolayer (Fig. 1). Directly

below the fungal colony and along its edge, endothelial cells appeared adherent, but completely spherical, whereas cells only a few diameters away exhibited a normal extended morphology. This morphological change interested me because in other ongoing studies in the laboratory, I had recently found that capillary cells will not grow when stimulated by any angiogenic factor, if cells are induced to round by preventing cell adhesion to extracellular matrix (later published in *35, 39–42*). This basic work was a direct extension of my dissertation research with J Jamieson at Yale, which centered on the importance of cell shape in the control of tissue growth and form (*52–54*). Folkman himself was a pioneer in this area having published an elegant study with A. Moscona (a medical student) in 1978 that was the first to show clearly that cell shape plays a key role in growth control (*55*). In fact, one of the major reasons I chose to carry out my postdoctoral research training in Folkman's laboratory (i.e., rather than complete a clinical residency) was his openness to this novel way of looking at cell growth regulation.

In any case, it was because of my unconventional view of cell growth control that I cultured these fungi. I also cultured the fungus in the third well, which was of similar size, yet it produced only minimal cell detachment or rounding as another control. All three contaminants were cultured in Sabouraud's agar tubes that "happened" to be sitting on a shelf in our mammalian tissue-culture laboratory. Amazingly, it took almost 2 mo before I communicated this finding to Folkman. Basically, I was ambivalent about launching yet another research project given the already overextended state of any postdoctoral fellow. Nevertheless, about 6 wk later in late December of 1985, I could no longer hold back my excitement, and I finally told Folkman of my "discovery." He was extremely enthusiastic and immediately saw the potential importance of this observation. In fact, he was so excited about the finding that he cultured the fungus, collected the medium, and set about testing it for angiogenesis inhibitory activity himself.

The question of whether this fungus might secrete a **soluble** inhibitor was a very important one. About a year before this incident, Folkman had tried to isolate an angiogenesis inhibitor from a fungus that had spontaneously settled on a CAM, apparently inducing capillary regression in that assay. However, that project was a complete failure; if the fungus did produce an angiogenesis inhibitor, it was not secreted in a soluble form that could be detected. The major lesson learned was that isolating a fungus in a mammalian cell tissue-culture laboratory can be a dangerous mistake: rampant contamination (and death) of many endothelial cell cultures resulted. In fact, this fear of contamination was another reason that I questioned whether I should tell Folkman about my discovery. It is also important to point out that the Sabbouraud's agar tubes I used would never have been available in our cell-culture laboratory, if it were not for this prior experience with fungi. Thus, chance played a key role at least twice in the early phase of this discovery process.

Once we cultured the fungus, we confirmed that it indeed secreted a soluble factor that inhibited embryonic angiogenesis in the CAM as well as capillary endothelial cell proliferation in vitro. When the fungus was sent for typing, it was identified as a relatively rare fungus, *Aspergillus fumigatus fresenius*. Conditioned medium from cultures of this fungus also induced cell rounding, and this activity was used as an initial bioassay during subsequent purification of the active molecule.

A third element of chance then came into play. Work in the Folkman laboratory had previously been funded in part by an industrial grant from the Monsanto Company. This grant ended at about this time, and thus, we actively sought funding from a number of different American pharmaceutical companies. When these companies came to review our work, we described our preliminary findings relating to this fungal contaminant. However, there was little interest. At least one of the executives that reviewed our findings very openly stated that he had little interest because he did not see anything "that would be in the clinic within the next six months."

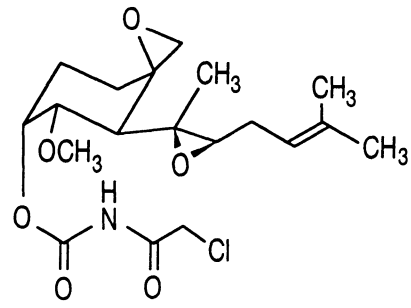
When all seemed to be lost, Folkman received a note from a Dr. Sugino, who had briefly visited the laboratory in the past. It turned out that Sugino now held a high position in a major pharmaceutical company in Japan called Takeda Chemical Industries, Limited. On hearing that Folkman was seeking new funding, Sugino immediately sent a delegation to review our proposal. Rather than send mostly businessman (the approach of the American companies at that time), Takeda sent a delegation that was filled primarily with research scientists. I have two memorable recollections of this meeting: (1) the Takeda scientists were extremely excited about the fungus because microbial analysis and culture were major strengths of their company, and (2) Takeda took a long-term view of the drug discovery process and clearly stated their belief that angiogenesis would represent a major target for therapeutic intervention in the near future. If it were not for Takeda's partnership and incredible talents in the areas of fungal culture, factor purification, and chemical synthesis, TNP-470 would never have been developed.

3.2. Identification of Fumagillin and Analog Development

Immediately after initiating our collaboration with Takeda, we mailed a sample of the fungus that induced a gradient of cell rounding to their scientists. They cultured it in large batches and set out to isolate the putative angiogenesis inhibitory factor. Eventually, they discovered that the active compound that was secreted by the fungus I had isolated was fumagillin, a known antibiotic that had been previously used to treat Amebiasis in humans. Purified fumagillin was shown to inhibit endothelial cell proliferation in vitro, embryonic angiogenesis, and tumor-induced neovascularization in an sc sponge model in mice (*52*). It also suppressed the growth of a number of mouse tumors. However, its effectiveness was limited because it produced severe weight loss.

For this reason, we then set out to synthesize and test fumagillin analogs that would retain the potent antiangiogenic activity of fumagillin, but lack its toxic side effects. The most potent of these analogs is shown in Fig. 2. It was originally called AGM-1470 (AngioGenesis Modulator-1470). However, Takeda changed its name to TNP-470 (Takeda Neoplastic Product-470) once it entered clinical trials. In this chapter, it will be referred to as TNP-470.

I should note that these studies, which extended over a period of years, were spearheaded by scientists at Takeda, including Katsuichi Sudo, Shoji Kishimoto, Tsuneo Kanamaru, and Takeshi Fugita. Many of the animal studies carried out at our institution were done by Harold Brem, a surgical resident who was working as a research fellow in Folkman's laboratory, along with a number of different surgical residents, technicians, and rotating medical students.



TNP-470

Fig. 2. The structure of the angiogenesis inhibitor, TNP-470 [*O*-(chloroacetyl-carbamoyl)fumagillol].

TNP-470 was found to be a potent inhibitor of endothelial cell proliferation in vitro (half-maximal growth inhibition at approx 100 pg/mL in both human umbilical vein and bovine capillary endothelium; *11*). It also inhibited embryonic and tumor-induced angiogenesis in a variety of models (e.g., CAM, rat and rabbit corneas, sc sponge implants, Matrigel) (*11,56*). Importantly, although TNP-470 prevented cultured endothelial cells from growing when added over a wide range of doses (10 pg to 1 μg/mL), it did not kill these cells. In other words, it produced cytostatic rather than cytotoxic inhibition (*11,57,58*). Furthermore, the same doses that inhibited capillary cell growth had little effect on cultured tumor cells. Nevertheless, TNP-470 effectively suppressed the growth of the same tumor cells when implanted in vivo (*11*). This work has now been extended to include over 30 different solid tumors in mice, rats, rabbits, dogs, and monkeys as well as human tumors in nude mice (*11,57,59–76*; and unpublished data). Almost every solid tumor tested so far has responded well to treatment with this compound, whereas a P388 tumor growing in an ascites (nonsolid) form was found to be relatively resistant (*11*).

Although TNP-470 usually only prevents tumor expansion when used alone, recent studies by B. Teicher (Dana Farber Cancer Institute) have shown that tumor regression can actually be obtained in a high percentage of animals (over 50% in mice bearing Lewis Lung Carcinoma) by combining TNP-470 with standard chemotherapeutic agents, such as cytoxan (*69*). Work at Takeda and in our laboratory also has shown that TNP-470 is a potent inhibitor of tumor metastasis (*59,73*). As expected, angiogenesis inhibition does not inhibit initial implantation of tumor cells; rather, it prevents further tumor expansion at these distant sites. For example, when mice are injected with tumor cells via tail vein and then treated with TNP-470, only small ($< 1\text{-mm}^3$) lesions are produced in the lungs.

Most importantly, in all of our studies in which mice were injected sc with pure TNP-470 dispersed in saline, we never observed any drug-related toxicities in the range of doses that were necessary to suppress tumor growth. In the thousands of mice tested with this compound, there was no loss of appetite, hair loss, infections, or drug-related deaths. There results were incredibly exciting, to say the least, especially given the history that led to the development of this drug.

3.3. Advantages over Other Anticancer Agents

In summary, based on a serendipitous finding we (the Takeda Scientists, Folkman, Brem, and I) developed a new class of molecules that appear to suppress tumor growth and metastasis by inhibiting angiogenesis. These antiangiogenic compounds are analogs of the fungal product, fumagillin (*11*). Importantly, this type of angiogenesis inhibitor offers many advantages over curently available anticancer therapies.

The first is low toxicity, which is largely based on the fact that TNP-470 does not kill capillary cells or cells within highly proliferative normal tissues, such as the gut epithelium or lymphoid cells. Rather, it prevents quiescent capillary cells from responding to any mitogenic stimulus. The specificity here comes from the fact that normal capillary cells have extremely low turnover rates, with half-lives on the order of years, whereas cells in tumor capillaries commonly turn over once every few days (*49*).

A second advantage is that TNP-470 inhibits the growth of both primary and metastatic tumors. Thus, this type of nontoxic compound could be used prophylactically, for example, following resection of a tumor of the breast or colon, which are known to be highly metastatic and, hence, to have a high rate of recurrence. If their low toxicity holds true in humans, then they also may be used in patients with premalignant lesions (e.g., carcinoma *in situ* of the cervix, patients with high serum PSA levels) or in otherwise healthy people who have a genetically high propensity for tumor development (e.g., as detected by genetic screening methods that are becoming increasingly available).

Another advantage is that the target of this drug, the endothelial cell, is not transformed, and thus, development of drug resistance should be less of a problem than with conventional therapies. In fact, this turns out to be true. Thus far, we have not observed development of drug resistance in any tumors, even when continuously administered in mice for more than 6 mo (Brem et al., unpublished data).

Finally, TNP-470 as well as other angiogenesis inhibitors have been shown to potentiate dramatically the anticancer effects of known chemotherapeutic agents (e.g., cytoxan; *68,69,77*). Simultaneous administration of multiple angiogenesis inhibitors also produced additive inhibitory effects (*60*). Thus, antiangiogenesis agents, such as TNP-470, are perfectly suited to act in concert with other drugs as part of a combined chemotherapeutic regimen.

3.4. Translation from the Lab Bench to the Bedside

Based on these advantages, Phase I clinical trials were initiated with TNP-470 in September 1992 at the National Cancer Institute in patients with Kaposi's sarcoma. More recently, these trials were extended to include adult patients with many different types of solid cancers as well as children with brain tumors.

It should be noted that in the transition to the clinic, an iv route was chosen for the first line of attack. To accomplish this, an entirely new drug formulation had to be developed, which involved adding additional chemicals to TNP-470 to ensure stability and optimize solubility. When this final formulation was administered into beagle dogs by iv bolus injection, toxic effects were observed at high doses in certain organs (e.g., microhemorrhages were visualized in histological specimens of the brain). This

toxicity seemed to be partially suppressed by slowing the rate of infusion, and no microhemorrhages could be detected when TNP-40 was infused into baboons (unpublished data). Thus, we currently do not know whether this toxicity, although relatively minor compared to conventional cytotoxic agents, relates to the new route or rate of drug administration, the additional chemical components added to the formulation, TNP-470 itself, the animal model being utilized, or combinations of these factors.

In any case, given the relatively minor toxicity observed compared to conventional anticancer agents, TNP-470 quickly moved forward into clinical trials. However, based on the toxicology studies, a slow rate of infusion (over 30 min rather than iv bolus) was incorporated into the clinical protocol. Nevertheless, the take-home message here is that when translating a discovery from the lab bench to the bedside, seemingly small decisions can potentially make an enormous difference. For example, the choice of chemical additives for a new drug formulation usually is not a significant issue for most anticancer agents, which are themselves very toxic. However, this could be a major concern for those interested in developing novel anticancer agents, such as angiogenesis inhibitors, that potentially can suppress tumor growth without significant systemic toxicity.

It is precisely because of concerns such as these that clinical trials involve an initial phase (Phase I), which focuses exclusively on defining dose-limiting toxicities. Animal studies may be useful to identify potential problem areas. However, only results of actual toxicology studies carried out in human patients receiving the final formulation using an appropriate administration route and dose schedule have real meaning in the long term. In the case of TNP-470, the toxicities in Phase I trials have been found to be relatively minor. In fact, this has been a problem in the sense that Phase I trials have extended much longer than is usually required for cytotoxic agents (e.g., the original trial that began in 1992 still continues as of today). Dose-limiting toxicity has only been determined in older male patients with prostate cancer. In these patients, TNP-470 appeared to induce confusion, although this effect reversed when therapy was stopped.

In hindsight, it is interesting that because of our initial toxicology results in beagle dogs, TNP-470 was initially restricted from use in patients with brain cancer, even though there is no effective therapy available for these highly vascular tumors. Given that internal bleeding has not been a problem so far in human testing, Phase I trials in children with brain tumors were recently initiated. Most importantly, Phase II trials also should begin in the near future. However, here again there is a concern. Most oncologists usually only view an anticancer drug as active and useful if they can measure a dramatic and rapid tumor "response," as defined by a measureable shrinkage in tumor size or mass. In contrast, based on our preclinical studies, we expect TNP-470 (and other angiogenesis inhibitors) to produce relatively minor growth suppression (rather than shrinkage) when used alone, especially in the short term. Furthermore, extensive clinical trials in children with hemangioma using another angiogenesis inhibitor, α-interferon, suggest that no clinical effect may be visible for many months, even though complete tumor regression can be obtained by 1 yr in most of these patients (*78,79*). In fact, this clinical experience with α-interferon provides probably the best indication to date of how antiangiogenesis therapy will need to be administered in the future. Thus, not only must an angiogenesis inhibitor be active, but the clinicians involved in the testing of the drug must also understand that they are deal-

ing with an entirely different type of therapeutic agent and, thus, develop totally new clinical end points. This, of course, may change once angiogenesis inhibitors are tested in combination with conventional chemotherapeutic agents, since dramatic and immediate shrinkage in tumor size should result.

In summary, we must await the publication of results of the ongoing TNP-470 studies and future clinical trials involving drug combinations before we can ascertain the importance of this discovery. There clearly have been many drugs that were promising in early testing and yet only minimally effective when examined in humans. However, I believe that the preclinical results obtained with TNP-470 and other angiogenesis inhibitors, at the very least, provide a glimpse of what an anticancer therapy based on antiangiogenesis might be like in the future.

3.5. Implications for the Future

Probably the most important lesson we have learned from past experience with cancer chemotherapy in human patients is that it is unlikely that there will be a single silver bullet that will cure all cancers. We also know that clinical cures can be obtained with certain types of cancer. However, these dramatic responses almost always require simultaneous administration of multiple drugs. As an example, in treatment of acute lymphocytic leukemia in children, three to four different active agents had to be administered in combination before complete remissions could be produced in almost all patients. However, even then, clinical cures were only obtained in about 15% of these cases. To increase the cure rate to over 75%, as it is today, six to seven different active agents had to be administered in concert. This is likely what a cure of adult human cancer will require in the future. Given our preclinical results with TNP-470, I believe it is likely that one or more angiogenesis inhibitors will be a key part of this type of combined regimen for "cancer management" in the future.

4. CONCLUSIONS

The discoveries in the field of angiogenesis inhibition that I described in this chapter may provide a lesson for those interested in learning how to identify new anticancer drugs. One point was raised repeatedly: the incredible importance of chance and serendipity in the drug discovery process. Contamination of the CAM assay by eggshell dust and of my endothelial culture dish by a fungus are two particular examples of how chance can intervene at a particular time and place. However, these serendipitous events did not occur in a vacuum. No drugs would come from these observations, if the investigators did not have a clear goal in mind and methods on hand to follow up their observations. If the investigators were in a laboratory where rigid focus and strict observance of a preplanned path of inquiry (e.g., as laid out in the specific aims of a government grant proposal) were the rule, these observations also would likely not be made, let alone pursued. In essence, these events would remain no more than cursory observations similar to those made every day in labs around the world, if the research environment was wrong.

In contrast, as you probably can appreciate from my description, the Folkman laboratory of the 1980s (and to this day) was one that was continually open to new ideas and unconventional ways of viewing biological problems, young people (who are not yet tied to convention) were always involved on the front lines, and bioassays

that were both novel and highly useful were freely accessible to all. There also was a rare clarity of focus that came directly from Folkman's own vision, and his incredible ability to communicate the excitement and importance of the clinical problem at hand. Finally, gelling all this was sustained financial support from both government (NIH) and industrial sponsors, as well as an active ongoing collaboration with top-notch industrial medicinal chemists and microbiologists. Serendipity without this type of receptive environment would be like a bulb without electricity; the potential for enlightenment would be there, but little light would fall.

In conclusion, if there is any message in this chapter, it is that to best facilitate drug discovery, the scientific and pharmaceutical communities must learn how to develop this type of research incubator in which all of the key elements that are essential for discovery are placed in close proximity in a stable environment. If the final goal is truly translation of basic discoveries directly to the bedside, then ongoing collaborations and paths of communication between academic researchers and industrial scientists also must be established and maintained. Academics have the freedom to change paths at will and to explore serendipitous or counterintuitive results. However, they usually do not understand when to patent a discovery or how to develop a "product" that can be manufactured and distributed worldwide. Industrial scientists, on the other hand, tend to understand the nuts and bolts of true drug development (e.g., toxicology, pharmacokinetics, formulation development, FDA approval, and so forth). Yet, they lack the freedom to change direction at will. The creation of this type of research incubator becomes increasingly more difficult as the funds available to basic researchers and to the biopharmaceutical community become increasingly hard to find. Nevertheless, this is the goal that we must collectively pursue to be sure that new discoveries, both rational and serendipitous, will continue to be made in years to come.

ACKNOWLEDGMENTS

I would like to especially thank Judah Folkman, because essentially none of the discoveries I described would have ever come about without his drive and energy. The work that I described from my laboratory was supported by grants from NIH, American Cancer Society, and Takeda Chemical Industries, Limited. The author is a recipient of a Faculty Research Award from the American Cancer Society.

NOTE ADDED IN PROOF

The first complete tumor regression induced by administration of TNP-470 was recently reported in a woman with metastatic cervical carcinoma (*80*). This patient has now remained free to tumor and toxicity for over a year of continuous therapy with this single angiogenesis inhibitor.

REFERENCES

1. Folkman J, Ingber DE. Inhibition of Angiogenesis. *Semin Cancer Biol* 1992; 3:89-96.
2. Moses MA, Klagsbrun M, Shing Y. The role of growth factors in vascular cell development and differentiation. *Int Rev Cytol* 1995; 161:1-48.
3. Folkman J. Angiogenesis in cancer, vascular, rhematoid and other disease. *Nature Med* 1995; 1:27-30.

4. Folkman J. Tumor angiogenesis: therapeutic implications. *New Engl J Med* 1971; 285:1182–1186.
5. Folkman J. Cole P, Zimmerman S. Tumor behavior in isolated perfused organs: in vitro growth and metastases of biopsy material in rabbit thyroid and canine intestinal segment. *Ann Surg* 1966; 164:491–501.
6. Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible for angiogenesis. *J Exp Med* 1971; 133:275–288.
7. Gimbrone MA Jr, Leapman SB, Cotran RS, Folkman J. Tumor dormancy in vivo by prevention of neovascularization. *J Exp Med.* 1972; 136:261–276.
8. Knighton D, Ausprunk D, Tapper D, Folkman J. Avascular and vascular phases of tumour growth in the chick embryo. *Br J Cancer* 1977; 35:347–356.
9. Holmgren L, O'Reilly MS, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature Med.* 1995; 1:149–153.
10. Folkman J, Langer R, Linhardt RJ, Haudenchild C, and Tayler S. Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science* 1983, 221:719–725.
11. Ingber DE, Fujita T, Kishimoto S, Kanamaru T, Sudo K, Brem H, Folkman J. Synthetic analogues of fumagillin which inhibit angiogenesis and suppress tumour growth. *Nature* 1990; 348: 555–557.
12. Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990; 82:4–6.
13. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994; 79:315–328.
14. Srivastava A, Laidler P, Davies RP, Horgan K, Hughes LE. The prognostic significance of tumor vascularity in intermediate-thickness (0.76–4.0 mm thick) skin melanoma. *Am J Pathol* 1988; 133:419–423.
15. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. *New Eng J Med* 1991; 324:1–8.
16. Folkman J. The vascularization of tumors. *Sci Am* 1976; 234:58–73.
17. Folkman J, Klagsbrun M. Angiogenic factors. *Science* 1987; 235:442–447.
18. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995; 146:1029–1039.
19. Banda MJ, Knighton DR, Hunt TK, Werb Z. Isolation of a nonmitogenic angiogenesis factor from wound fluid. *Proc Natl Acad Sci USA* 1982; 79:7773–7777.
20. Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol* 1992; 3:65–71.
21. Brem H, Folkman J. Inhibition of tumor angiogenesis mediated by cartilage. *J Exp Med* 1975; 141:427–439.
22. Langer R, Conn H, Vacanti J, Haudenschild C, Folkman J. Control of tumor growth in animals by infusion of an angiogenesis inhibitor. *Proc Natl Acad Sci USA* 1980; 77:4331–4335.
23. Moses MA, Sudhalter J, Langer R. Identification of an inhibitor of neovascularization from cartilage. *Science* 1990; 248:1408–1410.
24. Lee A, Langer R. Shark cartilage contains inhibitors of tumor angiogenesis. *Science* 1983; 221: 1185–1187.
25. Taylor S, Folkman J. Protamine is an inhibitor of angiogenesis. *Nature* 1982; 297:307–310.
26. Maione TE, Gray GS, Petro J, Hunt AJ, Donner AL, Bauer Sl, Carson HF, Sharpe RJ. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science* 1990; 247:77–79.
27. Azizkhan RG, Azizkhan JC, Zetter BR, Folkman J. Mast cell heparin stimulates migration of capillary endothelial cells in vitro. *J Exp Med* 1980; 152:931–935.
28. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science* 1984; 223:1296–1299.
29. Klagsbrun M, Shing Y. Heparin affinity of anionic and cationic capillary endothelial cell growth factors: analysis of hypothalamus-derived growth factors and fibroblast growth factors. *Proc Natl Acad Sci USA* 1985; 82:805–809.
30. Ausprunk DH, Folkman J. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc Res* 1977; 14:53–65.

31. Berman M, Winthrop S, Ausprunk, Rose J, Langer R, Gage J. Plasminogen activator (urokinase) causes vascularization of the cornea. *Invest Ophthalmol Vis Sci* 1982; 22:191-199.
32. Kalebic T, Garbisa S, Glaser B, Liotta LA. Basement membrane collagen degradation by migrating endothelial cells. *Science* 1983; 221:281-283.
33. Gross J, Azizkhan RG, Biswas C, Bruns RR, Hsieh DST, Folkman J. Inhibition of tumor growth, vascularization, and collagenolysis in the rabbit cornea by medroxyprogesterone. *Proc Natl Acad Sci USA* 1981; 78:1176-1180.
34. Davies B, Brown PD, East N, Crimmin MJ, Balkwill FR. A synthetic matrix metalloproteinase inhibitor decreases tumor burden and prolongs survival of mice bearing human ovarian carcinoma xenografts. *Cancer Res* 1993; 53:2087-2091.
35. Ingber DE, Madri JA, Folkman J. A possible mechanism for inhibition of angiogenesis by angiostatic steroids: induction of capillary basement membrane dissolution. *Endocrinology* 1986; 119:1768-1775.
36. Ingber DE, Folkman J. Inhibition of angiogenesis through inhibition of collagen metabolism. *Lab Invest* 1988; 59:44-51.
37. Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* 1994; 264:569-571.
38. Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, Cheresh DA. Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 1994; 79:1157-1164.
39. Ingber DE, Madri JA, Folkman J. Extracellular matrix regulates endothelial growth factor action through modulation of cell and nuclear expansion. *In Vitro Cell Dev Biol* 1987; 23: 387-394.
40. Ingber DE, Folkman J. Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: role of extracellular matrix. *J Cell Biol* 1989; 109:317-330.
41. Ingber DE. Fibronectin controls capillary endothelial cell growth by modulating cell shape. *Proc Natl Acad Sci USA* 1990; 87:3579-3583.
42. Ingber DE, Prusty D, Frangione J, Cragoe EJ Jr, Lechene C, Schwartz M. Control of intracellular pH and growth by fibronectin in capillary endothelial cells. *J Cell Biol* 1990; 110: 1803-1812.
43. Ingber DE. Extracellular matrix as a solid state regulator of angiogenesis: identification of new targets for anti-cancer therapy. *Semin Cancer Biol* 1992; 3:57-63.
44. D'Amato RJ, Loughnan MS, Flynn E, Folkman J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci USA* 1994; 91:4082-4085.
45. Folkman J, Weisz PB, Joullie MM, Li WW, Ewing WR. Control of angiogenesis with synthetic heparin substitutes. *Science* 1989; 243:1490-1493.
46. Crum R, Szabo S, and Folkman J. A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science* 1985; 230:1375-1378.
47. Folkman J, Ingber DE. Angiostatic steroids: method of discovery and mechanism of action. *Ann Surg* 1987; 206:373,374.
48. Li WW, Casey R, Gonzalez EM, Folkman J. Angiostatic steroids potentiated by sulfated cyclodextrins inhibit corneal neovascularization. *Invest Ophthalmol Vis Sci* 1991; 32:2898-2905.
49. Hobson B, Denekamp J. Endothelial proliferation in tumours and normal tissues: continuous labelling studies. *Br J Cancer* 1984; 49:405-413.
50. Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L. Genistein, a dietary-derived inhibitor of in vitro angiogenesis. *Proc Natl Acad Sci USA* 1993; 90:2690-2694.
51. D'Amato RJ, Lin CM, Flynn E, Folkman J, Hamel E. 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. *Proc Natl Acad Sci USA* 1994; 91:3964-3968.
52. Ingber DE, Madri JA, Jamieson JD. Role of basal lamina in the neoplastic disorganization of tissue architecture. *Proc Natl Acad Sci USA* 1981; 78:3901-3905.
53. Ingber DE, Madri JA, Jamieson JD. Basement membrane as a spatial organizer of polarized epithelia: exogenous basement membrane reorients pancreatic epithelial tumor cells in vitro. *Am J Pathol* 1986; 122:129-139.
54. Ingber DE, Jamieson JD. Cells as tensegrity structures: architectural regulation of histodifferen-

tiation by physical forces transduced over basement membrane. In: Andersson LC, Gahmberg CG, Ekblom P, eds. *Gene Expression During Normal and Malignant Differentiation.* Orlando, FL: Academic. 1985:13–32.
55. Folkman J, Moscona A. Role of cell shape in growth control. *Nature* 1978; 273:345–349.
56. Kusaka M, Sudo K, Fujita T, Marui S, Itoh F, Ingber DE, Folkman J. Potent antiangiogenic action of AGM-1470: comparison to the fumagillin parent. *Biochem Biophys Res Commun* 1991; 174:1070–1076.
57. Kusaka M, Sudo K, Matsutani E, Kozai Y, Marui S, Fujita T, Ingber D, Folkman J. Cytostatic inhibition of endothelial cell growth by the angiogenesis inhibitor, TNP-470 (AGM-1470). *Br J Cancer* 1994; 69:212–216.
58. Hori A, Ikeyama S, Sudo K. Suppression of cyclin D1 mRNA expression by the angiogenesis inhibitor TNP-470 (AGM-1470) in vascular endothelial cells. *Biochem Biophys Res Commun* 1994; 204:1067–1073.
59. Brem H, Ingber D, Blood CH, Bradley D, Urioste S, Folkman J. Suppression of tumor metastasis by angiogenesis inhibition. *Surg Forum* 1992; 42:439–441.
60. Brem H, Gresser I, Grosfeld J, Folkman J. The combination of antiangiogenic agents to inhibit primary tumor growth and metastasis. *J Pediatr Surg* 1993; 28:1253–1257.
61. Kamei S, Okada H, Inoue Y, Yoshioka T, Ogawa Y, and Toguchi H. Antitumor effects of angiogenesis inhibitor TNP-470 in rabbits bearing VX-2 carcinoma by arterial administration of microspheres and oil solution. *J Pharmacol Exp Ther* 1993; 264:469–474.
62. Kato T, Sato K, Kakinuma H, Matsuda Y. Enhanced suppression of tumor growth by combination of angiogenesis inhibitor *O*-(chloroacetyl-carbamoyl)fumagillol (TNP-470) and cytotoxic agents in mice. *Cancer Res* 1994; 54:5143–5147.
63. Morita T, Shinohara N, Tokue A. Antitumour effect of a synthetic analogue of fumagillin on murine renal carcinoma. *Br J Urol* 1994; 74:416–421.
64. Takechi A. Effect of angiogenesis inhibitor TNP-470 on vascular formation in pituitary tumors induced by estrogen in rats. *Neurol Medico-Chirurg* 1994; 34:729–733.
65. Takechi A, Uozumi T, Kawamoto K, Ito A, Kurisu K, Sudo K. Inhibitory effect of TNP-470, a new anti-angiogenic agent, on the estrogen induced rat pituitary tumors. *Anticancer Res* 1994; 14:157–162.
66. Takamiya Y, Brem H, Ojeifo J, Mineta T, Martuza RL. AGM-1470 inhibits the growth of human glioblastoma cells in vitro and in vivo. *Neurosurgery* 1994; 34:869–875.
67. Taki T, Ohnishi T, Arita N, Hiraga S, Saitoh Y, Izumoto S, Mori K, Hayakawa T. Antiproliferative effects of TNP-470 on human malignant glioma in vivo: potent inhibition of tumor angiogenesis. *J Neuro-Oncol* 1994; 19:251–258.
68. Teicher BA, Holden SA, Ara G, Northey D. Response of the FSaII fibrosarcoma to antiangiogenic modulators plus cytotoxic agents. *Anticancer Res* 1993; 13:2101–2106.
69. Teicher BA, Holden SA, Ara G, Sotomayor EA, Huang ZD, Chen YN, Brem H. Potentiation of cytotoxic cancer therapies by TNP-470 alone and with other antiangiogenic agents. *Int J Cancer* 1994; 57:920–925.
70. Toi M, Yamamoto Y, Imazawa T, Takayanagi T, Akutsu K, Tominaga T. Antitumor effect of the angiogenesis inhibitor AGM-1470 and its combination effect with tamoxifen in DMBA induced mammary tumors in rats. *Int J Oncol* 1993; 3:525–528.
71. Yamaoka M, Yamamoto T, Ikeyama S, Sudo K, Fujita T. Angiogenesis inhibitor TNP-470 (AGM-1470) potently inhibits the tumor growth of hormone-independent human breast and prostate carcinoma cell lines. *Cancer Res* 1993; 53:5233–5236.
72. Yamaoka M, Yamamoto T, Masaki T, Ikeyama S, Sudo K, Fujita T. Inhibition of tumor growth and metastasis of rodent tumors by the angiogenesis inhibitor *O*-(chloroacetyl-carbamoyl)fumagillol (TNP-470; AGM-1470). *Cancer Res* 1993; 53:4262–4267.
73. Yanase T, Tamura M, Fujita K, Kodama S, Tanaka K. Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines in vitro and in vivo. *Cancer Res* 1993; 53:2566–2570.
74. Yanai S, Okada H, Misaki M, Saito K, Kuge Y, Ogawa Y, Toguchi H. Antitumor activity of a medium-chain triglyceride solution of the angiogenesis inhibitor TNP-470 (AGM-1470) when administered via the hepatic artery to rats bearing Walker 256 carcinosarcoma in the liver. *J Pharmacol Exp Ther* 1994; 271:1267–1273.
75. Yamamoto T, Sudo K, Fujita T. Significant inhibition of endothelial cell growth in tumor

vasculature by an angiogenesis inhibitor, TNP-470 (AGM-1470). *Anticancer Res* 1994; 14:1-3.
76. Tanaka T, Konno H, Matsuda I, Nakamura S, Baba S. Prevention of hepatic metastasis of human colon cancer by angiogenesis inhibitor TNP-470. *Cancer Res* 1995; 55:836-839.
77. Teicher BA, Sotomayor EA, Huang ZD. Antiangiogenic agents potentiate cytotoxic cancer therapies against primary and metastatic disease. *Cancer Res* 1992; 52:6702-6704.
78. White CW, Sondheimer HM, Crouch EC, Wilson H, Fan LL. Treatment of pulmonary hemangiomatosis with recombinant interferon alfa-2a. *New Eng J Med* 1989; 320:1197-1200.
79. Ezekowitz A, Mulliken J, Folkman J. Interferon alpha therapy of haemangiomas in newborns and infants. *Br J Haematol* 1991; 79:67,68.
80. Levy T, Kudelka A, Verschraegen CF, Edwards CL, Freedman RS, Kaplan A, Kieback D, Steger M, Mante R, Gutterman J, Piamsomboon S, Termrungruanglert W, Kavanagh JJ. A Phase I study of TNP-470 administered to patients with advanced squamous cell cancer of the cervix. *Proc Amer Assoc Cancer Res* 1996; 37:166 (Abstract #1140).

15 Antisense Oligonucleotides

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CONTENTS

1. INTRODUCTION

The therapeutic use of oligonucleotides represents a new paradigm for drug discovery. The technology focuses on a class of chemicals, oligonucleotides, that have not been studied as potential drugs before and uses them to intervene in processes that, likewise, have not been studied as sites at which drugs might act. Although the field is still in its infancy, it has generated considerable enthusiasm because of the potential specificity of oligonucleotide drugs and the breadth of potential applications.

The enthusiasm about the technology has been tempered by appropriate reservations concerning the ability of the technology to deliver on its promise. In essence, the questions about the technology reduce to: can oligonucleotide analogs be created that have appropriate properties to be drugs; specifically, what are the pharmocokinetic, pharmacologic, and toxicologic properties of these compounds, and what are the scope and potential of the medicinal chemistry of oligonucleotides? Although much remains to be done, the answers to many of the questions are now available and suggest that the technology may yield important therapeutic advances.

This chapter will focus strictly on the antisense mechanism with particular emphasis on the potential role of this technology in the treatment of cancer.

2. THE ANTISENSE CONCEPT

2.1. History

The antisense concept derives from an understanding of nucleic acid structure and function, and depends on Watson-Crick hybridization (*1*). Thus, arguably, the demonstration that nucleic acid hybridization is feasible (*2*) and the advances in *in situ*

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

hybridization and diagnostic probe technology (*3*), lay the most basic elements of the foundation supporting the antisense concept.

However, the first clear enunciation of the concept of exploiting antisense oligonucleotides as therapeutic agents was in the work of Zamecnik and Stephenson in 1978 (*4*). In this publication, these authors reported the synthesis of an oligodeoxyribonucleotide 13 nucleotides long that was complementary to a sequence in the respiratory syncitial virus genome. They suggested that this oligonucleotide could be stabilized by 3′- and 5′-terminal modifications, and showed evidence of antiviral activity. More importantly, they discussed possible sites for binding in RNA and mechanisms of action of oligonucleotides.

Though less precisely focused on the therapeutic potential of antisense oligonucleotides, the work of Miller and Ts'o and their collaborators during the same period helped establish the foundation for antisense research and re-establish an interest in phosphate backbone modifications as approaches to improve the properties of oligonucleotides (*5,6*). Their focus on methylphosphotriester-modified oligonucleotides as a potential solution to pharmacokinetic limitations of oligonucleotides presaged a good bit of the medicinal chemistry to be performed on oligonucleotides.

Despite the observations of Miller and Ts'o and Zamecnik and colleagues, interest in antisense research was quite limited until the late 1980s, when advances in several areas provided technical solutions to a number of impediments. Since antisense drug design requires an understanding of the sequence of the RNA target, the explosive growth in availability of viral and human genomic sequences provided the information from which "receptor sequences" could be selected. The development of methods for synthesis of research quantitites of oligonucleotide drugs then supported antisense experiments on both phosphodiester and modified oligonucleotides (*7,8*). The inception of the third key component (medicinal chemistry) forming the foundation of oligonucleotide therapeutics, in fact, was the synthesis in 1969 of phosphorothioate poly rI:poly rC as a means of stabilizing the polynucleotide (*9*). Subsequently, Miller and Ts'o initiated studies on the neutral phosphate analogs, methylphosphonates (*5*), and groups at the National Institutes of Health and the Food and Drug Administration and the Worcester Foundation investigated phosphorothioate oligonucleotides (*10–14*). With these advances forming the foundation for oligonucleotide therapeutics and the initial studies suggesting in vitro activities against a number of viral and mammalian targets (*12,15–19*), interest in oligonucleotide therapeutics intensified.

2.2. Strategies to Induce Transcriptional Arrest

An alternative to the inhibition of RNA metabolism and utilization via an antisense mechanism is to inhibit transcription by interacting with double-stranded DNA in chromatin. Of the two most obvious binding strategies for oligonucleotides binding to double-stranded nucleic acids, strand invasion and triple-strand formation, triple-stranding strategies, until recently, attracted essentially all of the attention.

Triple helices have been known for polynucleotides since 1957 (*20*). Triple strands can form via non-Watson-Crick hydrogen bonds between the third strand and purines involved in Watson-Crick hydrogen bonding with the complementary strand of the duplex (for review, *see 21*). Thus, triple-stranded structures can be formed between a third strand comprised of pyrimidines or purines that interact with a homopurine strand in a homopurine-homopyrimidine strand in a duplex DNA. With the demon-

stration that homopyrimidine oligonucleotides could indeed form triplex structures (*22–24*), interest in triple-strand approaches to inhibit transcription heightened.

Although there was initially considerable debate about the value of triple-stranding strategies versus antisense approaches (*25*), there was little debate that much work remained to be done to design oligonucleotides that could form triple-stranded structures with duplexes of mixed sequences. Pursuit of several strategies has resulted in significant progress (for review, *see 21*), but resolution of many issues remains to be achieved before triplexing strategies can be used effectively to create pharmacological agents.

Strand invasion, an alternative approach to obstructing transcription by formation of triple strands, has been shown to be feasible if analogs with sufficient affinity can be synthesized. Recently, peptide nucleic acids (PNA) have been shown to have very high affinity and be capable of strand invasion of double-stranded DNA under some conditions (*26*). Again, much remains to be learned about this analog class and other high-affinity analogs before the pharmacological potential of transcriptional arrest via strand invasion is defined.

2.3. Ribozymes and Oligonucleotide-Mediated RNA Cleavage

Ribozymes are RNA molecules that catalyze biochemical reactions (*27*). Ribozymes cleave single-stranded regions of RNA via transesterification of hydrolysis reactions that cleave a phosphodiester bond (*28*). Clearly, a therapeutic oligonucleotide that could specifically bind to and cleave an RNA target should be attractive. Again, however, many technical hurdles must be overcome before modified ribozymes or synthetic oligonucleotides with ribozyme-like activity can be used as drugs.

2.4. Combinatorial Approaches to Oligonucleotide Therapeutics

At least two methods have been published by which oligonucleotides have been identified to interact with cellular proteins by combinatorial methods (*29–31*). The potential advantage of a combinatorial approach is that oligonucleotide-based molecules can be prepared to adopt various structures that support binding to nonnucleic acid targets as well as nucleic acid targets. These can then be screened for potential activities without knowledge about the cause of the disease or the structure of the target.

2.5. The Medicinal Chemistry of Oligonucleotides

Because it was apparent almost immediately that native phosphodiester oligodeoxy- or ribonucleotides are unsatisfactory as drugs because of rapid degradation (*32*), a variety of modifications have been prepared. As previously mentioned, perhaps the most interesting of the initial modifications were the phosphate analogs, the phosphorothioates (*9*) and the methylphosphonates (*5*). Both fully modified oligonucleotides and oligonucleotides "capped" at the 3′- and/or 5′-termini with phosphorothioate or methylphosphonate moieties were tested (*33*). However, studies from many laboratories demonstrated that capped oligonucleotides were relatively rapidly degraded in cells (*34–37*), nor were point modifications with intercalators that enhanced binding to RNA (*38,39*), cholesterol (*40*), or poly-L-lysine (*41,42*) sufficiently active or selective to warrant broad-based exploration.

Since the initial approaches to modifications of oligonucleotides, an enormous range of modifications, including novel bases, sugars, backbones, conjugates, and chimeric oligonucleotides, have been tested (for review, *see 43,44*). Many of these

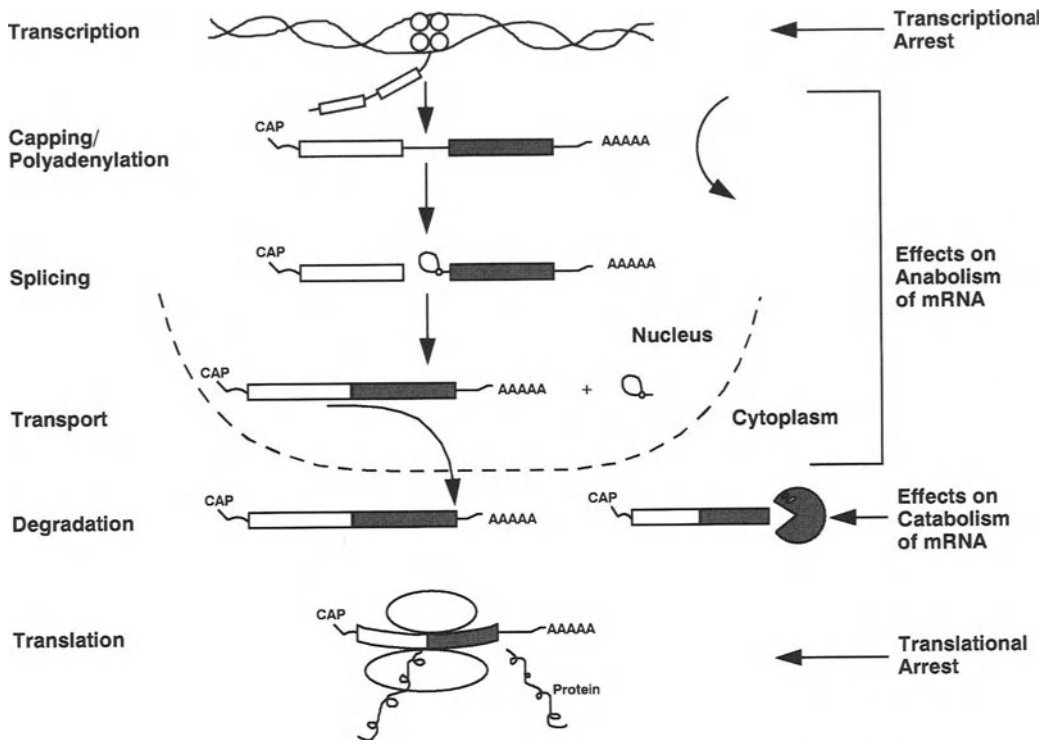


Fig. 1. RNA processing.

modifications have proven to be quite useful and are progressing in testing leading to clinical trials.

2.6. Pharmacological Rationalization of Therapeutic Oligonucleotides

Of central importance to the long-term future of therapeutic oligonucleotides has been the rationalization of oligonucleotides in the context of modern pharmacology (*45*). Progress has been gratifying in that most of the fundamental concerns about oligonucleotides are now answered. These include such issues as questions about the kinetics and characteristics of oligonucleotides binding to RNA targets, proof of activity and mechanism of action in vitro and in vivo, pharmacokinetic and toxicologic properties of oligonucleotides, and the breadth and value of oligonucleotide medicinal chemistry. With six oligonucleotides in clinical trials, numerous demonstrations of potent systemic activities in animals, and clarification of potential toxicities and the therapeutic index of phosphorothioate oligonucleotides, it is reasonable to conclude that substantial progress has been achieved.

2.7. RNA Intermediary Metabolism

Oligonucleotides are designed to modulate the information transfer from the gene to protein—in essence, to alter the intermediary metabolism of RNA. Figure 1 summarizes these processes.

RNA intermediary metabolism is initiated with transcription. The transcription initiation complex contains proteins that recognize specific DNA sequences and locally denature double-stranded DNA, thus allowing a member of the RNA polymerase

family to transcribe one strand of the DNA (the antisense strand) into a sense pre-mRNA molecule. Usually during transcription, the 5′-end of the pre-mRNA is capped by adding a methyl-guanosine, and most often by methylation of one or two adjacent sugar residues. This enhances the stability of the pre-mRNA and may play a role in a number of key RNA processing events (*46*). Between the 5′-cap and the site at which translation is initiated is usually a stretch of nucleotides (5′-untranslated region, 5′-UTR). This area may play a key role in regulating mRNA half-life (*47*).

Similarly, the 3′-end of the pre-mRNA usually has a stretch of several hundred nucleotides beyond the translation termination signal. This area often plays an important role in determining mRNA half-life. Moreover, posttranscriptionally, most pre-mRNA species are polyadenylated. Polyadenylation stabilizes the RNA, is important in transport of mature mRNA out of the nucleus, and may play important roles in the cytoplasm as well (*48,49*).

Because eukaryotic genes usually contain intervening sequences (introns), most pre-mRNA species must have these sequences excised and the mature RNA spliced together. Splicing reactions are complex, highly regulated, and involve specific sequences, small-mol-wt RNA species, and numerous proteins. Alternative splicing processes are often used to produce different mature mRNAs and, thus, different proteins. Even though introns are often considered waste, important sequences are conserved, and some introns may play a variety of regulatory roles.

Mature mRNA is exported to the cytoplasm and engages in translation. mRNA half-lives vary from a few minutes to many hours, and appears to be highly regulated (*47*).

Each step shown in the pathway is a composite of numerous steps and is theoretically amenable to intervention with oligonucleotides. For virtually no mRNA is the pathway fully defined; however, available information is insufficient to determine the rate-limiting steps in the intermediary metabolism of any mRNA species (*50,51*).

2.8. *Factors Influencing Antisense Activity*

2.8.1. Affinity

The affinity of oligonucleotides for their receptor sequences results from hybridization interactions. The two major contributors to the free energy of binding are hydrogen bonding (usually Watson-Crick base pairing) and base stacking in the double helix that is formed. Affinity is affected by ionic strength. In general, the higher the ionic strength, the higher the affinity of charged oligonucleotides for polynucleotides. As affinity results from hydrogen bond formation between bases and stacking occurs between coplanar bases, affinity increases as the length of the oligonucleotide receptor complex increases. Thus, the affinity per nucleotide unit and the number of hybridizing nucleotide pairs are crucial determinants of overall affinity. Affinity also varies as a function of the sequence in the duplex. Nearest neighbor rules allow the prediction of the free energy binding for DNA–DNA and RNA–RNA hybrids with relatively high precision (*52,53*). A common misconception is that DNA–RNA duplexes are more stable than DNA–DNA duplexes. In fact, the relative stability of these duplexes varies as a function of the sequence. RNA–RNA duplexes are typically the most stable (S. M. Freier, unpublished results).

As with other drug–receptor interactions, activity requires a minimum level of affinity. For many targets and types of oligonucleotides, the minimum length of an oligonucleotide may be 12–14 nucleotides.

Although theoretical affinities for oligonucleotide–single-strand nucleic acid interactions are very large, in practice, affinity constants are substantially lower. Several factors contribute to the differences between theoretical and realized affinities. Undoubtedly, the most important factor is that RNA can adopt a variety of secondary structures (for review, *see 54*). In addition to secondary structure, RNA can adopt tertiary structures. Tertiary structures result from the interactions of secondary structures in an RNA molecule with other secondary structural elements or single-stranded regions (*55*). A third factor that can potentially reduce the affinity of an oligonucleotide for its RNA receptor is that oligonucleotides can form secondary and tertiary structures themselves. To avoid duplex formation, oligonucleotides that contain self-complementary regions are usually not employed. However, other structures that were not well understood or expected have recently been described. Tetrameric complexes formed by oligonucleotides with multiple guanosines (*29,56–58*) and other base sequences (*59*) can be highly stable, clearly would prevent an antisense interaction, and have a number of biological effects that have confounded interpretation of experiments.

Since RNA and oligonucleotide structures are affected by ionic milieu and nonproductive interactions with proteins and polycations, the in vivo situation is, of course, considerably more complicated. Relatively little is understood about the interplay among all these factors and their effects on the true affinities of oligonucleotides for potential RNA targets.

2.8.2. Specificity for Nucleic Acid Sequences

Specificity derives from the selectivity of Watson-Crick or other types of base pairing. The decrease in affinity associated with a mismatched base pair varies as a function of the specific mismatch, the position of the mismatch in a region of complementarity, and the sequence surrounding the mismatch. In a typical interaction between complementary 18-mers, the $\Delta\Delta G^{\circ}_{37}$ or change in Gibbs free energy of binding induced by a single mismatch varies from +0.2 to +4.0 kcal/mol/modification at 100 m*M* NaCl. Thus, a single base mismatch could result in a change in affinity of approx 500-fold (*60*). Modifications of oligonucleotides may alter specificity.

At the genomic level, any sequence of 17 residues is expected to occur only once (*61*). Assuming a random distribution of sequences in RNA, any sequence of 13 residues is expected to occur once in the cellular RNA population and, if the nonrandom nature of mammalian RNA sequence is taken into account, an 11-mer oligonucleotide or perhaps smaller could identify and bind to a unique sequence (*62,63*).

To exploit fully the theoretical potential for specificity of an oligonucleotide in a therapeutic context, it is necessary to manipulate the length of the oligonucleotide and its concentration at target. The results of such an exercise have been reported (*64*). In this study, phosphorothioate oligodeoxynucleotides were designed to target the normal or codon 12-point mutation of Ha-*ras* mRNA. Predictions from hybridization experiments suggested that approximately a fivefold specificity for mutant compared to normal Ha-*ras* RNA was possible. By optimizing oligonucleotide length and the extracellular concentration of the oligonucleotide, nearly theoretical specificity was achieved in cells in tissue culture.

Other factors can also be used to enhance specificity. RNA secondary and tertiary structure assures that not all sequences are equally accessible. Design of oligonucleo-

tides to interact with sequences involved in maintenance of RNA structure can theoretically enhance specificity and, if the structure is essential to the stability or function of the RNA, potency. Furthermore, many RNA and DNA sequences interact with proteins, again assuring far more diversity in response to an oligonucleotide and, therefore, greater specificity than might be predicted solely on the basis of differences in nucleic acid sequence.

2.8.3. Therapeutic Specificity (Therapeutic Index)

Clearly, in a therapeutic context, the ability of an oligonucleotide to bind selectively to specific sequences in nucleic acid targets is an important factor in determining its therapeutic index. However, oligonucleotides and analogs can interact with other cellular components, and these interactions can have significant effects on the therapeutic index of oligonucleotides. The factors that determine the significance of nonnucleic interactions of oligonucleotides on the therapeutic index include the affinities for nonnucleic acid sites vs nucleic acids, the numbers of different nonnucleic acid binding sites, the concentrations of each of the binding sites, the biological importance of various binding sites, and kinetic factors. These are, of course, conceptually equivalent to the factors that affect the therapeutic index of drugs of all classes, but very little is understood about these potential interactions.

Chemical classes of oligonucleotides differ in their tendency to interact with various nonnucleic acid targets. For example, phosphorothioates tend to bind to a wide range of proteins with relatively low affinity (*36*). Nevertheless, detailed in vitro and in vivo toxicological studies have shown that these interactions probably reduce the therapeutic index of phosphorothioates less than perhaps was expected (*44,65*). We believe that this is owing to the fact that the phosphorothioates bind with very low affinity to a larger number of proteins and their potential toxic effects are consequently "buffered."

2.9. Mechanisms of Action of Antisense Oligonucleotides

The mechanisms by which interactions of oligonucleotides with nucleic acids may induce biological effects are complex and potentially numerous. Furthermore, very little is currently understood about the roles of various mechanisms or the factors that may determine which mechanisms are involved after oligonucleotides bind to their receptor sequences. Consequently, a discussion of mechanisms remains largely theoretical.

2.9.1. Occupancy-Only Mediated Mechanisms

Classic competitive antagonists are thought to alter biological activities because they bind to receptors preventing natural agonists from binding and inducing normal biological processes. Binding of oligonucleotides to specific sequences may inhibit the interaction of the RNA or DNA with proteins, other nucleic acids, or other factors required for essential steps in the intermediary metabolism of the RNA or its utilization by the cell.

2.9.1.1. Inhibition of Splicing. A key step in the intermediary metabolism of most mRNA molecules is the excision of introns. These "splicing" reactions are sequence-specific and require the concerted action of spliceosomes. Consequently, oligonucleotides that bind to sequences required for splicing may prevent binding of necessary

factors or physically prevent the required cleavage reactions. This then would result in inhibition of the production of the mature mRNA. Although there are several examples of oligonucleotides directed to splice junctions, none of the studies presented data showing inhibition of RNA processing, accumulation of splicing intermediates, or a reduction in mature mRNA, nor are there published data in which the structure of the RNA at the splice junction was probed and the oligonucleotides demonstrated to hybridize to the sequences for which they were designed (*66–69*). Activities have been reported for anti-c-*myc* and antiviral oligonucleotides with phosphodiester, methylphosphonate, and phosphorothioate backbones. Very recently, an oligonucleotide was reported to induce alternative splicing in a cell-free splicing system, and in that system, RNA analyses confirmed the putative mechanism (*70*).

2.9.1.2. Translational Arrest. The mechanism for which the majority of oligonucleotides have been designed is translational arrest by binding to the translation initiation codon. The positioning of the initiation codon within the area of complementarity of the oligonucleotide and the length of oligonucleotide used have varied considerably. Again, unfortunately, only in relatively few studies have the oligonucleotides, in fact, been shown to bind to the sites for which they were designed, and other data that support translation arrest as the mechanism have not been reported.

Target RNA species that have been reported to be inhibited include HIV (*71*), vesicular stomatitis virus (VSV) (*72*), n-*myc* (*73*), and a number of normal cellular genes (*74–77*).

In our laboratories, we have shown that a significant number of targets may be inhibited by binding to translation initiation codons. For example, ISIS 1082 hybridizes to the AUG codon for the UL13 gene of herpes virus types 1 and 2. RNase H studies confirmed that it binds selectively in this area. In vitro protein synthesis studies confirmed that it inhibited the synthesis of the UL13 protein, and studies in HeLa cells showed that it inhibited the growth of herpes type 1 and type 2 with an IC_{50} of 200–400 n*M* by translation arrest (*78*). Similarly, ISIS 1753, a 30-mer phosphorothioate complementary to the translation initiation codon and surrounding sequences of the E2 gene of bovine papilloma virus, was highly effective, and its activity was shown to be owing to translation arrest. ISIS 2105, a 20-mer phosphorothioate complementary to the same region in human papilloma virus, was shown to be a very potent inhibitor. Compounds complementary to the translation initiation codon of the E2 gene were the most potent of the more than 50 compounds studied complementary to various other regions in the RNA (*79*). We have shown inhibition of translation of a number of other mRNA species by compounds designed to bind to the translation codon as well.

In conclusion, translation arrest represents an important mechanism of action for antisense drugs. A number of examples purporting to employ this mechanism have been reported, and recent studies on several compounds have provided data that unambiguously demonstrate that this mechanism can result in potent antisense drugs.

2.9.1.3. Disruption of Necessary RNA Structure. RNA adopts a variety of three-dimensional structures induced by intramolecular hybridization, the most common of which is the stem loop. These structures play crucial roles in a variety of functions. They are used to provide additional stability for RNA and as recognition motifs for a number of proteins, nucleic acids, and ribonucleoproteins that participate in the intermediary metabolism and activities of RNA species. Thus, given the potential

general utility of the mechanism, it is surprising that occupancy-based disruption RNA has not been more extensively exploited.

As an example, we designed a series of oligonucleotides that bind to the important stem-loop present in all RNA species in HIV, the TAR element. We synthesized a number of oligonucleotides designed to disrupt TAR, and showed that several indeed did bind to TAR, disrupt the structure, and inhibit TAR-mediated production of a reporter gene (*31*). Furthermore, general rules useful in disrupting stem-loop structures were developed as well (*80*).

Although designed to induce relatively nonspecific cytotoxic effects, two other examples are noteworthy. Oligonucleotides designed to bind to a 17-nucleotide loop in *Xenopus* 28 S RNA required for ribosome stability and protein synthesis inhibited protein synthesis when injected into *Xenopus oocytes* (*81*). Similarly, oligonucleotides designed to bind to highly conserved sequences in 5.8 S RNA inhibited protein synthesis in rabbit reticulocyte and wheat germ systems (*82*).

2.9.2. Occupancy-Activated Destabilization

RNA molecules regulate their own metabolism. A number of structural features of RNA are known to influence stability, various processing events, subcellular distribution, and transport. It is likely that, as RNA intermediary metabolism is better understood, many other regulatory features and mechanisms will be identified.

2.9.2.1. 5′-Capping. A key early step in RNA processing is 5′-capping (Fig. 1). This stabilizes pre-mRNA and is important for the stability of mature mRNA. It also is important in binding to the nuclear matrix and transport of mRNA out of the nucleus. Since the structure of the cap is unique and understood, it presents an interesting target.

Several oligonucleotides that bind near the cap site have been shown to be active, presumably by inhibiting the binding of proteins required to cap the RNA. For example, the synthesis of SV40 T-antigen was reported to be most sensitive to an oligonucleotide linked to poly-L-lysine and targeted to the 5′-cap site of RNA (*83*). However, again, in no published study has this putative mechanism been rigorously demonstrated. In fact, in no published study have the oligonucleotides been shown to bind to the sequences for which they were designed.

In studies in our laboratory, we have designed oligonucleotides to bind to 5′-cap structures and reagents to cleave specifically the unique 5′-cap structure (*84*). These studies demonstrated that 5′-cap-targeted oligonucleotides were capable of inhibiting the binding of the translation initiation factor eIF-4α (*85*).

2.9.2.2. Inhibition of 3′-Polyadenylation. In the 3′-untranslated regions of pre-mRNA molecules are sequences that result in the posttranscriptional addition of long (hundreds of nucleotides) tracts of polyadenylate. Polyadenylation stabilizes mRNA and may play other roles in the intermediary metabolism of RNA species. Theoretically, interactions in the 3′-terminal region of pre-mRNA could inhibit polyadenylation and destabilize the RNA species. Although there are a number of oligonucleotides that interact in the 3′-untranslated region and display antisense activities (*86*), to date, no study has reported evidence for alterations in polyadenylation.

2.9.2.3. Other Mechanisms. In addition to 5′-capping and 3′-adenylation, there are clearly other sequences in the 5′- and 3′-untranslated regions of mRNA that affect the stability, localization, and translatability of the molecules. Again, there are a number of antisense drugs that may work by interfering with these processes.

2.9.3. Activation of RNase H

RNase H is an ubiquitous enzyme that degrades the RNA strand of an RNA–DNA duplex. It has been identified in organisms as diverse as viruses and human cells (for review, *see 87*). At least two classes of RNase H have been identified in eukaryotic cells. Multiple enzymes with RNase H activity have been observed in prokaryotics (*87*). Furthermore, there are data that suggest that in eukaryotic cells, there are multiple isozymes.

Although RNase H is involved in DNA replication, it may play other roles in the cells and is found in the cytoplasm as well as the nucleus (*88*). However, the concentration of the enzyme in the nucleus is thought to be greater, and some of the enzyme found in cytoplasmic preparations may be the result of nuclear leakage.

RNase H activity is quite variable. It is absent or minimal in rabbit reticulocytes (*89*), but present in wheat germ extracts (*87*). In HL60 cells, for example, the level of activity in undifferentiated cells is greatest, relatively high in DMSO and vitamin D-differentiated cells, and much lower in PMA-differentiated cells (Hoke, unpublished data).

The precise recognition elements for RNase H are not known. However, it has been shown that oligonucleotides with DNA-like properties as short as tetramers can activate RNase H (*90*). Changes in the sugar influence RNase H activation as sugar modifications that result in RNA-like oligonucleotides, e.g., 2′-fluoro- or 2′-*O*-methyl does not appear to serve as a substrate for RNase H (*91,92*). Alterations in the orientation of the sugar to the base can also affect RNase H activation, since α-oligonucleotides are unable to serve as substrates for RNase H or may require parallel annealing (*93,94*). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates do not serve as RNase H substrates (*95,96*). In contrast, phosphorothioates are excellent substrates (*78,97,98;* Hoke, unpublished data). Chimeric oligonucleotides that bind to RNA and activate RNase H have been studied (*99,100*). For example, oligonucleotides comprised of wings of 2′-*O*-methyl sugars and methylphosphonate backbone and a 5-base gap of deoxyoligonucleotides bind to their target RNA and activate RNase H (*99,100*). Furthermore, a single ribonucleotide in a sequence of deoxyribonucleotides was shown to be sufficient to serve as a substrate for RNase H when bound to its complementary deoxyoligonucleotide (*101*).

That it is possible to take advantage of chimeric oligonucleotides designed to activate RNase H and have greater affinity for their RNA receptors and to enhance specificity has also been demonstrated (*102,103*). In a recent study, RNase H-mediated cleavage of target transcript was much more selective when deoxyoligonucleotides comprised of methylphosphonate deoxyoligonucleotide wings and phosphodiester gaps were compared to full phosphodiester oligonucleotides (*103*).

Despite the information about RNase H and the demonstrations that many oligonucleotides may serve as RNase H substrates in lysate and purified enzyme asasys (*104–106*), relatively little is yet known about the role of structural features in RNA targets in activating RNase H and direct proof that RNase H activation is, in fact, the mechanism of action of oligonucleotides in cells.

Recent studies in our laboratories provide additional, although indirect, insights into these questions. ISIS 1939 is a 20-mer phosphorothioate complementary to a sequence in the 3′-untranslated region of ICAM-1 RNA (*86*). It inhibits ICAM-1 production in human umbilical vein endothelial cells, and Northern blots demonstrate

that ICAM-1 mRNA is rapidly degraded. A 2'-*O*-methyl analog of ISIS 1939 displays higher affinity for the RNA than the phosphorothioate, is stable in cells, but inhibits ICAM-1 protein production much less potently than ISIS 1939. It is likely that ISIS 1939 destabilizes the RNA and activates RNase H. In contrast, ISIS 1570, an 18-mer phosphorothioate that is complementary to the translation initiation codon of the ICAM-1 message, inhibited production of the protein, but caused no degradation of the RNA. Thus, two oligonucleotides that are capable of activating RNase H had different effects depending on the site in the mRNA at which they bound (*86*).

2.9.4. Covalent Modification of the Target Nucleic Acid by the Oligonucleotide

A large number of oligonucleotides conjugated by alkylating and photoactivable alkylating species have been synthesized and tested for effects on purified nucleic acids and intracellular nucleic acid targets (*106–111*). The potential disadvantages are obvious: non-specific alkylation may occur in vivo and result in toxicities.

A variety of alkylating agents have been used to modify covalently single-stranded DNA and shown to induce alkylation at sequences predicted by the complementary oligonucleotide to which they were attached (*107–111*). Similar alkylators have been employed to modify covalently double-stranded DNA after triplex formation (*112–115*).

Photoactivable crosslinkers and platinates have been coupled to oligonucleotides and shown to crosslink sequence specifically as well. Photoactivable crosslinkers coupled to phosphodiesters, methylphosphonates, and phosphorothioates have been shown to produce sequence-specific crosslinking (*116–123*). Photoreactive crosslinking has also been demonstrated for double-stranded DNA after triplex formation (*124,125*).

Preliminary data suggesting that covalent modifications of nucleic acids in cells are feasible and may enhance the potency of oligonucleotides have also been reported. Psoralen-linked methylphosphonate oligonucleotides were reported to be significantly more potent than methylphosphonate oligonucleotides in inhibiting rabbit globin mRNA in rabbit reticulocyte lysate assay (*126*). Psoralen-linked methylphosphonates were also reported to be more potent in inhibiting herpes simplex virus infection in HeLa cells in tissue culture (*67*). Additionally, although not producing covalent modification a 9-mer phosphodiester conjugated with an intercalator inhibited mutant Ha-*ras* synthesis in T-24 bladder carcinoma cells (*127*).

2.9.5. Oligonucleotide-Induced Cleavage of Nucleic Acid Targets

Another attractive mechanism by which the potency of oligonucleotides might be increased is to synthesize derivatives that cleave their nucleic acid targets directly. Several potential chemical mechanisms are being studied and positive results have been reported.

The mechanism that has been most broadly studied is to conjugate oligonucleotides to chelators of redox-active metals and generate free radicals that can cleave nucleic acids. Dervan and colleagues have developed EDTA-conjugated oligonucleotides that cleave double-stranded DNA sequence specifically after triplex formation (*22,128*). Dervan and others have also employed EDTA-oligonucleotide conjugates to cleave single-stranded DNA (*129,130*). It is thought that EDTA-chelated iron generates hydroxyl radicals via a fenton-like reaction that cleaves the DNA. However, the cleavage occurs at several oligonucleotides near the nucleotide at which EDTA is attached rather than with absolute specificity.

In the presence of copper, oligonucleotides conjugated to 2,10 phenanthroline also cleave DNA via a free radical mechanism with some sequence specificity (*131–135*) as do porphyrin-linked oligonucleotides when exposed to light (*136–138*). Porphyrin-linked oligonucleotides, however, oxidize bases and induce crosslinks, as well as cleave the phosphodiester backbone.

To date, no reports have demonstrated selective cleavage of an RNA or enhanced potency of oligonucleotides in cells using oligonucleotides with cleaving moieties that employ these mechanisms. However, it seems likely that studies in progress in a number of laboratories will explore this question shortly.

To date, no reports have demonstrated selective cleavage of an RNA or enhanced potency of oligonucleotides in cells using oligonucleotides with cleaving moieties that employ these mechanisms. However, it seems likely that studies in progress in a number of laboratories will explore this question shortly.

Another mechanism that may be intrinsically more attractive for therapeutic applications, particularly for cleavage of RNA targets, is a mechanism analogous to that used by many ribonucleases, nucleotidyltransferases, phosphotransferases, and ribozymes. Ribozymes are oligoribonucleotides or RNA species capable of cleaving themselves or other RNA molecules (*139*). Furthermore, the Tetrahymena ribozyme has been shown to cleave DNA, but at a slower rate than RNA (*134*). Although several classes of ribozymes have been identified and differ with regard to substrate specificity, the use of internal or external guanosine, and other characteristics, they all employ similar enzymatic mechanisms. Cleavage and ligation involve a Mg^{2+}-dependent transesterification with nucleophilic attack by the 3′-hydroxyl of guanosine (*140*).

Significant progress in achieving a number of key objectives that may result in the therapeutic application of ribozymes has been reported. In addition to studies to understand the basic mechanisms by which ribozymes cleave RNA (*141*), progress in reducing the size of ribozymes and stabilizing them has been reported. A hammerhead ribozyme (*142*) that has been reduced to a minimum length of 22 nucleotides has been shown to cleave RNA (*143*). Most ribonucleotides in several ribozymes have been shown to be nonessential and have been replaced by deoxynucleotides that may stabilize the ribozyme against RNases (*144,145*). Phosphorothioate moieties have been substituted in various positions without loss of activity (*146*). By substituting 2′-fluoro-2′-deoxy uridine for pyrimidines in the conserved region of a hammerhead ribozyme and four phosphorothioate moieties at the 3′-end of the ribozyme, a ribozyme that was as active at the parent ribozyme and stable in undiluted fetal calf serum was reported (*147*). Additionally, a ribozyme containing 2′-fluoro or 2′-amino nucleotides at all pyrimidine positions was reported to be highly active and stable in fetal calf serum (*148*). Other ribozymes containing 2′-modified adenosines and one with 2′-methoxy substituents in a contiguous oligonucleotide have been reported (*149,150*). Thus, it would appear that there is some scope for the application of medicinal chemistry to improve the pharmacokinetic properties of ribozymes.

Although the therapeutic utility of modified synthetic ribozymes is yet to be reported, the progress in this area is gratifying and suggests that this may be a viable approach.

Another approach to the exploitation of ribozyme activities involves the synthesis of oligonucleotides complementary to a target RNA and linked to a sequence that can serve as an external guide sequence for RNase P. RNase P is an enzyme that contains

a catalytic RNA (ribozyme) and uses a highly conserved sequence, the external guide sequence, to direct the catalytic RNA to the site of cleavage (*151*). Oligonucleotides incorporating an external guide sequence and expressed by transfection of appropriate plasmids were reported to cause RNase P cleavage of the RNA for chloroamiphenical acetyl transferase (*152*). Thus, it would seem feasible to design stabilized oligonucleotides with external guide sequences that could be employed as drugs.

The notion that a relatively small oligonucleotide could be designed that could interact with and cleave desired sequences as do ribozymes was given impetus by studies that showed activity for ribozymes as short as a 19-mer (*153*) and the demonstration that ribozyme activity can be retained after substitutions, such as phosphorothioates, are introduced (*140*). Consequently, creating oligonucleotides that cleave RNA targets by synthesizing oligonucleotides with appropriate tethers and functionalities positioned to catalyze degradation via acid-base mechanisms (Cook, unpublished data) is an attractive possibility.

2.9.6. Conclusions

In conclusion, an array of potential postbinding mechanisms has already been identified for oligonucleotides. However, for specific oligonucleotides, insufficient data are available to draw firm conclusions about mechanism, and it is likely that more than one mechanism may play a role in the activity of a given oligonucleotide.

Perhaps more importantly, it is clear that many additional mechanisms are likely to be identified as progress continues. It is important to consider the structure and function of receptor sequences in designing oligonucleotides, and to continue to study potential mechanisms in detail. Clearly, RNase H may play a role in the mechanisms of many oligonucleotides, but equally clearly, it is not critical for the activity of others. In the future, the mechanisms (and resulting efficacy) for which oligonucleotides are designed will probably be optimized for each drug target and chemical class of oligonucleotide.

3. THE SELECTION OF OPTIMAL RNA RECEPTOR SITES

The antisense mechanism begins with the binding of an oligonucleotide to a pre-mRNA or mRNA species via Watson-Crick hybridization. In essence then, antisense oligonucleotides are designed to alter the intermediary metabolism of RNA as described in the previous section. Therefore, at least four factors might influence the receptor sequence that results in the greatest antisense activity: accessibility of receptor sequences to oligonucleotide, RNA structure and the functions of the various structures, terminating mechanism of the oligonucleotide, and chemical properties of the oligonucleotide.

3.1. Factors Influencing Receptor Sequence Accessibility

3.1.1. Interactions with Proteins and Other Nucleic Acids

It is generally accepted that each RNA molecule interacts with multiple proteins at multiple sites, and that there are specialized sequences that interact with selected proteins and other nucleic acids. However, there is relatively little information about the overall accessibility of receptor sites in RNA species or the kinetics of the numerous

interactions that must take place between an RNA molecule and other cellular components as the RNA is processed and used. Moreover, there are likely multiple poorly understood cellular processes that affect targeting of antisense drugs that are even yet to be considered. For example, a number of studies have demonstrated that RNA species may be localized to different subcellular compartments. In addition to the obvious compartmentation into nuclear, nucleolar, and cytoplasmic loci, RNA species have been reported to be sorted into a number of different cytoplasmic sites, and this sorting has significant effects on the translation of the mRNA, the half-life, and other properties of the RNA (*154,155*). Clearly, such events should have a profound influence on the potential effects of antisense drugs. Consequently, the search for optimal sites for antisense interactions has been largely empirical, and at present, meaningful generalizations are not possible. Nevertheless, enough information has been developed to emphasize the complexity of these issues and their effects on antisense drug actions.

3.1.2. The Optimal Antisense Site Varies as a Function of the RNA Species

To determine whether the pattern of sites that are most sensitive to antisense effects varies depending on the RNA species, it is necessary to study a series of oligonucleotides of the same chemical class that have approximately equivalent theoretical affinities for their target sequences and have equivalent terminating mechanisms against a number of RNA species. We chose to study phosphorothioate oligodeoxynucleotides 20 nucleotides in length against a large number of RNA targets. Each RNA target was "scanned" using one or two concentrations of oligonucleotides targeted to various regions, and then for many oligonucleotides, full-dose response and time-course studies were performed.

Figure 2 shows the effects of phosphorothioate oligonucleotides targeted to different regions in two RNAs, ICAM, and E-selectin. As can be seen, even for oligonucleotides of a single chemical class with equivalent affinities and the ability to terminate RNA activity by serving as substrates for RNase H, enormous variations in potency was observed, depending on the position in the RNA targeted (*86,156–158*). For example, ISIS 2679 is a potent inhibitor of E-selectin production. Yet ISIS 2687, positioned a few nucleotides from ISIS 2679, is nearly inactive. Similar studies have been performed in our laboratories on many mammalian and viral genes, and the only generalization that is currently supported is that each RNA target (even closely related isotypes) is different. Table 1 represents a qualitative summary of some of our experience. The results are qualitative and represent the "best site" identified after studying multiple oligonucleotides. The number of oligonucleotides studied varies from target to target, as do assay conditions, cell type, and other factors. Consequently, this table and the next are meant to demonstrate the complexity of the problem only, not to provide a definitive statement about the "most active site" in any RNA. Table 2 (Dean et al., unpublished data) summarizes in more detail experience in our laboratories with regard to the activities of phosphorothioate oligodeoxynucleotides active against human and mouse pKC-α. Clearly, although the numbers of oligonucleotides tested are small, it is apparent that all regions may provide active receptor sites, and that mouse and human pKC-α may be different.

3.1.3. The Optimal Antisense Site Varies as a Function of Terminating Mechanism

To test whether the type of terminating mechanism an oligonucleotide may have influences the sites of optimal antisense activity, we have studied two groups of oligonucleotides, phosphorothioate oligodeoxynucleotides and phosphorothioate oligonucleotides fully modified in the 2′-position as either 2′-fluoro, methoxy, or propoxy. RNase H activation is a terminating mechanism available to the oligodeoxynucleotides. The fully 2′-modified oligonucleotides will not serve as substrates for RNase H. The 2′-fluoro and methoxy oligonucleotides have significantly greater affinity for RNA than the oligodeoxynucleotides. The propoxy oligonucleotides have slightly greater affinities (*45,159*).

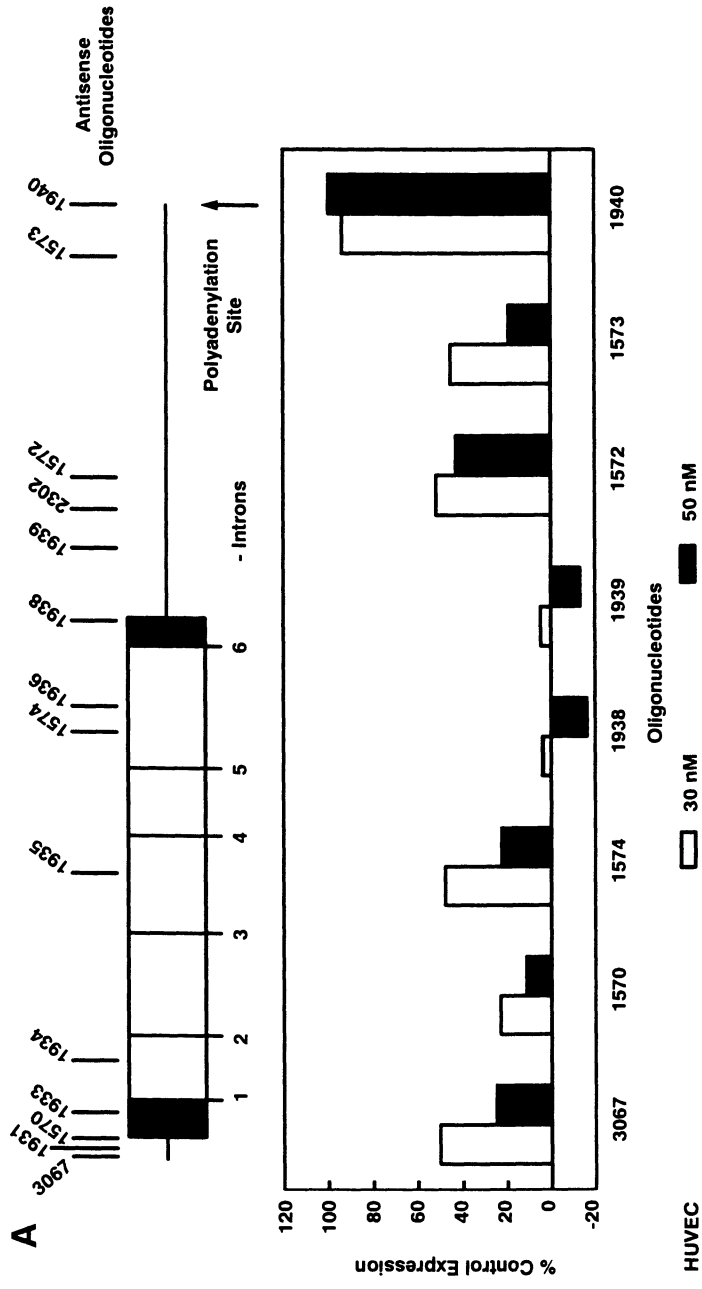
In a study on intercellular adhesion molecule-1 (ICAM-1), a phosphorothioate oligodeoxynucleotide, ISIS 1939, was shown to cause an RNase H-mediated reduction in ICAM-1 RNA. The 2′-methoxy analog is ISIS 1939 caused no reduction in RNA levels and was unable to inhibit ICAM protein synthesis (*86*). Thus, a position in the untranslated region of ICAM-1 RNA appeared to be sensitive only to an antisense oligonucleotide capable of supporting RNase H-mediated cleavage of the RNA. In contrast, the 2′-methoxy analog of ISIS 1570, a phosphorothioate oligodeoxynucleotide designed to bind to the translation initiation codon of ICAM-1 RNA, was almost as active as the parent molecule (*86*).

Figure 3 compares the most active receptor sequences in E-selectin RNA for oligodeoxynucleotides and fully modified 2′-propyl analogs (Bennett, unpublished results). There are quite obviously substantial differences in the positions of activity with the non-RNase H-dependent mechanisms displaying somewhat different patterns of activity compared to the oligodeoxynucleotide.

3.1.4. The Optimal Antisense Site May Vary as a Function of Affinity

Studies in our laboratory have shown that, in a series of compounds that bind to the same receptor sequence and have the same terminating mechanism, potency increases as affinity is enhanced (*102*). We have also reported that higher-affinity oligonucleotides are required to invade some RNA stem loops (*31,55,80,160*). It, therefore, is reasonable to speculate that higher-affinity oligonucleotides might be able to bind to receptor sequences unaccessible to lower-affinity compounds, thus producing a different pattern of activities.

One approach to addressing this question is to compare the patterns of activities for higher-affinity RNase H activity "gapmers" to oligodeoxynucleotides. The gapmers display higher affinity and support RNase H. Figure 4 shows the patterns of activities observed when murine VCAM-1 was studied. Some differences were observed (Bennett, unpublished observations). However, given the fact that the propoxy gapmers have only slightly greater affinities for RNA than their parents, relatively small differences were expected and observed. More substantial differences have been observed in patterns of activities for higher-affinity gapmers. For example, in a study of the E-7 mRNA of human papillomavirus, the overall pattern of activity for gapmers was similar to the oligodeoxyoligonucleotides, but a 2′-fluoro gapmer was found that was active in a region not identified by the oligodeoxynucleotides and


A
Antisense Oligonucleotides
1940
1573
1572
2302
1939
1938
1936
1574
1935
1934
1933
1570
1931
3067
Polyadenylation Site
- Introns
1
2
3
4
5
6
120
100
80
60
40
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0
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% Control Expression
3067
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1938
1939
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Oligonucleotides
30 nM
50 nM
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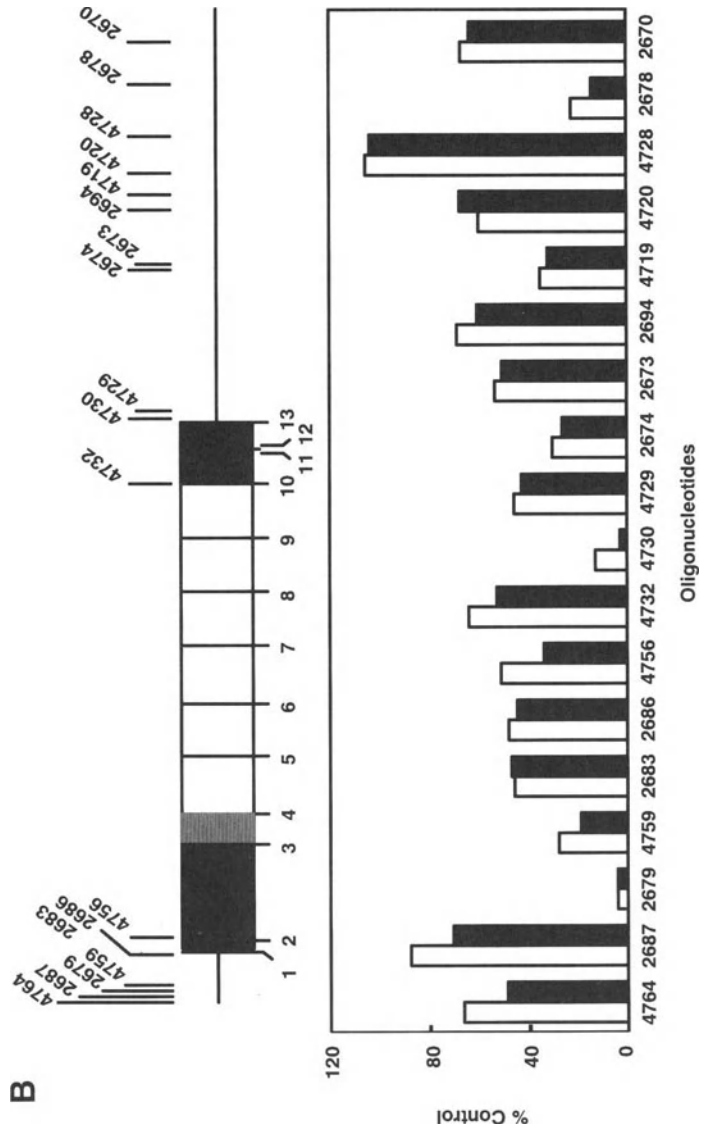

Fig. 2. Pattern of antisense activities for 20-mer phosphorothioate oligodeoxynucleotides. Antisense oligonucleotide target sites on: (**A**) ICAM-1 mRNA and (**B**) human E-selectin mRNA. Sites on human mRNA to which antisense oligonucleotides have been designed to hybridize are shown. Enlarged boxes correspond to translated regions. Activities determined by evaluating protein production as described in Bennett et al. (*158*).

Table 1
Most Active Receptor Sequence[a]

Target	*Site*	*RNA degradation*
HSV-UL13	AUG	None
HPV-E2	AUG	Possible
BPV-E2	AUG	—
H-ICAM-1	3′-UTR	Yes
	AUG	No
	5′-cap	No
M-ICAM-1	3′-UTR	Yes
H-ELAM	5′-UTR	No
	3′-UTR	Yes
H-Type II PLA_2	3′-UTR	Yes
5-LO	Coding	Yes
HIV	5′-UTR (TAR)	No
HIV-Rev	AUG	Yes
RAS	Coding region	Yes
	AUG	Yes
Influenza	Repetitive sequences	—
PKC-α	5′-UTR	Yes
	3′-UTR	Yes
	AUG	Yes
HCMV major immediate early region	Coding region	Yes
HCV polyprotein	5′-UTR and AUG	Yes

[a]The most active site(s) in each target gene is presented along with comments on whether degradation of the RNA was observed when an oligonucleotide that would form an RNA substrate was studied.

Table 2
Active Sites for Phosphorothioate Oligodeoxynucleotides in Protein Kinase C-α[a]

Target	*Oligos tested*	*Active oligos*	*% "Hit rate"*
Human pKC-α			
5′-UTR	1	1	100
AUG	3	2	66
ORF	6	1	16
3′-UTR	10	2	20
Mouse pKC-α			
5′-UTR	4	2	50
AUG	2	1	50
ORF	1	0	0
3′-UTR	11	5	45

[a]The activities of 23-mer P = S oligonucleotides against different sites on PKC-α in vitro in the presence of cationic lipids as reported by Dean and McKay, 1994. The "activity level" is, of course, somewhat arbitrary since some oligonucleotides are more active than others. However, all assays were conducted similarly, and the minimal activity label observed is biologically significant.

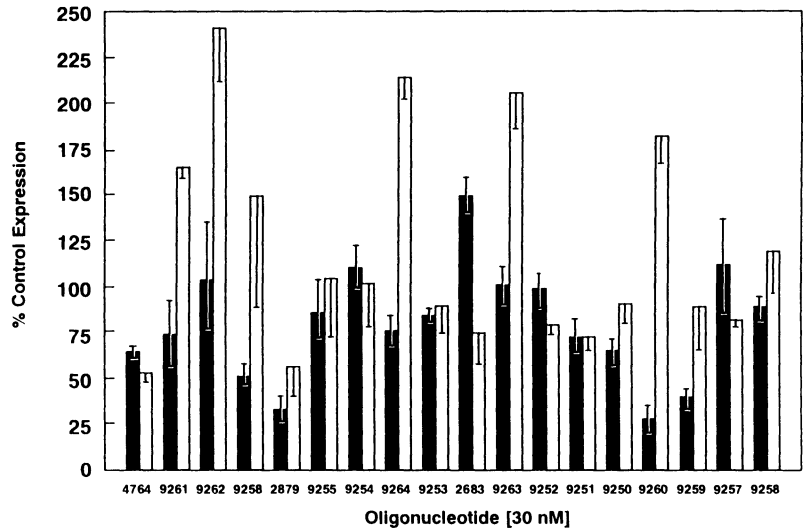

Fig. 3. Comparison of 2′-deoxy (■) with 2′-*O*-propyl (□) P-S oligo inhibition of E-selectin. Most active receptor sequences in E-selectin RNA for oligodeoxynucleotides and fully modified 2′-*O*-propyl analogs. Assays were as described in Bennett et al. (*158*). The positions of the oligonucleotides correspond to location in the RNA 5′- to 3′-ends as shown in the maps in Fig. 2.

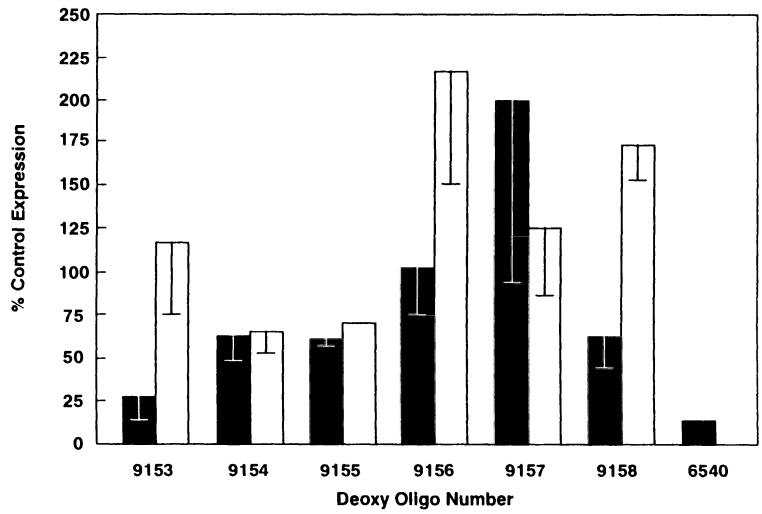

Fig. 4. Comparison of patterns of deoxy (■) and propyl gap (□) activities for higher-affinity RNase H activating gapmers observed when murine VCAM-1 was studied. Assays were as described in Bennett et al. (*158*). The positions of the oligonucleotides correspond to location in the RNA 5′- to 3′-ends in a manner similar to that shown in Fig. 2.

a propoxy gapmer analog of an active oligodeoxynucleotide was inactive (Cowsert, unpublished observations).

Clearly, much more thorough studies are required before firm conclusions about the effect of affinity on the patterns of antisense activities are determined, but it seems likely that affinity will play a major role.

4. CHARACTERISTICS OF PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES

Of the first-generation oligonucleotide analogs, the class that has resulted in the broadest range of activities and about which the most is known is the phosphorothioate class. Phosphorothioate oligonucleotides were first synthesized in 1969 when a poly rI:poly rC phosphorothioate was synthesized (*9*). This modification clearly achieves the objective of increased nuclease stability. In this class of oligonucleotides, one of the oxygen atoms in the phosphate group is replaced with a sulfur. The resulting compound is negatively charged as is a phosphodiester, but more resistant to nucleases (*161*).

4.1. Hybridization

The hybridization of phosphorothioate oligonucleotides to DNA and RNA has been thoroughly characterized (for review, *see 43,45,156*). The T_m of a phosphorothioate oligonucleotide for RNA is approx 0.5 °C less per nucleotide than for a corresponding phosphodiester oligodeoxynucleotide. This reduction in T_m per nucleotide is virtually independent of the number of phosphorothioate units substituted for phosphodiesters. However, sequence context has some influence as the $\triangle T_m$ can vary from −0.3 to 1.0 °C depending on sequence. Compared to RNA:RNA duplex formation, a phosphorothioate oligonucleotide has a T_m approx −2.2 °C lower per unit (*162*). This means that to be effective in vitro, phosphorothioate oligodeoxynucleotides must typically be 17–20 mer in length (*64,102*) and that invasion of double-stranded regions in RNA is difficult (*31,160*).

4.2. Interactions with Proteins

Phosphorothioate oligonucleotides bind to proteins. The interactions with proteins can be divided into nonspecific, sequence-specific, and structure-specific binding events, each of which may have different characteristics and effects. Nonspecific binding to a wide variety of proteins has been demonstrated. Exemplary of this type of binding is the interaction of phosphorothioate oligonucleotides with serum albumin. The affinity of such interactions is low. The K_d for albumin is approx 400 μM, thus, in a similar range with aspirin or penicillin (*163*). Phosphorothioate oligonucleotides can interact with nucleic acid binding protein, such as transcription factors and single-strand nucleic acid binding proteins. However, very little is known about these binding events. Additionally, it has been reported that phosphorothioates bind to an 80-kDa membrane protein that was suggested to be involved in cellular uptake processes (*164*). However, again, little is known about the affinities, sequence, or structure specificities of these putative interactions.

Phosphorothioates interact with nucleases and DNA polymerases. These compounds are slowly metabolized by both endo- and exonucleases (*45*) and inhibit these enzymes (*165*). The inhibition of these enzymes appears to be competitive, and this may account for some early data suggesting that phosphorothioates are almost infinitely stable to nucleases. In these studies, the oligonucleotide-to-enzyme ratio was very high, and thus, the enzyme was inhibited. A phosphodiester oligonucleotide added after degradation of the phosphorothioate plateaued was not degraded. Clearly, such effects could have significant pharmacokinetic consequences.

Phosphorothioates also bind to RNase H when in an RNA–DNA duplex, and the duplex serves as a substrate for RNase H (*166*). At higher concentrations, presumably by binding in a single-strand form to RNase H, phosphorothioates inhibit the enzyme (*166*). Again, the oligonucleotides appear to be competitive antagonists for the DNA–RNA substrate.

Phosphorothioates have been shown to be competitive inhibitors of DNA polymerase α and β with respect to the DNA template, and noncompetitive inhibitors of DNA polymerases γ and δ (*166*). Despite this inhibition, several studies have suggested that phosphorothioates might serve as primers for polymerases and be extended (*165,167,168*). In our laboratories, we have shown extensions of two to three nucleotides only. At present, a full explanation regarding why no longer extensions are observed is not available.

Phosphorothioate oligonucleotides have been reported to be competitive inhibitors for HIV reverse transcriptase (*169*) and inhibit reverse transcriptase-associated RNase H activity (*170*). They have been reported to bind to the cell-surface protein, CD4 (*171*), and to protein kinase C (*172*). Various viral polymerases have also been shown to be inhibited by phosphorothioates (for review, *see 167*). Additionally, we have shown potent, nonsequence-specific inhibition of RNA splicing by phosphorothioates (Hodges & Crooke, unpublished data).

Like other oligonucleotides, phosphorothioates can adopt a variety of structures. As a general rule, self-complementary oligonucleotides are avoided, if possible, to avoid duplex formation between oligonucleotides. However, other structures that are less well understood can also form. For example, oligonucleotides containing runs of guanosines can form tetrameric structures called G-quartets, and these appear to interact with a number of proteins with relatively greater affinity than unstructured oligonucleotides (*173*). ISIS 5320 is exemplary (Fig. 5).

4.3. Nuclease Stability

The principal metabolic pathway for oligonucleotides is cleavage via endo- and exonucleases. Phosphorothioate oligodeoxynucleotides, although quite stable to various nucleases (*32,37,174*), are competitive inhibitors of these same enzymes (*165,166*). Consequently, the stability of phosphorothioate oligonucleotides to nucleases is probably a bit less than initially thought, since high concentrations (that inhibited nucleases) of oligonucleotides were employed in the early studies. Similarly, phosphorothioate oligonucleotides are degraded slowly by cells in tissue culture with a half-life of 12–24 h (*37,165*) and are slowly metabolized in animals (*175*). The pattern of metabolites suggests primarily exonuclease activity with perhaps modest contributions by endonucleases.

4.4. In Vitro Cellular Uptake

Phosphorothioate oligonucleotides are taken up by a wide range of cells in vitro (*176–180*). In fact, very recently, uptake of phosphorothioate oligonucleotides into a prokaryote, *Vibrio parahaemoyticus,* has been reported (*181*). Uptake is time- and temperature-dependent. It is also influenced by cell type, cell-culture conditions, media and sequence, and length of the oligonucleotide (*178*). No obvious correlation between the lineage of cells, whether the cells are transformed or whether the cells are virally infected, and uptake has been identified (*178*), nor are the factors that result in

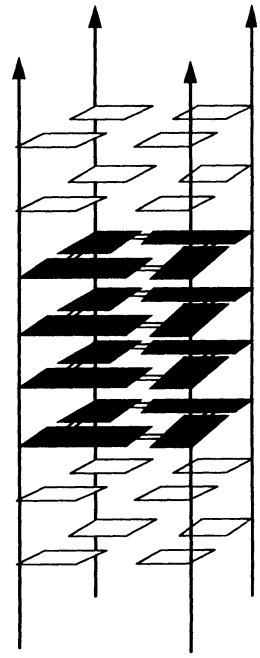

Fig. 5. The structure of ISIS 5320. Tetramer found of $T_sT_sG_sG_sG_sG_sT_sT$.

differences in uptake of different sequences of oligonucleotide understood. Although several studies have suggested that receptor-mediated endocytosis may be a significant mechanism of cellular uptake (*164*), the data are not yet compelling enough to conclude that receptor-mediated endocytosis accounts for a significant portion of the uptake in most cells.

Numerous studies have shown that phosphorothioate oligonucleotides distribute broadly in most cells once taken up (*178,179,182*). Again, however, significant differences in subcellular distribution among various types of cells have been noted.

Cationic lipids have been used to enhance uptake of phosphorothioate oligonucleotides in cells that take up little oligonucleotide in vitro (*183,184*). Again, however, there are substantial variations from cell type to cell type.

4.5. In Vivo Pharmacokinetics

Phosphorothioate oligonucleotides bind to serum albumin and α-2 macroglobulin. The apparent affinity for albumin is quite low (250–400 μM) and comparable to the low-affinity binding observed for a number of drugs, e.g., aspirin, penicillin (*163*). Serum protein binding, therefore, provides a repository for these drugs and prevents rapid renal excretion. Since serum protein binding is saturable, at higher doses, intact oligomer may be found in urine (*168,185*). Studies in our laboratory suggest that in rats, iv doses of 15–20 mg/kg saturate the serum protein binding capacity (Leeds, unpublished data).

Phosphorothioate oligonucleotides are rapidly and extensively absorbed after parenteral administration. For example, in rats, after an id dose 3.6 mg/kg of ^{14}C-ISIS 2105, a 20-mer phosphorothioate, approx 70% of the dose was absorbed within 4 h,

and total systemic bioavailability was in excess of 90% (*186*). After id injection in humans, absorption of ISIS 2105 was similar to that observed in rats (*187*).

Distribution of phosphorothioate oligonucleotides from blood after absorption or iv administration is extremely rapid. We have reported distribution half-lives of < 1 h (*175,186,187*), and similar data have been reported by others (*168,185*). Blood and plasma clearance is multiexponential with a terminal elimination half-life from 40 to 60 h in all species except humans. In humans, the terminal elimination half-life may be somewhat longer (*187*).

Phosphorothioates distribute broadly to all peripheral tissues. Liver, kidney, bone marrow, skeletal muscle, and skin accumulate the highest percentage of a dose, but other tissues display small quantitites of drug (*175,186*). No evidence of significant penetration of the blood–brain barrier has been reported. The rates of incorporation and clearance from tissues vary as a function of the organ studied, with liver accumulating drug most rapidly (20% of a dose within 1–2 h), and other tissues accumulating drug more slowly. Similarly, elimination of drug is more rapid from liver than any other tissue, e.g., terminal half-life from liver: 62 h; from renal medulla: 156 h.

At relatively low doses, clearance of phosphorothioate oligonucleotides is owing primarily to slow metabolism (*175,185,186*). Metabolism is mediated by exo- and endonucleases that result in shorter oligonucleotides, and ultimately, nucleosides that are degraded by normal metabolic pathways. Although no direct evidence of base excision or modification has been reported, these are theoretical possibilities that may occur. In one study, a larger-mol-wt radioactive material was observed in urine, but was not fully characterized (*168*). Clearly, the potential for conjugation reactions and extensions of oligonucleotides via these drugs serving as primers for polymerases must be explored in more detail. In a very thorough study, a pair of 20 nucleotide phosphodiester and phosphorothioate oligonucleotides were administered intravenously at a dose of 6 mg/kg to mice. The oligonucleotides were internally labeled with $^{3}H\text{-}CH_3$ by methylation of an internal deoxycytidine residue using HhaI methylase and *S*-[^{3}H] adenosyl methionine (*188*). The observations for the phosphorothioate oligonucleotide was entirely consistent with those made in our studies. Additionally, in this paper, autoradiographic analyses showed drug in renal cortical cells (*188*).

One study of prolonged infusions of a phosphorothioate oligonucleotide to human beings has been reported (*189*). In this study, five patients with leukemia were given 10-d iv infusions at a dose of 0.05 mg/kg/h. Elimination half-lives reportedly varied from 5.9 to 14.7 d. Urinary recovery of radioactivity was reported to be 30–60% of the total dose, with 30% of the radioactivity being intact drug. Metabolites in urine included both higher- and lower-mol-wt compounds. Obviously, these data differ from observations in other studies. At present, the data are insufficient to determine if the pharmacokinetics of prolonged iv infusions are truly substantially different from the pharmacokinetic behavior of iv bolus injections.

We have also performed oral bioavailability experiments in rodents treated with an H2 antagonist to avoid acid-mediated depurination or precipitation. In these studies, very limited (< 10%) bioavailability was observed.

In summary, pharmacokinetic studies of several phosphorothioates demonstrate that they are well absorbed from parenteral sites, distribute broadly to all peripheral tissues, do not cross the blood–brain barrier, and are eliminated primarily by slow metabolism. In short, once a day or every other day systemic dosing should be feasible. Although the similarities between oligonucleotides of different sequences are far

greater than the differences, additional studies are required before determining whether there are subtle effects of sequence on the pharmacokinetic profile of this class of drugs.

4.6. *Pharmacological Properties*

4.6.1. Molecular Pharmacology

Antisense oligonucleotides are designed to bind to RNA targets via Watson-Crick hybridization. Since RNA can adopt a variety of secondary structures via Watson-Crick hybridization, one useful way to think of antisense oligonucleotides is as competitive antagonists for self-complementary regions of the target RNA. Obviously, creating oligonucleotides with the highest affinity per nucleotide unit is pharmacologically important, and a comparison of the affinity of the oligonucleotide to a complementary RNA oligonucleotide is the most sensible comparison. In this context, phosphorothioate oligodeoxynucleotides are relatively competitively disadvantaged, since the affinity per nucleotide unit of oligomer is less than RNA ($> -2.0\,°C$ T_m/U) (*190*). This results in a requirement of at least 15–17 nucleotides in order to have sufficient affinity to produce biological activity (*64*).

Phosphorothioate oligonucleotides have also been shown to have effects inconsistent with the antisense mechanism for which they were designed. Some of these effects are owing to sequence- and structure-specific, as well as nonspecific interactions with proteins. These effects are particularly prominent in in vitro tests for antiviral activity since often high concentrations of cells, viruses, and oligonucleotides are coincubated (*191,192*). Human immune deficiency virus (HIV) is particularly problematic, since many oligonucleotides bind to the gp120 protein and other proteins of the virus (*173*). In addition to protein interactions, other factors, such as overrepresented sequences of RNA and unusual structures that may be adopted by oligonucleotides, can contribute to unexpected results (*173*).

Given the variability in cellular uptake of oligonucleotides, the variability in potency as a function of binding site in an RNA target and potential nonantisense activities of oligonucleotides, careful evaluation of dose–response curves and clear demonstration of the antisense mechanism are required before drawing conclusions from in vitro experiments. Nevertheless, numerous well-controlled studies have been reported in which antisense activity was conclusively demonstrated. Since many of these studies have been reviewed previously (*43–45,170,193*), suffice it to say that antisense effects of phosphorothioate oligodeoxynucleotides against a variety of targets are well documented.

4.6.2. In Vivo Pharmacological Activities

Table 3 summarizes a number of the published reports (abstracts and full publications) demonstrating in vivo activity of oligonucleotides. Local effects have been reported for phosphorothioate and methylphosphonate oligonucleotides. A phosphorothioate oligonucleotide designed to inhibit c-*myb* production and applied locally was shown to inhibit intimal accumulation in the rat carotid artery (*194*). In this study, a Northern blot showed a significant reduction in c-*myb* RNA in animals treated with the antisense compound, but no effect by a control oligonucleotide. Similar effects were reported for phosphorothioate oligodeoxynucleotides designed to inhibit cylin-dependent kinases (CDC-2 and CDK-2). Again, the antisense oligonucleotide inhibited intimal thickening and cylin-dependent kinase activity, whereas a control

Table 3
Reported Activities of Antisense Drugs in Animals

Target	*Animal*	*Reference*
HSV-1	Mouse	*67*
p120 Oncogene	Mouse	*197*
c-*myb*	Rat	*194*
Interleukin 1 receptor	Mouse	*201*
NF-kB	Mouse	*202*
CDC-2 and CDK-2	Rat	*195*
N-*myc*	Mouse	*196*
Y-Y1 receptors	Rat	*198*
NMDA-R1 receptor channel	Rat	*199*
Symptosomal-associated protein 25	Rat	*200*
NF-kB	Mouse	*203*
Intercellular adhesion molecule 1	Mouse	*213*
pKC-α	Mouse	*206*
BCR-ABL	Mouse	*204*
c-*myb*	Mouse	*205*

oligonucleotide had no effect (*195*). Additionally, local administration of a phosphodiester oligonucleotide designed to inhibit N-*myc* resulted in reduction in N-*myc* expression and slower growth to a subcutaneously transplanted human tumor in nude mice (*196*).

Local antitumor effects of phosphorothioate oligodeoxynucleotides have also been reported. An antisense oligonucleotide designed to inhibit the expression of the p120 protein was shown to inhibit the growth of a human tumor transplanted intraperitoneally in nude mice when the compound was administered intraperitoneally (*197*).

Antisense oligonucleotides administered intraventricularly have been reported to induce a variety of effects in the central nervous system. Intraventricular injection of antisense oligonucleotides to neuropeptide-Y-Y1 receptors reduced the density of the receptors and resulted in behavioral signs of anxiety (*198*). Similarly, an antisense oligonucleotide designed to bind to NMDA-R1 receptor channel RNA inhibited the synthesis of these channels and reduced the volume of focal ischemia produced by occlusion of the middle cerebral artery in rats (*199*).

Injection of antisense oligonucleotides to synaptosomal-associated protein-25 into the vitreous body of rat embryos reduced the expression of the protein and inhibited neurite elongation by rat cortical neurons (*200*).

In addition to local and regional effects of antisense oligonucleotides, a growing number of well-controlled studies have demonstrated systemic effects of phosphorothioate oligodeoxynucleotides. Expression of interleukin-1 in mice was inhibited by systemic administration of antisense oligonucleotides (*201*). Oligonucleotides to the NF-kB p65 subunit administered intraperitoneally at 40 mg/kg every 3 d slowed tumor growth in mice transgenic for the human T-cell leukemia viruses (*202*). Similar results with other antisense oligonucleotides were shown in another in vivo tumor model after either prolonged sc infusion or intermittent sc injection (*203*).

Two recent reports further extend the studies of phosphorothioate oligonucleotides as antitumor agents in mice. In one study, phosphorothioate oligonucleotide directed

to inhibition of the BCR-ABL oncogene was administered at a dose of 1 mg/d for 9 d iv to immunodeficient mice injected with human leukemic cells. The drug was shown to inhibit the development of leukemic colonies in the mice and to reduce selectively BCR-ABL RNA levels in peripheral blood lymphocytes, spleen, bone marrow, liver, lungs, and brain (*204*). In the second study, a phosphorothioate oligonucleotide antisense to the proto-oncogene *myb*, inhibited the growth of human melanoma in mice. Again, *myb* mRNA levels appeared to be selectively reduced (*205*).

Thus, there is a growing body of evidence suggesting that antisense oligonucleotides (in most cases, phosphorothioate oligodeoxynucleotides) can produce local, regional, and systemic effects at nontoxic doses in vivo. Although proof of mechanism of action is difficult, in most cases, studies with control oligonucleotides demonstrate that the effects are consistent with the proposed antisense mechanism. In the important series of studies by Dean and McKay (*206*), an antisense mechanism and isotype selectivity have been conclusively demonstrated after systemic administration of an antisense oligonucleotide designed to inhibit protein kinase C-α (pKC-α) in mice.

All of these data suggest that phosphorothioate oligodeoxynucleotides may have therapeutic potential.

4.6.3. In Vivo Toxicological Properties

The acute LD_{50} dose in mice of all phosphorothioates tested to date is in excess of 500 mg/kg (Kornbrust, unpublished observations). Although there may be differences among different oligonucleotide sequences and the LD_{50} may be influenced by route of administration, these factors appear to result in minimal variation. Several phosphorothioate oligodeoxynucleotides have been studied for potential fetal toxicities, and to date, no significant adverse effects have been noted (Kornbrust, unpublished observations; *207*).

Although there is no evidence of antigenicity or induction of delayed-type hypersensitivity in animals or humans given multiple doses of phosphorothioate oligonucleotides (*156;* Kornbrust, unpublished observations), multiple doses of these drugs clearly affect the immune system in animals, with rats being the most sensitive species. The manifestations of these toxicities that are observed at lowest doses are increases in spleen weight, production of IgM and IgG, and expansion of B-cell populations in spleens (Kornbrust, unpublished observations; *208*). Although the sequence of the oligonucleotides can affect the dose at which these effects are produced and an antisense sequence designed to inhibit the 65-kDa subunits of NF-kB was reported to be devoid of such effects (*209*), in our experience, all thoroughly tested phosphorothioate oligodeoxynucleotides induced these effects. As a general rule, the doses required to induce these effects have been substantially greater than those demonstrating pharmacologic activity.

In monkeys, several phosphorothioate oligodeoxynucleotides have been shown to cause acute hypotensive events (Kornbrust, unpublished observations; *210*). These effects are transient, if managed appropriately, relatively uncommon, and typically appear to occur in response to the first dose given to an animal. Recent studies suggest that one mechanism responsible for this may be related to complement activation, and that this toxicity can be avoided by giving iv infusions rather than bolus injections. We have evaluated the mechanisms by which phosphorothioate oligonucleotides might induce complement activation and phosphorothioates appear to

affect the alternative pathway. In large part, the effects of phosphorothioates are similar to those associated with other polyanions, such as heparin. Complement regulatory factors, such as Factor H and D, appear to be most influenced by these drugs. Based on predictions about studies on animal models, toxicological studies in monkeys, and unpublished studies on systemically administered phosphorothioates currently in the clinic, the therapeutic index relative to this potential toxicity would appear to be likely to be acceptable.

We have also noted prolongation of prothrombin, partial thromboplastin, and bleeding times in monkeys. Again, these effects are plasma concentration-dependent and appear to occur at doses that are sufficient to saturate serum albumin binding sites. The effects on partial thromboplastin time are much more pronounced than those on prothrombin time. The effects appear to be primarily on the extrinsic pathway with clear inhibition of thrombin activity demonstrated. No effects on Factors VIII–XI have been observed. Again, based on predictions from studies in monkeys, the doses likely to produce this toxicity seem substantially greater than the predicted therapeutic doses. Clearly, only carefully controlled clinical trials will define the human toxicities and therapeutic indices of these drugs.

4.6.4. Clinical Activities

To date, we have studied several hundred patients given multiple doses of three phosphorothioate oligonucleotides and encountered no significant toxicities. Other oligonucleotides are being studied in human beings by other groups, and to date, no significant or dose-related toxicities have been reported.

ISIS 2922, a 20-mer phosphorothioate designed to inhibit cytomegalovirus, has been administered intravitreally to patients with advanced AIDS and advanced drug-resistant CMV retinitis, and showed impressive antiviral activity (*211*).

In this study, three dose groups were studied in patients that had advanced CMV retinitis and had failed ganciclovir and/or foscarnet therapy. The median CD4 count in these patients was 4, so they were extremely immunocompromised. The median time from diagnosis of CMV retinitis was 10 mo. Two patients were treated in the 2-μM dose group and failed. Three of four eyes treated at 4 μM and six of eight eyes at 8 μM responded. The responses were rapid, long-lasting, and substantial. The only adverse event observed was increased inflammation (*212*).

ISIS 2105 is a 20-mer phosphorothioate oligonucleotide designed to inhibit the replication of human papilloma viruses 6 and 11, the viruses responsible for genital warts. Plot ascending-dose multiple-dose studies as primary therapy of genital warts have shown the drug locally and systemically well tolerated after id administration. At doses of 2.5 mg/wart twice weekly and greater, the drug has been associated with resolution of genital warts. In a pivotal placebo-controlled trial of patients treated by surgical removal of the warts and administration of single doses of 0.3 or 1.0 mg/wart ISIS 2105 at the time of surgery, reductions in wart recurrence were observed that were not statistically significant, but suggestive of antiviral activity as a surgical adjuvant.

Development of ISIS 2922 is proceeding with Phase III trials in patients with CMV retinitis. Development of ISIS 2105 is proceeding with a multiple-dose Phase II trial designed to confirm its activity and evaluate its potential utility.

ISIS 2302 is a 20-mer designed to inhibit ICAM (*158,179*). We have completed a single-dose Phase I study in normal volunteers given this drug by 2 h iv infusion at doses up to 2 mg/kg. No significant toxicities were observed.

Table 4
Phosphorothioate Oligonucleotides

Limits
Pharmacodynamic
Low affinity per nucleotide unit
Inhibition of RNase H at high concentrations
Pharmacokinetic
Limited bioavailability
Limited blood-brain barrier penetration
Dose-dependent pharmacokinetics
Toxicologic
Release of cytokines
Complement-associated effects on blood pressure?
Clotting effects

4.6.5. Conclusions

Phosphorothioate oligonucleotides have perhaps outperformed many expectations. They display attractive parenteral pharmacokinetic properties. They have produced potent systemic effects in a number of animal models and, in many experiments, the antisense mechanism has been directly demonstrated as the hoped-for selectivity. Further, these compounds appear to display satisfactory therapeutic indices for many indications.

Nevertheless, phosphorothioates clearly have significant limits (Table 4). Pharmacodynamically, they have relatively low affinity per nucleotide unit. This means that longer oligonucleotides are required for biological activity and that invasion of many RNA structures may not be possible. At higher concentrations, these compounds inhibit RNase H as well. Thus, the higher end of the pharmacologic dose-response curve is lost. Pharmacokinetically, phosphorothioates do not cross the blood-brain barrier, are not significantly orally bioavailable, and may display dose-dependent pharmacokinetics. Toxicologically, clearly the release of cytokines, activation of complement, and interference with clotting will pose dose limits if they are encountered in the clinic.

Since several clinical trials are in progress with phosphorothioates and since others will be initiated shortly, we shall soon have more definitive information about the activities, toxicities, and value of this class of antisense drugs in human beings.

5. POTENTIAL IN CANCER CHEMOTHERAPY

Against a growing number of human tumor xenografts, phosphorothioates and more novel analogs directed to a number of targets have been shown to have potent antitumor effects (for review, *see 44*; Dean, unpublished data; Monia, unpublished data). In a variety of studies, we have observed potent antitumor effects consistent with an antisense mechanisms at doses 2–3 orders of magnitude lower than minimally toxic doses. Furthermore, we have also observed enhanced potency with chimeric analogs (Monia, manuscript in preparation). Thus, it would seem that cautious optimism with regard to the potential of such compounds in the treatment of malignancies is warranted.

A further benefit is the opportunity to use antisense agents to evaluate the roles of specific targets in malignancies.

ACKNOWLEDGMENTS

I am pleased to acknowledge the outstanding assistance in the preparation of this manuscript by Colleen Matzinger. Thanks also to Frank Bennett for his thoughtful review.

REFERENCES

1. Watson JD, Crick FHC. Molecular structure of nucleic acids: A structure for deoxyribose nucleic acid. *Nature* 1953; 171:737.
2. Gillespie D, Spiegelman S. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J Mol Biol* 1965; 12:829–842.
3. Thompson JD, Gillespie D. Current concepts in quantitative molecular hybridization. *Clin Biochem* 1990; 23:261–266.
4. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotides. *Proc Natl Acad Sci USA* 1978; 75:280.
5. Ts'o POP, Miller PS, Greene JJ. Nucleic acid analogs with targeted delivery at chemotherapeutic agents. In: Cheng YC, Goz B, Minkoff M, eds. *Development of Target-Oriented Anticancer Drugs.* New York: Raven. 1983:189.
6. Barrett JC, Miller PS, Ts'o POP. Inhibitory effect of complex formation with oligodeoxyribonucleotide ethyl phosphotriesters on transfer ribonucleic acid aminoacylation. *Biochemistry* 1974; 13:4897.
7. Caruthers MH. Gene synthesis machines: DNA chemistry and its uses. *Science* 1985; 230:281.
8. Alvarado-Urbina G, Sathe GM, Liu WC, et al. Automated synthesis of gene fragments. *Science* 1981; 214:270.
9. De Clercq E, Eckstein F, Merigan TC. Interferon induction increased through chemical modification of a synthetic polyribonucleotide. *Science* 1969; 165:1137.
10. Marcus-Sekura CJ, Woerner AM, Shinozuka K, et al. Comparative inhibition of chloramphenicol acetyltransferase gene expression by antisense oligonucleotide analogues having alkyl phosphotriester, methylphosphonate and phosphorothioate linkages. *Nucleic Acids Res* 1987; 15:5749.
11. Matsukura M, Shinozuka K, Zon G, et al. Phosphorothioate analogs of oligodeoxyribonucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. *Proc Natl Acad Sci USA* 1987; 84:7706.
12. Agrawal S, Goodchild J, Civeira MP, et al. Oligodeoxynucleoside phosphoramidates and phosphorothioates as inhibitors of human immunodeficiency virus. *Proc Natl Acad Sci USA* 1988; 85:7079.
13. Goodchild J, Agrawal S, Civeira MP, et al. Inhibition of human immunodeficiency virus replication by antisense oligonucleotides. *Proc Natl Acad Sci USA* 1988; 85:5507.
14. Sarin PS, Agrawal S, Civeira MP. Inhibition of acquired immunodeficiency syndrome virus by oligodeoxynucleotide methylphosphonates. *Proc Natl Acad Sci USA* 1988; 85:7448.
15. Gao W, Stein CA, Cohen JS, et al. Effect of phosphorothioate homo-oligodeoxynucleotides on herpes simplex virus type 2-induced DNA polymerase. *J Biol Chem* 1989; 264:11,521.
16. Smith CC, Aurelian L, Reddy MP, et al. Antiviral effect of an oligo(nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type 1 immediate early pre-mRNAs 4 and 5. *Proc Natl Acad Sci USA* 1986; 83:2787.
17. Agris CH, Blake KR, Miller PS, et al. Inhibition of vesicular stomatitis virus protein synthesis and infection by sequence-specific oligodeoxyribonucleoside methylphosphonates. *Biochemistry* 1986; 25:6268.
18. Heikkila R, Schwab G, Wickstrom E, et al. A c-myc antisense oligonucleotide inhibits entry into S phase but not progress from GO to G1. *Nature* 1987; 328:445.

19. Wickstrom EL, Bacon TA, Gonzalez A, et al. Anti-c-myc DNA increases differentiation and decreases colony formation by HL-60 cells *in vitro*. *Cell Dev Biol* 1989; 25:297.
20. Felsenfeld G, Davies DR, Rich A. Formation of a three-stranded polynucleotide molecule. *J Am Chem Soc* 1957; 79:2023.
21. Helene C. Control of gene expression by triplex-helix-forming oligonucleotides. The antigene strategy. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC. 1993:375.
22. Moser HE, Dervan PB. Sequence-specific cleavage of double helical DNA by triple helix formation. *Science* 1987; 238:645.
23. Le Doan T, Perrouault L, Praseuth D, et al. Sequence-specific recognition, photocrosslinking and cleavage of the DNA double helix by an oligo-[α]-thymidylate covalently linked to an azidoproflavine derivative. *Nucleic Acid Res* 1987; 15:7749.
24. Cooney M, Czernuszewicz G, Postel EH, et al. Site-specific oligonucleotide binding represses transcription of the human c-myc gene *in vitro*. *Science* 1988; 241:456.
25. Mirabelli CK, Crooke ST. Antisense oligonucleotides in the context of modern molecular drug discovery and development. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC. 1993:7.
26. Nielsen PE, Egholm M, Berg RH, et al. Peptide nucleic acids (PNA): Oligonucleotide analogs with a polyamide backbone. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC. 1993:363.
27. Cech T. Self-splicing of group 1 introns. *Annu Rev Biochem* 1990; 59:543.
28. Uhlenbeck OC. Using ribozymes to cleave RNAs. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC. 1993:83.
29. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 1990; 249:505.
30. Ellington AD, Szostak JW. In vitro selection of RNA molecules that bind specific ligands. *Nature* 1990; 346:818.
31. Vickers T, Baker BF, Cook PD, et al. Inhibition of HIV-LTR gene expression by oligonucleotides targeted to the TAR element. *Nucleic Acids Res* 1991; 19:3359.
32. Wickstrom E. Oligodeoxynucleotide stability in subcellular extracts and culture media. *J Biochem Biophys Methods* 1986; 13:97.
33. Tidd DM, Warenieus HM. Partial protection on oncogene, antisense oligodeoxynucleotides against serum nuclease degradation using terminal methylphosphonate groups. *Br J Cancer* 1989; 60:343.
34. Dagle JM, Walder JA, Weeks DL. Targeted degradation of mRNA in Xenopus oncytes and embryos directed by modified oligonucleotides: studies of An2 and cylin in embryogenesis. *Nucleic Acids Res* 1990; 18:4751.
35. Dagle JM, Weeks DL, Walder JA. Pathways of degradation and mechanism of action of antisense oligonucleotides in Xenopus laevis embryos. *Antisense Res Dev* 1991; 1:11.
36. Crooke RM. *In vitro* toxicology and pharmacokinetics of antisense oligonucleotides. *Anti-Cancer Drug Design* 1991; 6:609.
37. Hoke GD, Draper K, Freier SM, et al. Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection. *Nucleic Acids Res* 1991; 19:5743.
38. Asseline U, Delarue M, Lancelot G, et al. Nucleic acid-binding molecules with high affinity and base sequence specificity: intercalating agents covalently linked to oligonucleotides. *Proc Natl Acad Sci USA* 1984; 81:3297.
39. Cazenave C, Stein CA, Loreau N, et al. Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides. *Nucleic Acids Res* 1989; 17:4255.
40. Letsinger RL, Zhang G, Sun DK. Cholesteryl-conjugated oligonucleotides: synthesis, properties and activity as inhibitors of replication of human immunodeficiency virus in cell culture. *Proc Natl Acad Sci USA* 1989; 86:6553.
41. Lamaitre M, Bayard B, Lebleu B. Specific antiviral activity of a poly (L-lysine)-conjugated oligodeoxyribonucleotide sequence complementary to vesicular stomatitis virus N protein mRNA initiation site. *Proc Natl Acad Sci USA* 1987; 84:648.
42. Leonetti JP, Rayner B, Lemaitre M, et al. Antiviral activity of conjugates between poly(L-lysine) and synthetic oligodeoxyribonucleotides. *Gene* 1988; 72:323.

43. Crooke ST, Lebleu B, eds. *Antisense Research and Applications.* Boca Raton, FL: CRC, 1993.
44. Crooke ST. Oligonucleotide Therapeutics. In: Wolff ME, ed. *Burger's Medicinal Chemistry and Drug Discovery,* vol. 1, 5th ed. New York: Wiley Interscience. 1995:863.
45. Crooke ST. Therapeutic applications of oligonucleotides. *Annu Rev Pharmacol Toxicol* 1992; 32:329.
46. Mizumoto K, Kaziro Y. Messenger RNA capping enzymes from eukaryotic cells. *Prog Nucleic Acids Res Mol Biol* 1987; 34:1.
47. Ross J. Messenger RNA turnover in eukaryotic cells. *Mol Biol Med* 1988; 5:1.
48. Friedman DI, Imperiale MJ. RNA 3′ end formation in the control of gene expression. *Annu Rev Genet* 1987; 21:453.
49. Manley JL. Polyadenyation of mRNA precursors. *Biochim Biophys Acta* 1988; 950:1.
50. Padgett RA, Grabowski PJ, Konarska MM, et al. Splicing of messenger RNA precursors. *Annu Rev Biochem* 1986; 55:1119.
51. Green MR. Pre-mRNA splicing. *Annu Rev Genet* 1986; 20:671.
52. Breslauer KJ, Frank R, Blocker H. Predicting DNA duplex stability from base sequence. *Proc Natl Acad Sci USA* 1986; 83:3746.
53. Freier SM, Kierzek R, Jaeger J, et al. Improved free-energy parameters for predictions of RNA duplex stability. *Proc Natl Acad Sci USA* 1986; 83:9373.
54. Chastain M, Tinoco I. RNA structure as related to antisense drugs. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications.* Boca Raton, FL: CRC. 1993:55.
55. Ecker DJ. Strategies for invasion of RNA secondary structure. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications.* Boca Raton, FL: CRC. 1993:387.
56. Wyatt JR, Vickers TA, Roberson JL, et al. Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelop-mediated cell fusion. *Proc Natl Acad Sci USA* 1994; 91:1356.
57. Wang KY, McCurdy S, Shea RG, et al. A DNA aptamer which binds to and inhibits thrombin exhibits a new structural motif for DNA. *Biochemistry* 1993; 32:1899.
58. Leroy J-L, Gehring K, Kettani A, et al. Acid multimers of oligodeoxycytidine strands: stoichiometry, base-pair characterization and proton exchange properties. *Biochemistry* 1993; 32: 6019.
59. Gehring K, Ley J-L, Gueron M. A tetrameric DNA structure with protonated cytosine-cytosine base pairs. *Nature* 1993; 363:561.
60. Freier SM, Lima WF, Sanghvi YS, et al. Thermodynamics of antisense oligonucleotide hybridization. In: Izant J, Erickson R, eds. *Gene Regulation by Antisense Nucleic Acids.* New York: Raven. 1992:95.
61. Thein SL, Wallace RB. The use of synthetic oligonucleotides as specific hybridization probes in the diagnosis of genetic disorders. In: Davies KE, ed. *Human Genetic Diseases: A Practical Approach.* Oxford: IRL. 1986:33.
62. Helene C, Toulme JJ. Control of gene expression by oligonucleotides covalently linked to intercalating agents and nucleic acid-cleaving reagents. In: Cohen JS, ed. *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression.* Boca Raton, FL: CRC. 1989:137.
63. Helene C, Toulme JJ. Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochim Biophys Acta* 1990; 1049:99.
64. Monia BP, Johnston JF, Ecker DJ, et al. Selective inhibition of mutant Ha-ras mRNA expression by antisense oligonucleotides. *J Biol Chem* 1992; 267:19,954.
65. Crooke ST. Progress in antisense therapeutics. *Hematol Pathol* 1995; 9:59.
66. McManaway ME, Neckers LM, Loke SL, et al. Tumour-specific inhibition of lymphoma growth by an antisense oligodeoxynucleotide. *Lancet* 1990; 335:808.
67. Kulka M, Smith C, Aurelian L, et al. Site specificity of the inhibitory effects of oligo(nucleoside methylphosphonates) complementary to the acceptor splice junction of herpes simplex virus type 1 immediately early mRNA. *Proc Natl Acad Sci USA* 1989; 86:6868.
68. Zamecnik PC, Goodchild J, Taguchi Y, et al. Inhibition of replication and expression of human T-cell lymphotropic virus type III in cultured cells by exogenous synthetic oligonucleotides complementary to viral RNA. *Proc Natl Acad Sci USA* 1986; 83:4143.
69. Smith CC, Aurelian L, Reddy MP, et al. Antiviral effect of an oligo(nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type 1 immediate early pre-mRNAs 4 and 5. *Proc Natl Acad Sci USA* 1985; 83:2787.

70. Dominski Z, Kole R. Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proc Natl Acad Sci USA* 1993; 90:8673.
71. Agrawal S, Goodchild J, Civeira MP, et al. Oligodeoxynucleoside phosphoramidites and phosphorothioates as inhibitors of human immunodeficiency virus. *Proc Natl Acad Sci USA* 1988; 85:7079.
72. Lemaitre M, Bayard B, Lebleu B. Specific antiviral activity of a poly(L-lysine)-conjugated oligodeoxyribonucleotide sequence complementary to vesicular stomatitis virus N protein mRNA initiation site. *Biochemistry* 1987; 84:648.
73. Rosolen A, Whitesell L, Olegalo M, et al. Antisense inhibition of single copy N-myc expression results in decreased cell growth without reduction of c-myc protein in a neuroepithelioma cell line. *Cancer Res* 1990; 50:6316.
74. Vasanthakumar G, Ahmed NK. Modulation of drug resistance in a daunorubicin resistant subline with oligonucleotide methylphosphonates. *Cancer Commun* 1989; 1:225.
75. Sburlati AR, Manrow RE, Berger SL. Prothymosin α antisense oligomers inhibit myeloma cell division. *Proc Natl Acad Sci USA* 1991; 88:253.
76. Zheng H, Sahai BM, Kilgannon P, et al. Specific inhibition of cell-surface T-cell receptor expression by antisense oligodeoxynucleotides and its effect on the production of an antigen-specific regulatory T-cell factor. *Proc Natl Acad Sci USA* 1989; 86:3758.
77. Maier JAM, Voulalas P, Roeder D, et al. Extension of the life-span of human endothelial cells by an interleukin-1α antisense oligomer. *Science* 1990; 249:1570.
78. Mirabelli CK, Bennett CF, Anderson K, et al. *In vitro* and *in vivo* pharmacologic activities of antisense oligonucleotides. *Anti-Cancer Drug Design* 1991; 6:647.
79. Cowsert LM, Fox MC, Zon G, et al. In vitro evaluation of phosphorothioate oligonucleotides targeted to the E2 mRNA of papillomavirus: Potential treatment of genital warts. *Antimicrob Agents and Chemotherapy* 1993; 37:171.
80. Ecker DJ, Vickers TA, Bruice TW, et al. Pseudo-half knot formation with RNA. *Science* 1992; 257:958.
81. Saxena SK, Ackerman EJ. Microinjected oligonucleotides complementary to the α-sarcin loop of 28 S RNA abolish protein synthesis in xenopus oocytes. *J Biol Chem* 1990; 265:3263.
82. Walker K, Elela SA, Nazar RN. Inhibition of protein synthesis by anti-5.8 S rNA oligodeoxyribonucleotides. *J Biol Chem* 1990; 265:2428.
83. Westerman P, Gross B, Hoinkis G. Inhibition of expression of SV40 virus large T-antigen by antisense oligodeoxyribonucleotides. *Biomed Biochim Acta* 1989; 48:85.
84. Baker B. Decapitation of a 5′ capped oligoribonucleotide by ortho-Phenanthroline:Cu(II). *J Am Chem Soc* 1993; 115:3378.
85. Baker BF, Miraglia L, Hagedorn CH. Modulation of eurkaryotic initiation factor-4E binding to 5′ capped oligoribonucleotides by modified antisense oligonucleotides. *J Biol Chem* 1992; 267:11,495.
86. Chiang MY, Chan H, Zounes MA, et al. Antisense oligonucleotides inhibit ICAM-1 expression by two distinct mechanisms. *J Biol Chem* 1991; 266:18,162.
87. Crouch RJ, Dirksen M-L. Ribonucleases H. In: Linn SM, Roberts RJ, eds. *Nucleases.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1985:211.
88. Crum C, Johnson JD, Nelson A, et al. Complementary oligodeoxynucleotide mediated inhibition of tobacco mosaic virus RNA translation *in vitro*. *Nucleic Acids Res* 1988; 16:4569.
89. Haeuptle MT, Frank R, Dobberstein B. Translation arrest by oligodeoxynucleotides complementary to mRNA coding sequences yields polypeptides of predetermined length. *Nucleic Acids Res* 1986; 14:1427.
90. Doris-Keller H. Site specific enzymatic cleavage of RNA. *Nucleic Acid Res* 1979; 7:179.
91. Kawasaki AM, Casper MD, Freier SM, et al. Uniformly modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides as nuclease resistant antisense compounds with high affinity and specifity for RNA targets. *J Med Chem* 1993; 36:831.
92. Sproat BS, Lamond AL, Beijer B, et al. Highly efficient chemical synthesis of 2′-*O*-methyloligoribonucleotides and tetrabiotinylated derivatives; novel probes that are resistant to degradation by RNA or DNA specific nucleases. *Nucleic Acids Res* 1989; 17:3373.
93. Morvan F, Rayner B, Imbach J-L. α-Oligonucleotides: A unique class of modified chimeric nucleic acids. *Anti-Cancer Drug Design* 1991; 6:521.

94. Gagnor C, Rayner B, Leonetti JP, et al. α-DNA IX. Parallel annealing of α-anomeric oligodoxyribonucleotides to natural mRNA is required for interference in RNaseH mediated hydrolysis and reverse transcription. *Nucleic Acids Res* 1989; 17:5107.
95. Maher JL, III, Wold B, Dervan PG. Inhibition of DNA binding properties by oligonucleotide-directed triple helix formation. *Science* 1989; 245:725.
96. Miller PS. Non-ionic antisense oligonucleotides. In: Cohen JS, ed. *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*. Boca Raton, FL: CRC. 1989:79.
97. Stein CA, Cohen JS. Phosphorothiote oligodeoxynucleotide analogues. In: Cohen JS, ed. *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*. Boca Raton, FL: CRC. 1989:97.
98. Cazenave C, Stein CA, Loreau N, et al. Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides. *Nucleic Acids Res* 1989; 17:4255.
99. Quartin R, Brakel C, Wetmur J. Number and distribution of methylphosphonate linkages in oligodeoxynucleotides affect exo- and endonuclease sensitivity and ability to form RNase H substrates. *Nucleic Acids Res* 1989; 17:7253.
100. Furdon P, Dominski Z, Kole R. RNase H cleavage of RNA hybridized to oligonucleotides containing methylphosphonate, phosphorothioate and phosphodiester bonds. *Nucleic Acids Res* 1989; 17:9193.
101. Eder PS, Walder JA. Ribonuclease H from K562 human erythroleukemia cells. *J Biol Chem* 1991; 206:6472.
102. Monia BP, Lesnik EA, Gonzalez C, et al. Evaluation of 2′ modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem* 1993:268.
103. Giles RV, Tidd DM. Increased specificity for antisense oligodeoxynucleotide targeting of RNA cleavage of RNase H using chimeric methylphosphonodiester structures. *Nucleic Acids Res* 1992; 20:763.
104. Walder RY, Walder JA. Role of RHase H in hybrid-arrested translation by antisense oligonucleotides. *Proc Natl Acad Sci USA* 1988; 85:5011.
105. Minshull J, Hunt T. The use of single-stranded DNA and RNase H to promote quantitative hybrid arrest of translation of mRNA/DNA hybrids in reticulocyte lysate cell-free translations. *Nucleic Acids Res* 1986; 14:6433.
106. Gagnor C, Bertrand J, Thenet S, et al. Alpha-DNA VI: Comparative study of alpha- and beta-anomeric oligodeoxyribonucleotides in hybridization to mRNA and in cell free translation inhibition. *Nucleic Acids Res* 1987; 15:10419.
107. Knorre DG, Vlassov VV, Zarytova VF. Reactive oligonucleotide derivatives and sequence-specific modification of nucleic acids. *Biochimie* 1985; 67:785.
108. Knoore DG, Vlassov VV, Zarytova VF. Oligonucleotides linked to reactive groups. In: Cohen JS, ed. *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*. Boca Raton, FL: CRC. 1989:173.
109. Vlassov VV, Zarytova VF, Kutyavin IV, et al. Sequence-specific chemical modification of a hybrid bacteriophage M13 single-stranded DNA by alkylating oligonucleotide derivatives. *FEBS Lett* 1988; 231:352.
110. Summerton J, Bartlett, PA. Sequence-specific crosslinking agents for nucleic acids. Use of 6-bromo-5,5-dimethoxyhexanohydrazide for crosslinking cytidine to guanosine and crosslinking RNA to complementary sequences of DNA. *J Mol Biol* 1978; 122:145.
111. Webb TR, Matteucci MD. Hybridization triggered crosslinking of leoxynucleotides. *Nucleic Acids Res* 1986; 14:7661.
112. Cooney M, Czernuszewicz G, Postel EH, et al. Site-specific oligonucleotide binding represses transcription of the human c-myc gene in vitro. *Science* 1988; 241:456.
113. Moser HE, Dervan PB. Sequence specific cleavage of double helical DNA by triple helix formation. *Science* 1987; 238:650.
114. Le Doan T, Perrouault L, Praseuth D, et al. Sequence-specific recognition, photocrosslinking and cleavage of the DNA double helix by an oligo-[alpha]-thymidylate covalently linked to an azidoproflavine derivative. *Nucleic Acids Res* 1987; 15:7749.
115. Federova OS, Knorre DG, Podust LM, et al. Complementary addressed modification of double-stranded DNA within a ternary complex. *FEBS Lett* 1988; 228:273.
116. Praseuth D, Perrouault L, Le Doan T, et al. Sequence-specific binding and photocrosslinking

of [alpha] and [beta]-oligodeoxynucleotides to the major groove of DNA via triple helix formation. *Proc Natl Acad Sci USA* 1988; 85:1349.

117. Praseuth D, Doan TL, Chassignol M, et al. Sequence-targeted photosensitized reactions in nucleic acids by oligo-α-deoxynucleotides and oligo-β-deoxynucleotides covalently linked to proflavin. *Biochemistry* 1988; 27:3031.
118. Le Doan T, Perrouault L, Chassignol M, et al. Sequence-targeted chemical modifications of nucleic acids by complementary oligonucleotides covalently linked to porphyrins. *Nucleic Acids Res* 1987; 15:8643.
119. Le Doan T, Perrouault L, Thuong NT, et al. Sequence-specific chemical and photochemical reactions on nucleic acids by oligonucleotides linked to porphyrins. *J Inorg Biochem* 1989; 36:274 (abstract).
120. Le Doan T, Praseuth D, Perrouault L, et al. Sequence-targeted photochemical modifications of nucleic acids by complementary oligonucleotides covalently linked to porphyrins. *Bioconj Chem* 1990; 1:108.
121. Lee BL, Blake KR, Miller PS. Interaction of psoralen-derivatized oligodeoxyribonucleoside methylphosphonates with synthetic DNA containing a promoter for T7 RNA polymerase. *Nucleic Acids Res* 1988; 16:10,681.
122. Lee BL, Murakami A, Blake KR, et al. Interaction of psoralen-derivatized oligodeoxyribonucleoside methylphosphonates with single-stranded DNA. *Biochemisty* 1988; 27:3197.
123. Praseuth D, Chassignol M, Takasugi M, et al. Double helices with parallel strands are formed by nuclease-resistant oligo-[alpha]-deoxynucleotides and oligo-[alpha]-deoxynucleotides covalently linked to an intercalating agent with complementary oligo-[beta]-deoxynucleotides. *J Mol Biol* 1987; 196:939.
124. Perrouault L, Asseline U, Rivalle C, et al. Sequence-specific artificial photo-induced endonucleases based on triple-helix forming oligonucleotides. *Nature* 1990; 344:358.
125. Helene C. Artificial control of gene expression by oligonucleotides covalently linked to intercalating agents. *Br J Cancer* 1989; 60:157.
126. Kean JM, Murakami A, Blake KR, et al. Photochemical cross-linking of psoralen-derivatized oligonucleotide methylphosphonates to rabbit globin messenger RNA. *Biochemistry* 1988; 27:9113.
127. Saison-Behmoaras T, Tocque B, Rey I, et al. Short modified oligonucleotides directed against Ha-ras point mutation induce selective cleavage of the mRNA and inhibit T24 cells proliferation. *Embo J* 1991; 10:1111.
128. Dervan PB. Oligonucleotide recognition of double-helical DNA by triple-helix formation. In: Cohen JS, ed. *Oligodeoxynucleotide: Antisense Inhibitors of Gene Expression*. Boca Raton, FL: CRC. 1989:197.
129. Chu BCF, Orgel LE. Nonenzymatic sequence-specific cleavage of single-stranded DNA. *Proc Natl Acad Sci USA* 1985; 82:963.
130. Boutorin AS, Vlassov VV, Kazakov SA, et al. Complementary addressed reagents carrying EDTA-Fe(II) groups for directed cleavage of single-stranded nucleic acids. *FEBS Lett* 1984; 172:43.
131. Francois JC, Saison-Behmoaras T, Chassignol M, et al. Periodic cleavage of poly(dA) by oligothymidylates covalently linked to ^{1,10}-phenanthroline-copper complexes. *Biochemistry* 1988; 27:2272.
132. Francois JC, Saison-Behmoaras T, Barbier C, et al. Sequence-specific recognition and cleavage of duplex DNA via triple helix formation by oligonucleotides covalently linked to a phenanthroline-copper chelate. *Proc Natl Acad Sci USA* 1989; 86:9702.
133. Francois JC, Saison-Behmoaras T, Chassignol M, et al. Sequence-targeted cleavage of single- and double-stranded DNA by oligothymidylates covalently linked to ^{1,10}-phenanthroline. *J Biol Chem* 1989; 264:5891.
134. Sun JS, Francois JC, Lavery R, et al. Sequence-targeted cleavage of nucleic acids by oligo-[α]-thymidylate-phenanthroline conjugates: parallel and anti-parallel double helices are formed with DNA and RNA, respectively. *Biochemistry* 1988; 27:6039.
135. Chen C.-HB, Sigman DS. Nuclease activity of 1,10-phenanthroline-copper: sequence-specific targeting. *Proc Natl Acad Sci USA* 1986; 83:7147.

136. Helene C, Le Doan T, Thuong NT. Sequence-targeted photochemical reactions in single-stranded and double-stranded nucleic acids by oligonucleotide-photosensitizer conjugated. In: Nielson PE, ed. *Photochemical Probes in Biochemistry*. Kluwer Academic Publishers. 1989:219.
137. Helene C, Thuong NT. Oligodeoxynucleotides covalently linked to intercalating agents and to nucleic acid-cleaving reagents. New families of gene regulatory substances. In: Chagas C, Pullman B, eds. *Working Group on Molecular Mechanisms of Carcinogenic and Antitumor Activity*. Vatican City: Pontificaiae Academiae Scientarium Scripta Varia. 1987:205.
138. Helene C, Thuong NT. Control of gene expression by oligonucleotides covalently linked to intercalating agents. *Genome* 1989; 31:413.
139. Cech TR. The chemistry of self-splicing RNA and RNA enzymes. *Science* 1987; 236:1532.
140. McSwiggen JA, Cech TR. Stereochemistry of RNA cleavage by the *tetrahymena* ribozyme and evidence that the chemical step is not rate-limiting. *Science* 1989; 244:679.
141. Uhlenbeck OC. Using ribozymes to cleave RNAs. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC. 1993:83.
142. Haseloff J, Gerlach WL. Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature* 1988; 334:585.
143. McCall MJ, Hendry P, Jennings PA. Minimal sequence requirements for ribozyme activity. *Proc Natl Acad Sci* 1992; 89:5710.
144. Chowrira BM, Berzal-Harranz A, Keller CF, et al. Four ribose 2′-hydroxyl groups essential for catalytic function of the hairpin ribozyme. *J Biol Chem* 1993; 268:19,458.
145. Perrouault J-P, Wu T, Cousineau B, et al. Mixed deoxyrib- and ribo-oligonucleotides with catalytic activity. *Nature* 1990; 344:565.
146. Koizumi M, Ohtsuka E. Effects of phosphorothioate and 2-amino groups in hammerhead ribozymes on cleavage rates and Mg^{2+} binding. *Biochemistry* 1991; 30:5145.
147. Heidenreich O, Benseler F, Fahrenholz A, et al. High activity and stability of hammerhead ribozymes containing 2′-modified pyrimidine nucleosides and phosphorothioates. *J Biol Chem* 1993; 269:2131.
148. Pieken WA, Olsen DB, Benseler F, et al. Kinetic characterization of ribonuclease-resistant 2′-modified hammerhead ribozymes. *Science* 1991; 253:314.
149. Olsen DB, Benseler F, Aurup H, et al. Study of a hammerhead ribozyme containing 2′-modified adenosine residues. *Biochem* 1991; 30:9735.
150. Goodchild J. Enhancement of ribozyme catalytic activity by a contiguous oligodeoxynucleotide (facilitator) and by 2′-*O*-methylation. *Nucleic Acids Res* 1992; 20:4607.
151. Forester AC, Altman S. External guide sequences for an RNA enzyme. *Science* 1990; 249:783.
152. Yuan Y, Hwang E-S, Altman S. Targeted cleavage of mRNA by human RNase P. *Proc Natl Acad Sci* 1992; 89:8006.
153. Herschlag D, Cech TR. DNA cleavage catalyzed by the ribozyme from *Tetrahymena*. *Nature* 1990; 344:405.
154. Wilhelm JE, Vale RD. RNA on the move: The mRNA localization pathway. *J Cell Biol* 1993; 123:269.
155. Kislauskis EH, Li Z, Singer RH, et al. Isoform-specific 3′-untranslated sequences sort α-cardiac and β-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *J Cell Biol* 1993; 123:165.
156. Crooke ST. Progress toward oligonucleotide therapeutics: Pharmacodynamic properties. *FASEB J* 1993; 7:533.
157. Bennett CF, Crooke ST. Oligonucleotide-based inhibitors of cytokine expression and function. In: Henderson B, Bodmer M, eds. *Therapeutic Modulation of Cytokines*. Boca Raton, FL: CRC. 1995; in press.
158. Bennett CF, Condon TP, Grimm S, et al. Inhibition of endothelial cell adhesion molecule expression with antisense oligonucleotides. *J Immunol* 1994; 152:3530.
159. Cook PD. Medicinal chemistry strategies for antisense research. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. 1993:149.
160. Lima WF, Monia BP, Ecker DJ, et al. Implication of RNA structure on antisense oligonucleotide hybridization kinetics. *Biochemistry* 1992; 31:12,055.
161. Cohen JS. Phosphorothioate oligodeoxynucleotides. In: Crooke ST, Lebleu B, eds. *Antisense*

Research and Applications. Boca Raton, FL: CRC. 1993:205.
162. Freier SM. Hybridization: Considerations affecting antisense drugs. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC. 1993:67.
163. Joos RW, Hall WH. Determination of binding constants of serum albumin for penicillin. *J Pharmacol Exp Ther* 1969; 166:113.
164. Loke SL, Stein CA, Zhang XH, et al. Characterization of oligonucleotide transport into living cells. *Proc Natl Acad Sci* 1989; 86:3474.
165. Graham MJ, Cummins L, Cooke ME, et al. *In vitro* metabolism of phosphorothioate antisense oligonucleotides. 1994, submitted.
166. Gao W-Y, Han F-S, Storm C, et al. Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: Implications for antisense technology. *Mol Pharm* 1991; 41:223.
167. Stein CA, Cheng Y-C. Antisense oligonucleotides as therapeutic agents—Is the bullet really magic? *Science* 1993; 261:1004.
168. Agrawal S, Temsamani J, Tang JY. Pharmacokinetics, biodistribution and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc Natl Acad Sci* 1991; 88:7595.
169. Majumdar C, Stein CA, Cohen JS, et al. Stepwise mechanism of HIV reverse transcriptase: primer function of phosphorothioate oligodeoxynucleotide. *Biochem* 1989; 28:1340.
170. Cheng Y-C, Gao W, Han F. Against human immunodeficiency virus and herpes viruses. *Nucleosides & Nucleotides* 1991; 10:155.
171. Stein CA, Neckers LM, Nair BC, et al. Phosphorothioate oligodeoxycytidine interferes with binding of HIV-1 with gp120 to CD4. *J AIDS* 1991; 4:686.
172. Fields AP, Bednarik DP, Hess A, et al. Human immunodeficiency virus induces phosphorylation of its cell surface receptor. *Nature* 1991; 333:278.
173. Wyatt JR, Vickers TA, Roberson JL, et al. Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelop-mediated cell fusion. *Proc Natl Acad Sci* 1994; 91:1356.
174. Campbell JM, Bacon TA, Wickstrom E. Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid. *J Biochem Biophys Methods* 1990; 20:259.
175. Cossum PA, Sasmor H, Dellinger D, et al. Disposition of the ^{14}C-labeled phosphorothioate oligonucleotide ISIS 2105 after intravenous administration to rats. *J Pharmacol Exp Ther* 1993; 267:1181.
176. Crooke RM. *In vitro* toxicology and pharmacokinetics of antisense oligonucleotides. *Anti-Cancer Drug Design* 1991; 6:609.
177. Crooke RM. *In vitro* and *in vivo* toxicology of first generation analogs. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC. 1993:471.
178. Crooke RM, Graham MJ, Cooke ME, et al. *In vitro* pharmacokinetic analysis of tritiated ISIS 2105 and other phosphorothioate antisense oligonucleotides. Submitted, 1994.
179. Nestle FO, Mitra RS, Bennett CF, et al. Cationic lipid is not required for uptake and selective inhibitory activity of ICAM-1 phosphorothioate antisense oligonucleotides in keratinocytes. *J Invest Dermatol* 1994; 103:569.
180. Neckers LM. Cellular internalization of oligodeoxynucleotides. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC. 1993:451–460.
181. Chrissey LA, Walz SE, Pazirandeh M, et al. Internalizing of oligodeoxyribonucleotides by *Vibrio parahaemolyticus*. *Antisense Res Dev* 1993; 3:367.
182. Crooke RM. Cellular uptake, distribution and metabolism of phosphorothiote, phosphorodiester and methylphosphonate oligonucleotides. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications*. Boca Raton, FL: CRC. 1993:427.
183. Bennett CF, Chiang M-Y, Chan H, et al. Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol Pharm* 1992; 41:1023.
184. Bennett CF, Chiang M-Y, Chan H, et al. Use of cationic lipids to enhance the biological activity of antisense oligonucleotides. *J Liposome Res* 1993; 3:85.
185. Iversen P. *In vivo* studies with phosphorothioate oligonucleotides: pharmacokinetic prologue. *Anti-Cancer Drug Design* 1991; 6:531.

186. Cossum PA, Truong L, Owens SR, et al. Pharmacokinetics of a ^{14}C labeled phosphorothioate oligonucleotides, ISIS 2105, after intradermal administration to rats. *J Pharmacol Exp Ther* 1994; 269:89.
187. Crooke ST, Grillone LR, Tendolkar A, et al. A pharmacokinetic evaluation of ^{14}C labeled afovirsen sodium in genital wart patients. *Clin Pharm Ther* 1994; 56:641-646.
188. Sands H, Gorey-Feret LJ, Cocuzza AJ, et al. Biodistribution and metabolism of internally ^{3}H-labeled oligonucleotides. I. Comparison of a phosphodiester and a phosphorothioate. *Mol Pharm* 1994; 45:932.
189. Bayever E, Iversen PL, Bishop MR, et al. Systemic administration of a phosphorothioate oligonucleotide with a sequence complementary to p53 for acute myelogenous leukemia and myelodysplastic syndrome: initial results of a phase 1 trial. *Antisense Res Dev* 1993; 3:383.
190. Cook PD. Medicinal chemistry strategies for antisense research. In: Crooke ST, Lebleu B, eds. *Antisense Research and Applications.* Boca Raton, FL: CRC. 1993:303.
191. Azad RF, Driver VB, Tanaka K, et al. Antiviral activity of a phosphorothioate oligonucleotide complementary to RNA of the human cytomegalovirus major immediate-early region. *Antimicrob Agents and Chemother* 1993; 37:1945.
192. Wagner RW, Matteucci MD, Lewis JG, et al. Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science* 1993; 260:1510.
193. Nagel KM, Holstad SG, Isenberg KE. Oligonucleotide pharmacotherapy. An antigene stratety. *Pharmacotherapy* 1993; 13:177.
194. Simons M, Edelman ER, DeKeyser J-L, et al. Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation *in vivo. Nature* 1992; 359:67.
195. Abe J, Zhou W, Taguchi J, et al. Suppression of neointimal smooth muscle cell accumulation *in vivo* by antisense CDC2 and CDK2 oligonucleotides in rat carotid artery. *Biochem Biophys Res Commun* 1994; 198:16.
196. Whitesell L, Rosolen A, Neckers LM. *In vivo* modulation of N-myc expression by continuous perfusion with an antisense oligonucleotide. *Antisense Res Dev* 1991; 1:343.
197. Perkaly L, Saijo Y, Busch RK, et al. Growth inhibition of human tumor cell lines by antisense oligonucleotides designed to inhibit p120 expression. *Anti-Cancer Drug Design* 1993; 8:3.
198. Wahlestedt C, Pich EM, Koob GF, et al. Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides. *Science* 1993; 259:528.
199. Wahlestedt C, Golanov E, Yamamotop S, et al. Antisense oligodeoxynucleotides to NMDA-R1 receptor channel protect cortical neurons from excitotoxicity and reduce focal ischaemic infarctions. *Nature* 1993; 363:260.
200. Osen-Sand A, Catsicas M, Staple JK, et al. Inhibition of axonal growth by SNAP-25 antisense oligonucleotides *in vitro* and *in vivo. Nature* 1993; 364:445.
201. Burch RM, Mahan LC. Oligonucleotides antisense to the interleukin 1 receptor mRNA block the effects of interleukin 1 in cultured murine and human fibroblasts and in mice. *J Clin Invest* 1991; 88:1190.
202. Kitajima I, Shinohara T, Bilakovics J, et al. Ablation of transplanted HTLV-1 tax-transformed tumors in mice by antisense inhibition of NF-kB. *Science* 1992; 258:1792.
203. Higgins KA, Perez JR, Coleman TA, et al. Antisense inhibition of the p65 subunit of NF-kB blocks tumorigenicity and causes tumor regression. *Proc Natl Acad Sci* 1993; 90:9901.
204. Skorski T, Nieborowska-Skorska M, Nicolaides NC, et al. Suppression of Ph^{1} leukemia cell growth in mice by BCR-ABL antisense oligodeoxynucleotide. *Proc Natl Acad Sci* 1994; 91:4504.
205. Hijiya N, Zhang J, Ratajczak MZ, et al. Biological and therapeutic significance of MYB expression in human melanoma. *Proc Natl Acad Sci* 1994; 91:4499.
206. Dean NM, McKay R. Inhibition of PKC-α expression in mice after systemic administration of phosphorothioate antisense oligonucleotides. *Proc Natl Acad Sci* 1994; 91:11,762-11,766.
207. Guadette MF, Hampikian G, Metelev V, et al. Effect on embryos of injection of phosphorothioate-modified oligonucleotides into pregnant mice. *Antisense Res Dev* 1993; 3:391.
208. Branda RF, Moore AL, Mathews L, et al. Immune stimulation by an antisense oligomer complementary to the *rev* gene of HIV-1. *Biochem Pharmacol* 1993; 45:2037.
209. McIntyre KW, Lombard-Gilooly K, Perez JR, et al. A sense phosphorothioate oligonucleotide directed to the initiation codon of transcription factor NF-kB p65 causes sequence-specific

immune stimulation. *Antisense Res Dev* 1993; 3:309.
210. Cornish KG, Iversen P, Smith L, et al. Cardiovascular effects of a phosphorothioate oligonucleotide with sequence antisense to p53 in the conscious rhesus monkey. *Pharmacol Commun* 1993; 3:239.
211. Crooke ST. Advances in oligonucleotide therapeutics. *1994 Experimental Biology Meeting, American Society for Pharmacology and Experimental Therapeutics,* Anaheim, CA, April 24–28, 1994. Abstract.
212. Palestine AG, Cantrill HL, Luckie AP, et al. Intravitreal treatment of CMV retinitis with an antisense oligonucleotide, ISIS 2922. *Tenth International Conference on AIDS.* Yokohama, Japan, August 7–12, 1994. Abstract.
213. Stepkowski SM, Tu Y, Condon TP, et al. Blocking of heart allograft rejection by intercellular adhesion molecule-1 antisense oligonucleotides alone or in combination with other immunosuppressive modalities. *J Immunol* 1994; 153:5336.

16 Growth Factors and Growth Factor Inhibitors

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CONTENTS

1. INTRODUCTION

This chapter will outline novel treatment strategies predicated on the modulation of growth factor or cytokine-mediated signal transduction pathways. Several excellent recent monographs have addressed this tissue from the point of view of inhibitors of individual pathway constituents (*1,2*). The current focus will be on defining the pathways potentially relevant to the common neoplasms, followed by a consideration of approaches to modulating processes common to the action of various pathways, with mention of individual agents as they exemplify these strategies. In addition, opportunities for interdigitation of "growth-factor directed" and "traditional" therapeutic agents will be considered.

One view of neoplasia (*3*) is that it is fundamentally a disorder of cellular communication induced by somatic mutations of growth regulatory genes. An extension of this hypothesis is the "autocrine" promotion of neoplasia, first put forth in response to the realization that a prominent aspect of the transformed phenotype in tissue culture or other artificial systems is a reduction in the requirement for exogenous growth factors usually supplied in the form of serum (*4*). Thus, as illustrated in Fig. 1, tumor cells could be viewed as secreting their own "autocrine" factors, or responding to

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

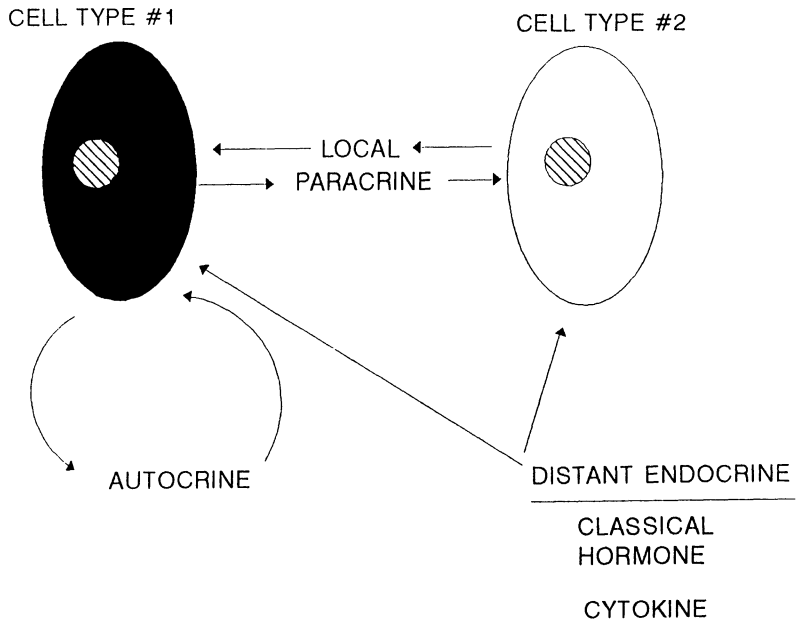

Fig. 1. Growth factors act in autocrine, paracrine, or endocrine styles.

"paracrine" factors secreted by adjacent tumor cells or stroma. The pathways activated by these factors would act in parallel or in addition to the traditional endocrine influences, although with the elucidation of the structures of multiple cytokines, it is clear that the concept of endocrine control of tumor cell growth may have to be altered to include cytokines.

A corollary of this thinking is that manipulation of growth-factor-directed pathways may offer inroads into the therapeusis of neoplasia that actually manipulate neoplastic physiology, rather than "simply" seeking cytotoxicity. From a strategic perspective, an enhanced therapeutic index and more specific targeting of the tumor are the hoped-for consequences, although the tools to begin this endeavor in a clinical sense are only now emerging.

2. SIGNAL TRANSDUCTION PATHWAYS AND MECHANISMS

2.1. Tyrosine Kinase-Linked Signaling

Soon after the elucidation of the structure of Rous sarcoma virus (RSV) genome, it became apparent that neoplastic transformation was caused by a protein kinase activity directed at tyrosine residues detectable in immunoprecipitates using serum from tumor-bearing animals or antibodies raised against the putative v-*src* transforming protein (*5*). Although phosphotyrosine was a relatively minor phosphoamino acid in normal cells, it was possible to demonstrate that RSV-transformed cells had increased levels of phosphotyrosine. The transforming oncogenes of numerous acute transforming viruses were found to have a general structure illustrated in Fig. 2. The SH1(src *h*omology) domain refers to the ATP binding region, and two other islands of homology defined the SH2 and SH3 domains represented in the *abl, fps, fgr, yes, lyn, fyn, lck,* and other nonreceptor-linked tyrosine kinases (reviewed in ref. *6*).

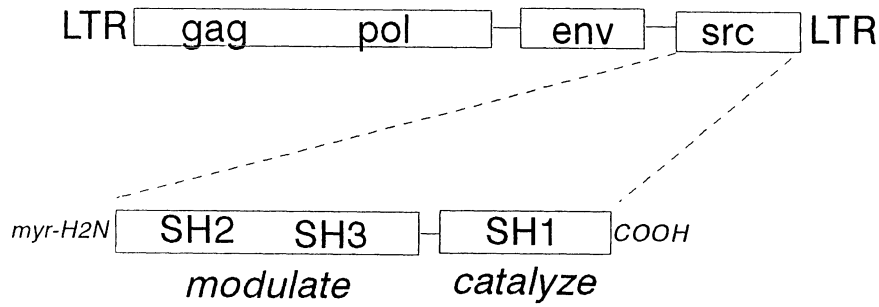

Fig. 2. Overview of non-receptor tyrosine kinase organization. The v-*src* protein arises from the *src* gene present in the RSV genome, downstream of the *gag*, p01, and *env* genes. The SH1 region (*src*-homology) encodes the catalytic domain, and the SH2 and SH3 regions encode regulatory domains.

The importance of these entities to human neoplasia was graphically defined by the demonstration that in certain diseases, pathogenetic alterations of tyrosine kinases could be demonstrated. For example, the Philadelphia chromosome, long known as a cytogenetic marker in chronic myelogenous leukemia (CML) and certain cases of acute lymphoblastic leukemia (ALL), was shown to arise from the translocation of the c-*abl* gene on chromosome 9 into the "breakpoint cluster region" (*bcr*) on chromosome 22 to create a chimeric fusion protein *bcr-abl* with dramatically increased tyrosine kinase activity in comparison to normal c-*abl* (*7–9*). Elevated expression of c-*src* activity was detected in colon carcinomas (*10*), and c-*lck* was aberrantly expressed in certain colon and lung carcinoma cells (*11*). The enzymatic activity of nonreceptor-linked tyrosine kinases can be linked to stimulation of cells by external stimuli. For example, stimulation of T-cells by antigen resulted in increased *lck* activity, and it was possible to demonstrate a physical association between *lck* and the CD4 cell-surface molecule (*12*) that participates in T-cell activation.

Growth factors may be defined as proteins or peptides that stimulate the growth of tissue culture cells in vitro or in organ culture systems. These activities had been defined over the preceding 50 years based primarily on the biologic assay system in which their activity was first manifest (reviewed in *13*). For example, epidermal growth factor (EGF) was first defined as a factor that promoted the development of the neonatal mouse, with potent growth-stimulatory activity for squamous cells. Platelet-derived growth factor (PDGF) was defined as a factor present in platelets that stimulated fibroblast growth. The relation of increased tyrosine kinase activity to growth factor action was strikingly illustrated by the demonstration that the transforming oncogene from the avian erythroblastosis virus consisted of two gene products, one of which, v-*erbB2*, had homology to the EGF-receptor (EGF-R) (*14,15*). A tyrosine kinase activity was clearly encoded by the EGF-R gene, and addition of EGF to cells bearing the EGF-R induced the phosphorylation of numerous intracellular proteins on tyrosine. Subsequent studies revealed this to be a common occurrence after addition of a variety of growth factors and cytokines. Such "growth-factor receptor-linked tyrosine kinases" had common structural features (reviewed in *16*), including an extracellular ligand binding domain, a transmembrane domain, an SH1 domain, and a variety of potential regulatory structural motifs presumed to allow specific signaling. Comparison of sequences of transforming genes from other animal tumors defined the EGF-R as the prototype for a family of growth factor receptors, including c-*erbB2* (*17*). The

clinical importance of the EGF-R family is evident from the frequent elevated expression or amplification of the EGF-R in squamous tumors (*18–20*) or association of adverse prognosis with expression of c-*erbB2* (*21*).

The most immediate consequence of tyrosine kinase action, altered phosphorylation of proteins in growth factor-stimulated cells, was initially difficult to relate to a specific pathway leading to cellular proliferation, since the phosphorylated molecules included molecules as diverse as cytoskeletal elements and "housekeeping proteins" of uncertain relevance to neoplasia. However, genetic evidence indicated that *ras* oncogene function was required for transformation by oncogenes of the *src* family (*22*), and moreover, it was possible to demonstrate that soon after addition of certain growth factors that acted by tyrosine kinase activation, there was increased activity of the c-*raf* oncogene product (*23*), a serine/threonine kinase. Thus, the concept emerged that there are hierarchical arrangements of protein kinase activities in growth factor-stimulated cells, with growth factor-receptor-linked tyrosine kinases "upstream" of "downstream" serine/threonine kinases.

Three independent approaches were undertaken to define the correct relationships in this signaling pathway. The first was directed at understanding how tyrosine kinases activated their substrates and was based ultimately on the demonstration that transforming oncoproteins existed, such as v-*crk*, which clearly increased the tyrosine phosphate content of transformed cells, but which did not contain an SH1 (phosphoryltransfer) domain (*24*). By definition, therefore, such a protein could act only by influencing the action of endogenous kinases. However, v-*crk* did contain an SH2-like domain, raising the possibility that SH2 domains could regulate the phosphorylation of substrates for tyrosine kinases. How this might occur was suggested by the demonstration that polyoma middle T-antigen, a tyrosine kinase, could be coimmunoprecipitated with an enzymatic activity, phosphatidylinositol (PI) 3′-kinase, which could also be detected in PDGF- and EGF-stimulated cells. The structure of PI-3′-kinase revealed that it possessed an SH2 domain (*25,26*). This result suggested that SH2 domains could form a physical association with substrates for tyrosine kinases, and indeed, extensive studies have documented that SH2 domains serve as a molecular "docking mechanism" to cause association of phosphotyrosine-containing proteins, including the autophosphorylated growth factor receptors or autophosphorylated tyrosine kinases with the SH2-domain-bearing proteins. In the case of PI-3′-kinase, this association allows a mechanism whereby its activity may be increased after binding to activated signaling molecules (*27*). An analogous mechanism was demonstrated to operate in the case of PDGF-R and EGF-R activation of phospholipase-C-γ (PLC-γ) (*28–30*). The latter finding provided a direct mechanism by which a tyrosine kinase-linked receptor could activate the serine/threonine protein kinase C (PKC), since the product of the activated PLC-γ, diacylglycerol (DAG), is a known endogenous regulator of PKC. The capacity of SH2 domains to subserve an "adapter" function was most dramatically illustrated by the use of a phosphorylated EGF-R as a probe to define proteins that bound phosphorylated tyrosine. This defined a protein *grb2* (which "grabbed" phosphotyrosine) (*31*) with a unique structure consisting of a single SH2 domain flanked by two SH3 domains and no kinase activity. *Grb2* in turn could be demonstrated to be homologous to proteins defined in invertebrate systems as participating in a tyrosine kinase-regulated developmental pathway (*32*), and to form a complex through its SH3 domain with the mammalian homolog

of the *son of sevenless* (*Sos*) protein (*33,34*), which was known to regulate *ras* function by causing *ras* proteins to exchange GDP for GTP. Thus, a means for tyrosine kinases to influence *ras* action was demonstrated.

The second approach to define the proper sequence of protein kinase activation after growth factor action was based ultimately on genetic experiments that c-*raf* could be proposed to act "downstream" of *ras*, since "dominant-negative" *ras* mutants did not affect *raf*-transformed systems. This implied a potential interaction between *ras* and *raf*. Such an interaction could be directly demonstrated by high concentrations of cloned and expressed *ras* and *raf* alleles, and ultimately by physical association between *ras* and *raf* in cells (*35,36*). Once *ras* exchanges GDP for GTP, assuming an "activated" conformation, c-*raf* is "recruited" to the membrane, where it undergoes an as yet poorly defined "activation" process, resulting in an increase in the enzymatic activity of c-*raf* (*37,38*).

The final approach that ultimately clarified the downstream effectors of tyrosine kinase action resulted from an examination of the hypothesis that if the "kinase cascade" of growth factor action was correct, then serine/threonine kinase activities should increase following the application of a stimulus of tyrosine kinase activity. Thus, it was possible to demonstrate that after application of EGF, a variety of activities increased, including an activity that phosphorylated a *M*icrotubule *A*ssociated *P*rotein (MAP) kinase and increased its kinase activity for the MAP substrate (*39–41*). This activity was unique in that the MAP kinase could be shown to require both tyrosine and threonine phosphorylation for activity. This allowed the definition of a "dual-specificity" MAP kinase kinase (MAPKK) (*42*), which was itself regulated by c-*raf* kinase (*43*), thus linking tyrosine kinase action ultimately to the activation of MAP kinase. The significance of this observation is that MAP kinase could be demonstrated to phosphorylate several nuclear proteins, including the *myc* and *jun* oncoproteins, cytoskeletal elements, and transcription factors. Thus, a cell-surface ligand–receptor interaction is linked through a series of direct and indirect steps to the expression of specific genes (*41*).

The current view of tyrosine kinase-mediated signaling is summarized in Fig. 3, where either engagement of a growth factor receptor in quiescent cells or stimulation of a nonreceptor-linked tyrosine kinase through cell-surface molecules with which it forms a noncovalent association leads to stimulation of *ras* activity, which in turn activates c-*raf* and ultimately MAP kinase. The action of the latter protein causes increased transcription of regulatory molecules, which allows either entry into or normal progression through the G1 phase of the cell cycle.

2.2. G-Protein-Linked Signaling

A distinct class of growth factor receptors uses receptors that function not by directly inducing covalent modification of substrate proteins, as is the case with the tyrosine protein kinase-linked receptors, but rather by the elaboration of "second messengers" that allosterically modify the function of effector molecules, including protein kinases and phospholipases. The paradigm for humoral mediators of this class is the β-adrenergic receptor, which activates adenylate cyclase and thereby catalyzes the conversion of ATP to cyclic AMP (cAMP). cAMP in turn binds to regulatory subunits of cAMP-

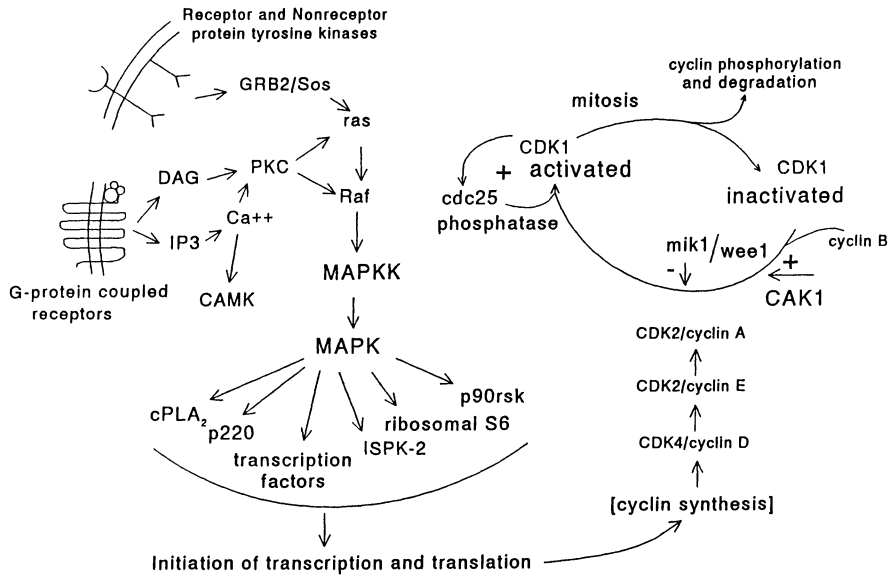

Fig. 3. Growth factor action induces a phosphorylation cascade. Signaling through growth factor receptors leads to activation of *ras* through the adapter molecule grb2 and the GDP release protein *sos*. Activated *ras*, in turn, increases *raf* activity, which then leads to activation of MAP kinase. Transcription "factors" and other signaling pathways are activated, following entry of cells in a path that results in DNA synthesis.

dependent protein kinase (PKA) (*44*). The molecular "switch" that allows ligand-bound receptor to activate adenylate cyclase is a heterotrimeric "G-protein" that consists of a heterotrimer. Ligand-activated receptor (*see* Fig. 4) releases GDP from the $G_s\alpha$ subunit, which then binds GTP, causing the release of $G_s\alpha$ from the $\beta\gamma$ subunits. The GTP·$G_s\alpha$ subunit stimulates adenylate cyclase until the GTP is hydrolyzed, allowing re-formation of the $G_s\alpha\beta\gamma$ heterotrimer, bearing GDP, ready for induction of another catalytic cycle.

The structures of receptors that utilize this general mechanism are all similar in that they have an extracellular domain, seven transmembrane segments, which course in and out of the membrane in a "serpentine" fashion, and an intracellular "loop," which with its carboxyl-terminal portion interacts with G proteins and is the site of receptor-directed regulatory mechanisms, including desensitization and control of receptor internalization controls (reviewed in *45,46*). G proteins exist that, as indicated above, stimulate adenylate cyclase (G_s), inhibit adenylate cyclase (G_i), modulate ion channel activity (G_s and G_o), stimulate phospholipase-Cβ (G_q and G_{16}), and couple photoreceptors to cGMP phosphodiesterase (G_T). Of special relevance to targeting therapeutic measures at the pathways activated by these transducers, G_s is known as the target for cholera toxin, which covalently modifies it to assume a persistently activated state; G_i is the target for pertussis toxin, which causes an inhibitory covalent modification. Note that the heterotrimeric G proteins do have limited homology to the monomeric GTP binding proteins, such as members of the *ras* oncoprotein superfamily in the GTP binding domain and in the overall scheme of catalysis, but are distinctive in that the ras alleles in higher eukaryotes have never been shown to regulate

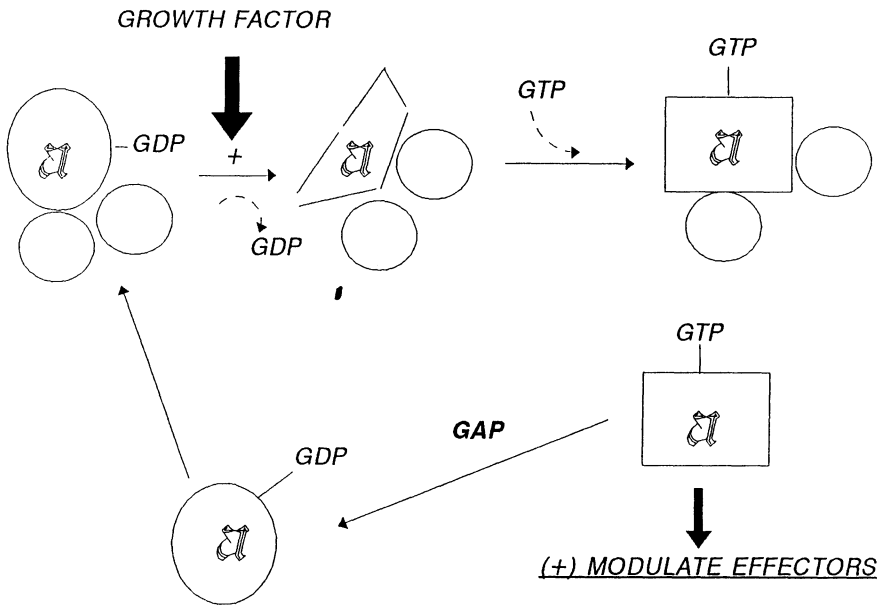

Fig. 4. G protein cycle. The inactive G protein, bearing GDP, is caused to release GDP under the influence of a growth factor or hormone. A transient inactive "empty" subunit exists, but then binds GTP, activating the α subunit to modulate positively effectors, such as adenylate cylase. Hydrolysis of the GTP to GDP under the influence of a GTPase-activating protein (GAP) returns the α subunit to its basal state, bound to G protein β and γ subunits (empty circles).

directly the action of downstream soluble effectors, such as cAMP, but as indicated above, appear to engage in protein–protein interactions in the activated state.

G protein-coupled receptors and their downstream effectors are of potential relevance to a number of human neoplasms. Neuropeptides of the bombesin family, of possible importance in the pathogenesis of lung, breast, and prostate neoplasms, have serpentine receptors (*47*) that activate phospholipase-C (*48*). The consequence of that interaction is elevation of intracellular Ca^{2+} and diacylglycerol, the latter serving in particular to activate PKC. Pituitary tumors have been shown to have "activating" mutations in $G_s\alpha$ (*49*), and in model systems, transfectants from immortalized cell lines bearing heterologous serpentine receptors can apparently cause tumors in a ligand-dependent fashion (*50*). In addition to adrenergic receptors, this class includes receptors for chemotactic cytokines or chemokines (e.g., IL-8, RANTES, IL-10), for f-met-leu-phe, and for autocrine motility factor, which is involved in directed migration of tumor cells (*51*).

2.3. Other Signal Transduction Mechanisms

The tyrosine kinases and the G protein-linked mechanisms account for the transduction mechanisms employed by hundreds of humoral mediators, including growth factors, autocoids, and cytokines. However, in a few specialized instances, other receptor mechanisms have been defined that also have potential therapeutic relevance, although in many cases, aspects of their mechanisms have not been as extensively defined as in the former two receptor types.

2.3.1. A Serine-Kinase-Linked Receptor: Transforming Growth Factor-β (TGF-β)

TGF-β has recently been demonstrated to engage a receptor (receptor II), a constitutively active transmembrane serine/threonine kinase (*52*), which after binding of TGF-β engages a "receptor I" into a noncovalent complex, allowing propagation by receptor I of a serine/threonine kinase signal to downstream substrates. The definition of those downstream effectors will be of interest to define, since TGF-β is a negative growth regulator of a variety of cell types, perhaps by acting as a negative regulator of cyclin-dependent kinases (*53*).

2.3.2. Tumor-Necrosis Factor (TNF) Receptors

The TNF family consists of TNF-α (cachectin), TNF-β (lymphotoxin), and lymphotoxin B, which are now recognized as major inflammatory cytokines that may also be paracrine effectors in a variety of tumors. Two distinct receptors (p55 and p75) have been identified (*54*), and evidence exists that either receptor can activate biologic responses to TNF through a mechanism that involves receptor complexing on the cell surface. TNF-treated cells show activation of PKC, but without increases in Ca^{2+}, suggesting that a phospholipase-C is not responsible. Recently, evidence has been presented that the TNF system can activate a sphingomyelinase (*55*) with the resulting ceramide acting as a second messenger to activate a ceramide-dependent protein kinase. Also of interest has been the recent recognition that receptors related to TNF, including Fas/APO-1 (CD95), can participate in the activation of an endogenous apoptotic death program important in the physiologic regulation of the immune system, but also with relevance to the therapy of neoplasms, such as gliomas, which express the receptor (*56*). Other members of this receptor family include CD40, CD30, nerve growth factor receptor, and CD27. These receptors have more limited tissue distribution, but may be manipulable for therapeutic goals.

2.3.3. Interferon Receptors and the JAK-STAT Pathway

The interferons are a diverse family of factors originally recognized by their ability to confer resistance to viral infection through activation of discrete genes in cells bearing receptors specific for each interferon subtype. Recent insights into interferon-induced signal transduction have demonstrated that engagement of either IFN-α or IFN-γ receptors activates so-called Janus kinases (tyrosine kinases with two kinase domains, only one of which is active), which then phosphorylate a p91 molecule, which can then bind another p91, p84, or p113 molecule. The protein complex then undergoes rapid ("STAT") transport to the nucleus, where transcription of IFN-responsive genes occurs (*57–60*). This recently defined mechanism appears to have relevance not only to the action of the IFNs, but also to IL-6, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor, oncostatin M, IL-2, IL-4, IL-7, IL-9, IL-15, growth hormone prolactin, erythropoietin, and GM-CSF.

2.3.4. Phosphatase Signaling Mechanisms: CD45

CD45 was originally defined as an antigen differentially expressed on a variety of hematopoietic lineages, whose epitope derived from a transmembrane glycoprotein with a complex origin from alternative splicing to yield a family of molecules from 180–220 kDa (*61*). However, definition of the structure of a protein tyrosine phos-

phate phosphatase revealed homology, and it rapidly followed that CD45 is itself a transmembrane tyrosine phosphate phosphatase, implying a role in the regulation of signal transduction in hematopoietic cell signaling. Construction of CD45 deletion mutants revealed notable defects in antigen-mediated signaling, implying that the functional outcome of CD45 activity is a positive signal in generation of the immune response (*62*). Although this is of obvious significance to normal T-cell function, since there is a growing list of tyrosine phosphate phosphatases identified by homology cloning (*63*), the formal possibility is that their action similarly may propagate a "positive" signal.

2.3.5. Phosphatase Signaling Mechanisms: Cyclosporin

The immunosuppressants cyclosporin and rapamycin bind to a family of intracellular binding proteins that are peptidyl-prolyl isomerases, perhaps important in protein folding (*64*). However, it also appears that the cyclosporin binding protein complex results in inhibition of the phospho-serine/threonine phosphatase calcineurin, abundant in hematopoietic cells and brain (*65*), which is postulated to regulate transcription factor action. In a way not completely understood, this ultimately results in selective inhibition of signaling in T cells through pathways that normally stimulate cytokine and cytokine receptor gene expression, thus accounting for the observed immunosuppression. However, it is likely that other high-affinity drug binding proteins operate on signals important to cell proliferation, since rapamycin in particular (*66*) can mediate inhibition of tumor cell growth. The direct relationship of this effect to inhibition of a particular phosphatase or to other signaling mechanism action is currently unclear.

2.3.6. Ganglioside Signaling Mechanisms

Gangliosides are glycosphingolipids containing both hydrophobic ceramide and hydrophilic carbohydrate moieties that include sialic acid residues. Gangliosides are generally represented by the letter G followed by M or D to define whether they contain one or two sialic acid residues, respectively, followed by a number or letter to distinguish monosialic acid and disialic acid members from one another. Gangliosides are present in many cell membranes, but are most abundant in nervous tissue, neural crest, and lymphocytes.

Gangliosides can bind to a number of extracellular proteins, including cholera toxin and diphtheria toxin; however, their physiological ligands are not yet defined. The use of monoclonal antibodies (MAb) to mimic ligand–ganglioside interactions has revealed that gangliosides can initiate a wide variety of intracellular signaling mechanisms (*67*). For example, a subset of T cells expresses the GD3 ganglioside. Antibody to this ganglioside induces tyrosine phosphorylation, nuclear translocation of c-rel, but not NFκB, upregulation of IL-2 receptors and HLA-DR, and secretion of interferon-γ (*68*). In addition, T cells stimulated through GD3 proliferate and develop cytotoxic activity. These effects can be inhibited by cyclosporin, staurosporin, and herbimycin A, implying the involvement of cyclophilin/calcineurin, PKC, and tyrosine kinases in this response. T cells normally require two signals to become activated. No other single stimulus exerts such a wide range of biologic effects on T cells. Similar changes have been noted in tumor cells expressing gangliosides.

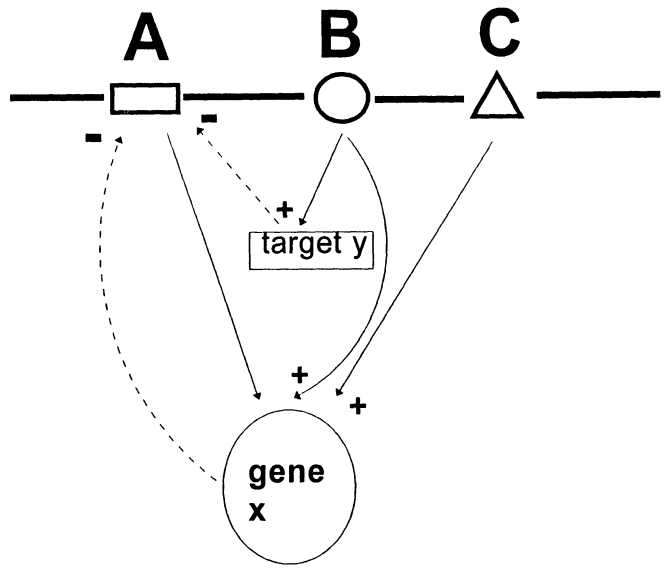

Fig. 5. Regulation of growth factor cascades by stimulation of receptors with negative influence on receptor activation.

3. IMPLICATIONS OF GROWTH FACTOR-DRIVEN CASCADES/NETWORKS

It is apparent that the growth factor-driven signal transduction systems described above do not function independently, but rather act in parallel as well as intersecting arrays. The implications of this arrangement include the possibility that entire pathways may be diminished or augmented in their biologic effect by the action of another pathway at a critical regulatory point in the former pathway. This possibility is illustrated in Fig. 5. Signals propagate from three input receptors and stimulate a particular transcription unit that gives rise to a product that decreases the expression of one of the receptor types. Hence, the result of operation of the signal from ligand A might feedback to decrease response to itself; this is homologous desensitization; however, it is apparent that ligands B and C could also heterologously desensitize the system to the actions of A. In addition, ligand B has a protein-mediated negative regulatory effect on receptor A. This example is not contrived in that, for example, the affinity of EGF-R for EGF is known to be diminished by activated PKC (*67*). The latter could arise from stimulation of EGF-R through PLC-γ, or could arise through activation of G protein kinase-coupled receptors through the action of PLCβ. Another mechanism of receptor desensitization is the modification of the receptor, for example, by phosphorylation, such that its affinity for its ligand is altered or its coupling to its signal transduction mechanism is blocked.

These principles have not been incorporated at all into the design of therapeutically relevant strategies. There has been much focus, as will be shown below, in the design of **specific** antagonists of **specific** targets. Yet it should be clear that narrow therapeutic windows might allow limited biologic effect. What should be pursued are strategies that **decrease** or inappropriately **increase** the activation state of entire pathways in a way that is cell-specific, depending on the array of receptors present.

The most obvious way to effect this goal is to use combinations of growth modulatory large molecules or their antagonists, in conjunction with the small molecules, which although not absolutely specific, may be augmented in their effect or by their action, increase the effect of growth factors or their antagonists acting on their cognate receptors. Such an approach would attempt to capitalize on the pharmacologic tractability of small molecules with the specificity of receptor-mediated processes. Specific types of therapeutic intervention predicated on modulation of growth-factor-related signaling will now be considered.

4. GROWTH FACTORS, ANALOGS, AND ANTAGONISTS

4.1. Suramin

Suramin is a polysulfonated naphthylurea (with a structure that can be viewed as reminiscent of heparan sulfate) that emerged from a screening survey early in the 20th Century to find noncolored analogs of the trypan blue series of antitrypanosomal compounds. Interest in polyanions, such as suramin, as potential antineoplastic agents was renewed following the demonstration that it could reverse simian sarcoma virus transformation (*68*) and alter the binding of PDGF to its receptor (*69*). Thus, it was proposed that suramin could antagonize the growth of tumors that depended on autocrine or paracrine mechanisms (*70*). Antitumor responses in patients have been attributed to suramin in prostate carcinoma, although the exact contribution of suramin to concomitant hormonal manipulation (*71*), including flutamide withdrawal in that series, remains to be clarified.

An additional difficulty is that the concentration–effect relationship for the inhibition of growth factor binding to receptors is variable with, for example, EGF being somewhat insensitive and FGFs somewhat more sensitive to suramin action. However, it is also clear that the concentrations even where the drug can inhibit the binding of "sensitive" growth factors overlap where effects on a variety of glycosaminoglycan enzymes are also seen, with the consequent risk of anticoagulation.

Nonetheless, it remains formally interesting to consider the development of agents that can specifically bind to growth factors, and suramin is clearly a "lead structure" for this goal with respect to the FGFs in particular. In addition, design of such compounds must take into account the toxicities associated with suramin use, including adrenal injury, neurotoxicity, and susceptibility to infection. Suramin in vivo causes lymphocytopenia and can antagonize IL-2 binding to its receptor (*72*). It has been incompletely studied in cytokine-driven neoplasms.

4.2. Bombesin-like Peptides

Evidence has been presented that bombesin-like peptides, which in humans include gastrin-releasing peptide and neuromedin-B, function as autocrine growth factors in human small-cell lung carcinoma (*73*). These peptides function through serpentine receptors (*47*) coupled to phospholipase-C, with release of Ca^{2+} and phosphatidylinositol turnover (*74,75*) demonstrable after addition of exogenous peptide. In addition, there is evidence that tumors of diverse histologies, including breast, prostate, and gastrointestinal sites, can respond to peptide addition with similar responses. Since the pharmacophore for this series has been defined and consists of only the C-terminal octapeptide, there has been great interest in developing antagonists (*76*). SCLC xenografts in athymic mice are susceptible to bombesin antagonists (*77*). Schally and col-

laborators have demonstrated antitumor activity in vivo for example, in xenografts of colon, pancreatic, breast, gastric, and CNS origin (*78–80*). Of concern, however, in developing this concept further is the fact that cells have multiple pathways for activation of the Ca^{2+}/phosphatidylinositol signaling pathway (as has been amply demonstrated for SCLC) (*81,82*) and that, therefore, use of alternative receptors for this goal could be accomplished readily in human tumors.

Efforts have also been undertaken to produce antibodies against bombesin peptides, and evidence for some antitumor activity has been observed in SCLC xenografts in athymic mice (*73*). Antibodies to peptides do not localize to tumor masses in vivo in humans and have not been shown to induce responses in a significant fraction of patients. The poor capacity of macromolecules to penetrate tumor masses only heightens the concern that efforts to block Ca^{2+}/PI signaling may elude current therapeutic tools to accomplish this goal by efforts directed at a single-receptor system.

4.3. Somatostatin and Analogs

Lamberts et al. have recently reviewed (*83*) the mechanisms by which somatostatin or its agonists may cause an antitumor effect, including inhibition of the secretion of hormones, such as growth hormone and insulin; direct or indirect inhibition of insulin-like growth factor 1; inhibition of angiogenesis, and a direct antiproliferative effect. The receptors for somatostatin (of which four have been defined) are G protein-coupled receptors that in some systems activate adenylate cyclase. Direct antitumor effects of somatulin have been demonstrated in lung carcinoma xenografts (*84*).

4.4. LHRH Antagonists

In both breast and endometrial carcinoma cells, evidence has been presented that the LHRH antagonist [Ac-D-Nal(2)1,D-Phe(4C1)2,D-Pal(3)3,D-Cit6,D-Ala10]LHRH can inhibit IGF-driven cell proliferation, associated with a decrease in IGF-2 elaboration. A postreceptor mechanism has been proposed (*85,86*), but the nature of the receptor by which the peptide elicits its effect has not been defined.

4.5. Transforming Growth Factor-α

This growth factor is produced by a variety of tumors (e.g., *87,88*) where it signals through the EGF receptor. Efforts to interdict this signaling have included the development of specific antireceptor antibodies (*89,90*) and the synthesis of TGF-α fragments. Most interesting has been the recent observation in model systems (*91,92*) that the combination of an anti-EGF-R antibody plus either cis-platin or doxorubicin appears to increase the likelihood of benefit (*vide intra*). Rieman et al. (*93*) devised a mechanism-based screen of ligand binding to a cell line that overexpresses the receptor, and have examined a series of natural product extracts. One compound series emerged, the methyl pheophorbides, which appear to give evidence not only of blocking the interaction of TGF-α congeners, but also in PDGF and IL-1β assays. In addition, the pheophorbides are related to the protoporphyrins and are dependent on light for efficient action on cells. Although the molecular basis for these effects is not clear, pheophorbides are important as a lead structure for compounds that can "dampen" or eliminate ligand and receptor interaction. Trapidil (*94*) is another compound that, in meningioma cells, inhibits PDGF-R mediated DNA synthesis, yet can also antagonize EGF-driven cell growth in fibroglasts.

4.6 Insulin-like Growth Factor-I (IGF-I)

Convincing evidence by Baserga has recently been summarized (*95*) that IGF-I can function as an important mediator of the transformed state in model systems, and in the maintenance of the transformed phenotype in glioblastoma, breast cancer, small-cell lung cancer, and melanoma cell lines. This system is of interest because in addition to inhibition of IGF-I action by antibodies or potential antagonists, there is a large family of IGF binding proteins (*96,97*) that are coexpressed with IGF-I receptor in, for example, certain breast carcinoma cell lines and, therefore, could represent an alternative strategy to modulating IGF-I action.

4.7. PDGF

The PDGF-R clearly has the potential to transmit an oncogenic signal, since the v-*sis* oncogene product is homologous to the PDGF-β chain. Of interest, evidence has been presented for stimulation of the PDGF-R both at the cell surface and with presentation of the mitogenic signal to the receptor in an intracellular compartment in model systems. Recently, convincing evidence has been presented that an autocrine pathway involving PDGF can drive glioblastoma cell growth (*98*). This result is intriguing because it has been further demonstrated that synthetic peptides can be created that fuse peptide segments from disparate segments of the PDGF molecule to create antagonists of moderate affinity (*99*). Interestingly, these antagonist peptides disrupted receptor dimerization, and although this resulted in clear diminution of receptor autophosphorylation, a decrease in [^{3}H]-thymidine incorporation, which was not restricted to that produced by PDGF, was noted. This finding delineates a potential strategy for the design of antagonists that can affect signals through more than one growth factor system by altering the capacity to dimerize, thus potentially inducing aberrant dimerization.

4.8 Cytokines

Although a comprehensive treatment of this subject would be outside the scope of this chapter, certain cytokine pathways are of clear relevance in considering strategies for clinical exploitation.

The IL2-R consists of the α (T-cell activation antigen; Tac), β, and γ, chains, which together form a heterotrimeric receptor linked to the activation of tyrosine kinase cascades (*100–102*). This receptor system is expressed in a variety of hematopoietic neoplasms (*103*). Current strategies are seeking to target radiolabels or toxins to the IL2-R, either employing antibodies or chimeric fusion proteins (*104–107*) as targeting agents. The latter approach has produced clinical responses in neoplasms bearing the IL2-R (*108–111*). An important feature of the IL2 signaling system is that the γ-chain of the IL2-R is actually shared with the IL4 and IL7 receptors (*112*). A consequence of this is that exposure to IL2 can potentially modulate signaling and mitogenic responses to the other two cytokines. This has been shown to be of functional consequence in cutaneous T-cell lymphoma cells exposed to IL7 (*113*). Of interest to develop are preclinical studies with some of the modulators of protein kinase action to be described below in conjunction with IL2, IL4, or IL7 in neoplasms bearing these receptors.

In multiple myeloma, some evidence exists for an autocrine or paracrine pathway activated by IL6, and although efforts are under way to derive analogous targeting

approaches using this cytokine receptor, its expression is more ubiquitous than the IL2 receptor system (*114*), and therefore, the likelihood of a useful therapeutic index is lower. In contrast, the CD30 antigen is a cell-surface molecule with homology to members of the TNF receptor family whose expression is largely restricted to hematopoietic cells, and it is prominently expressed on the Reed-Sternberg cells in Hodgkin's disease. Although it has served as a target for the development of "traditional" toxin or antibody-based targeting strategies, it would be useful to clarify whether TNF-like intracellular signals are elicited by this molecule, since efforts to deregulate their expression could lead to additional therapeutic strategies (*115*).

Cytokine receptors may also be of relevance to the treatment of nonhematopoietic neoplasms. For example, interferon and IL-1 have been clearly demonstrated as potentially useful modulators of the growth of certain gynecologic tumor cells. Recent evidence has been accumulating that IL-1 can inhibit the growth of ovarian carcinoma cells (*116*). IL-1 has also been found to regulate secretion of collagenase, important in mediating invasiveness (*117*) in choriocarcinoma cells. Interferons can also act directly to inhibit the growth of ovarian carcinoma cells (*118*).

5. MODULATION OF CYTOKINE OR GROWTH FACTOR RECEPTOR EXPRESSION

A prominent effect of numerous stimuli for cell activation is an altered display or regulation of cell-surface expression. Examples where this strategy may be of value in developing novel therapeutic strategies include the following cases.

5.1 Interferons

Interferons α and γ can cause marked alterations in apparent expression of EGF-R, perhaps by altering the rate of internalization (*119*). Evidence has been presented for the existence of cell types that, in response to interferons, modulate (*120–122*) expression of the EGF receptor.

5.2. TNF

TNF can act as an autocrine and paracrine (*123*) stimulator of the growth of ovarian carcinoma cells. A striking potential mechanism to consider in explaining these phenomena has recently been demonstrated in pancreatic carcinoma cells, where exposure to TNF caused a decrease in c-*erbB2* expression, but an increase in EGF-R RNA. The decrease in c-*erbB2* mRNA was accompanied by decreased protein expression (*124*). Thus, in addition to direct signaling through its own receptor-linked pathways, TNF can clearly influence signaling through these tyrosine kinase linked pathways. The generality of this phenomenon will be of interest to clarify in greater detail.

5.3. Steroids and Retinoids

In breast carcinoma cells, there is considerable evidence that part of the stimulation of growth attributable to estrogens proceeds by activation of growth factor secretion (*125*) with the consequent autocrine or paracrine action of those growth factors on adjacent tumor cells or stroma promoting the growth of the tumor cells. For example, estrogen-treated breast carcinoma cells elaborate TGF-α, FGF, and IGF-like species (*125,126*). Conversely, antiestrogens, such as tamoxifen, droloxifene, and tore-

mifene, can be shown to induce the production of TGF-β, a negative growth regulatory influence (*127*), as well as to oppose the growth-stimulating effects of IGF-1 (*128*). Most interesting is the observation that tamoxifen decreases c-*erbB2* expression in biopsy specimens from estrogen receptor(-) tumor specimens in patients treated for 3 wk before surgery (*129*). These results raise the possibility that the antiestrogen can decrease c-*erbB2* expression by hormone receptor-linked and hormone receptor-independent mechanisms, although this point has not yet been suggested in studies with cell lines in vitro.

Retinoids are of clear importance in delaying or abrogating the onset of second aerodigestive tract neoplasms in a population at risk; however, the mechanism for this effect is not clear. Recent evidence has been presented that although retinoic acid can enhance EGF-related signaling in normal keratinocytes, human epidermoid carcinoma cells have been described in which retinoids suppress the transcription of the EGF-R through a specific element in the EGF-R promoter that can be related to the activity of the RAR-γ (*130*). This finding may also have relevance to breast neoplasms, where exposure of mammary carcinoma cells to retinol resulted in a diminished tyrosine phosphorylation of the EGF-R with decreased TGF-α stimulation of the PLC-γ isoform (*131*). In addition, retinoic acid decreases the estrogen-induced increase in TGF-α secretion by MCF-7 cells (*132*). Taken together, these molecular mechanisms clearly justify clinical studies of retinoid plus antiestrogen combinations in the treatment or prevention of breast neoplasms.

APL represents an important paradigm for the development of differentiation agents as therapeutic agents, since as a single agent all trans retinoic acid can elicit valuable clinical response. In vitro, HL60 cells or patient-derived APL specimens demonstrate enhanced expression of the GM-CSF receptor after differentiation by the retinoid (*133*). Whether this is cause or effect has not yet been demonstrated, but modulation of cytokine responsiveness may be a clinically exploitable effect of retinoid exposure (*134*).

6. MODULATION OF INTRACELLULAR SIGNAL TRANSDUCTION PATHWAYS

6.1. Protein Kinases

Much effort has been expended to design specific antagonists of protein kinases. Yet consideration of Fig. 3 will suggest that a very specific kinase antagonist may be circumvented by the action of additional pathways of cellular activation, or a kinase antagonist directed at a key "final common pathway" kinase, such as PKC, MAP kinase, or the cyclin-dependent kinases, will not have the requisite therapeutic index. The latter consideration is of special relevance, since as discussed by Hunter (*135*), the catalytic mechanism of all protein kinases studied to date is similar in a way that would suggest descent from a common ancestral gene.

Thus, the design features that should be considered in pursuing protein kinase antagonists for therapeutic development should incorporate not only their ability to function as enzymatic inhibitors, but as deregulators of cellular signal transduction in a way that would achieve the desired end point, which in the case of neoplasia is cell growth inhibition or death. In addition to merely designing "potent" enzymatic inhibitors, one must consider the cellular consequences of that inhibition.

6.2. *Tyrosine Protein Kinases*

Two general classes of tyrosine protein kinase signaling antagonists are conceivable: those that inhibit the act of phosphoryl transfer, and those that interfere with the protein–protein associations of tyrosine kinase substrates through interruption of SH2 and SH3 domain function. The former are revealed by a decrease in tyrosine phosphates in treated cells and are the class that has received the greatest developmental attention over the past 20 years, whereas the latter are at a very preliminary stage of development, but could afford means of interrupting tyrosine kinase signaling pathways without necessarily affecting the catalytic activity of the kinase. Burke (*136*) and Fry (*137*) have recently reviewed in detail the various chemical classes that have been found to cause tyrosine kinase inhibition. We shall focus here on biological issues pertinent to their development.

6.2.1. Inhibitors of Phosphoryl Transfer

6.2.1.1. Natural Products. Inhibition of $p60^{src}$ phosphoryltransfer by flavonoid compounds, such as quercetin, was first described by Graziani et al. (*138*). The structure–activity and kinetic analysis as summarized by ref. (*139*) defines these compounds to be competitive with ATP. Accordingly, there is potential interference with a large number of ATP-requiring enzymatic reactions, and the specificity of this class of compounds can be legitimately questioned. Quercetin is actually a better inhibitor of many serine/threonine kinases in comparison to tyrosine kinases. Genistein, an isoflavone again present in many plants, particularly soy products, shares the general kinetic mechanism, but exhibits greater specificity for tyrosine as compared to serine/threonine kinases (*140*).

Erbstatin (Fig. 6) was isolated from a *Streptomyces* culture filtrate and has a structure clearly relatable to tyrosine. It was found to be a potent inhibitor of the EGF-receptor tyrosine kinase (*141*), as well as the activity of the $pp60^{src}$ in immunoprecipitates from Rous sarcoma virus-infected cells. However, the cellular effects of the agent are less clear. It inhibits the growth of L1210 leukemia cells, and evidence for induction of apoptosis in these cells has recently been presented (*142*). The mechanism of this effect is not clear, but in sea star oocytes, erbstatin can prevent the activation by 1-methyladenine-induced activation of $p34^{cdc2}$, $p44^{mpk}$, and ribosomal S6 kinase (*143*). A general criticism of the use of erbstatin and synthetic analogs is that a clear delineation of the responsible mechanism leading to growth inhibition and/or cell death has not been achieved.

Lavendustin was also originally isolated from a *Streptomyces*, and produces inhibition that is of mixed type with respect to both ATP and substrate. The compound is of interest because both amine and nonamineanalogs have demonstrated in one case nanomolar potency vs $p56^{lck}$. A difficulty with many compounds of this type is exemplified by the recent series (*144,145*) where there is discordance between the compounds' ability to inhibit enzymatic activities as compared to their ability to inhibit cell growth, emphasizing the need to develop this type of inhibitor with a consideration of biologic as well as chemical effects of the drug.

Recently, a novel chemical class of tyrosine kinase antagonist was discovered from marine sponge extracts. Halistanol trisulfdate is a sulfated steroid derivative with micromolar potency for $pp60^{src}$ (*146*). More detailed studies are required to assess the capacity of this class of compound to inhibit cell growth.

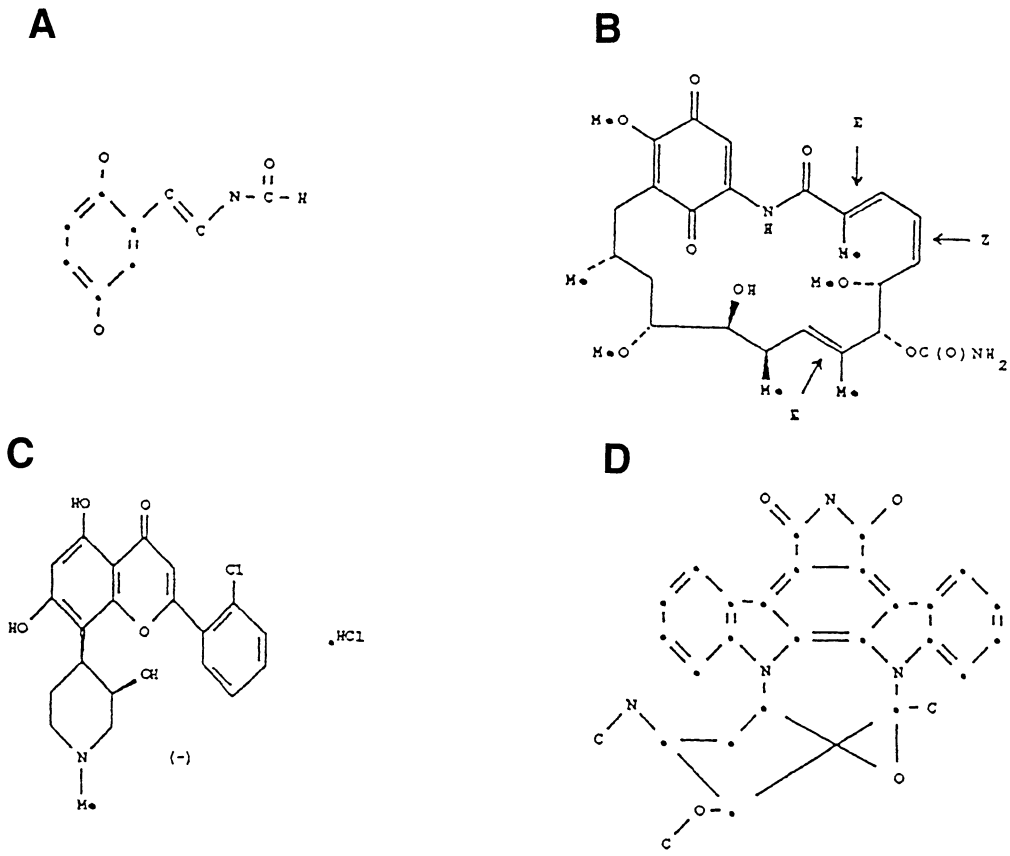

Fig. 6. Structures of protein kinase antagonists. (**A**) Erbstatin (NSC no. 606641). (**B**) Geldanamycin (NSC no. 122750). (**C**) Flavopiridol (NSC no. 649890). (**D**) UNC-01 (NSC no. 638850).

The benzoquinoid ansamycins, including herbimycin A and geldanamycin (Fig. 6), are of interest because recent experiments have suggested novel ways in which interaction of a drug with either its target directly or accessory molecules can lead to apparent modulation of protein kinase activity. The parent compound of the series, herbimycin A, was originally described as an activity isolated from a *Streptomyces* species with activity in a pesticide screening assay (*147*). However, Uehara et al. (*148*) were able to demonstrate that herbimycin caused striking phenotypic reversion of RSV-transformed cells, and pp60^{src} prepared from the cells was inactive in phosphorylating substrate, but the drug added in vitro could not efficiently inhibit the kinase activity of pp60^{src} from untreated cells. This finding suggested to these workers that herbimycin might be acting indirectly to alter the normal cellular function of pp60^{src}. The capacity of herbimycin to reverse many of the protean effects of pp60^{src} in transformed cells (*149*) with relatively few effects on nontransformed cells only heightened interest in the compound, as did documentation of antiangiogenic activity (*150*). However, Uehara et al. (*151*) went on to show that the decreased kinase activity of pp60^{src} was actually accompanied by a decrease in the level of pp60^{src} protein, suggesting that the drug could affect the turnover of the target. This general type of observation has been extended to EGF-R (*152*) and p185$^{c\text{-}erbB2}$ (*153*), and thus not simply inhibition of kinase activity *per se*, but a change in the normal dynamics of the target must be considered in assessing activity.

Whitesell et al. (*154*) made a distinction between relatively rapid cytocidal action of herbimycin and geldanamycin in "sensitive" cell types at concentrations below where *src* kinases would be inhibited, again suggesting a cellular target in addition to the kinases alone. Whitesell went on to demonstrate recently (*155*) that the heat-shock protein hsp-90 could form a complex with immobilized geldanamycin, and that soluble benzoquinoid ansamycins inhibited the previously described complex between the *src* kinase and hsp-90. These findings therefore suggest that hsps, pleiotropically involved in a cell's stress response, may also be regarded as the "real" target for the action of the benzoquinoid ansamycins, with indirect effects on apparent tyrosine kinase activity. Although the implications of these findings are continuing to be assessed, it is clear that attempts to develop benzoquinoid ansamycins clinically might profitably consider use of strategies for long exposure to relatively low concentrations, a strategy that has been suggested to cause differentiation of neuroblastoma (*156*) and leukemia cells (*157*). Such a usage may allow function through altered assuration of heat-shock proteins with regulatory kinases.

6.2.1.2. Synthetic Inhibitors of Phosphoryl Transfer. Modeled on the structure of erbstatin, several groups have produced small molecules with the capacity to discriminate between different tyrosine protein kinases. Yaish et al. (*158*) coined the term "tyrphostin" to describe compounds that were modeled on erbstatin and that were designed to inhibit phosphoryl transfer by competing with substrate as opposed to compounds, such as genistein, which were competitive with ATP. A large number of tyrphostins have been synthesized (*159,160*), and as summarized by Levitzki (*161,162*), these compounds can be divided into four structural classes: the malononitriles, the *S*-arylmalononotriles, the bisubstrate quinoline (where the quinoline portion acts as a pseudo-ATP mimetic), and a series of compounds related to lavendustin. Although the earliest tyrphostins were synthesized with the intention of providing molecules competitive with substrate, actual analysis of the kinetic behavior of tyrphostins suggests that, depending on the particular kinase, there may be mixed inhibition (competitive with respect to both substrate and ATP).

Two immediate problems with this class of compounds is that in many instances, although they have the capacity to discriminate among different tyrosine kinases in in vitro kinase reactions, evidence of activity in living cells in some cases occurs at widely discordant concentrations. Second, clear examples of where these structures actually act in cells to inhibit growth with moderate to good potency, but without reference to inhibition of tyrosine kinases have been demonstrated. For example, the tyrphostin AG 555 is a good inhibitor of topoisomerase I (*163*).

Despite the caveat, therefore, that the mechanism of a tyrphostin in living cells has to be carefully established as relating to the inhibition of a tyrosine kinase, it is possible to show in several systems that cell growth can be blocked through mechanisms that may in some way relate to inhibition of the kinase. For example, Lyall et al. (*164*) showed that tyrphostin-like compounds directed against the EGF receptor could inhibit cell growth in a way that was arguably related to the inhibition of the EGF-R kinase, and could act in athymic mouse xenografts expressing high levels of EGF-R to inhibit tumor growth (*165*). However, the capacity of such compounds to inhibit "outbred" real human tumors in such xenograft systems is less clear.

A number of such tyrphostins that inhibit the $p210^{bcr\text{-}abl}$ fusion protein have been described (*166*), including those that correlatively inhibit CML cell growth (*167*) or

cause differentiation (*168*) of these cells. Such compounds could conceivably be used as an adjunct to bone marrow transplantation in CML or ALL. An additional system with clear evidence of biologic effect on the part of tyrphostins includes PDGF-R induced mitogenesis, with consequent great interest in employing tyrphostin-like compounds to inhibit endothelial cell growth (*169,170*).

A structure reminiscent of, but clearly distinct from the tyrphostins, PD153035, is an EGF-R (*171*) tyrosine kinase antagonist with inhibitory potency in the picolmolar range, and the capacity to inhibit other kinase systems thus far tested only at the micromolar range. It is competitive with ATP, and successfully reverts Swiss 3T3 cells transformed by overexpression of EGF-R. Evidence of activity in animals bearing human tumor xenografts has not yet been reported.

6.2.2. Inhibitors Based on SH2 and SH3 Domains

As discussed above, a consequence of tyrosine kinase action is the creation of tyrosine phosphates that serve as anchoring points for attachment of docking or adapter proteins, which bind to the tyrosine phosphates through SH2 domains. The determinants of binding to these approx 100 amino acid domains present on a wide variety of biologically relevant signaling molecules have been explored. In addition to the tyrosine phosphate, amino acids on either side of the tyrosine phosphate are important in determining high-affinity binding to the SH2 domain (*172,173*). This finding encouraged the development of phosphotyrosyl peptide mimics that in principle would interrupt the normal ability of SH2 domains to which the peptide had affinity from forming complexes with its "normal" repertoire of tyrosine-phosphorylated binding partners. Two problems relevant to the further development of this concept include the susceptibility of such phosphopeptides to hydrolysis and their ability to enter cells. A series of phosphonomethyl-peptides were designed (*174*) that were successful in blocking complexes between c-Src or polyoma middle T-antigen and PI-3′-kinase. Subsequent efforts to develop this class of inhibitors will require efforts to cyclize or attach these molecules to molecules that internalize efficiently.

6.3. PKC

The different structural classes of PKC antagonist, as summarized by Harris et al. (*175*) include antagonists with respect to phospholipid or diacylglycerol, including calphostins and various lipid analogs; antagonists of peptide substrates, including various pseudo-substrate peptides, as well as the natural product chelerythrine and ATP competitors.

Antagonists of PKC that compete with ATP include the widely used isoquinoline sulfonamides H7 and H8, whose selectivity for PKC is not very impressive, and a series of indolocarbazoles and 2,3-bisinolylmaleimides. The lead structure for the latter two classes is staurosporine, an alkaloid originally isolated from a *Streptomyces*. This entity was originally shown to inhibit brain PKC potently (*176*), but in actual fact, a variety of other serine/threonine and tyrosine kinases are inhibited as similar concentrations (*177*). UCN-01 (Fig. 6) (7-OH staurosporin) has improved selectivity for PKC, and kinetic experiments have identified a clear selectivity of UCN-01 for the α, β, γ isoforms of PKC (*178*). Although UCN-01 does show evidence of potent antiproliferative effects in vitro (*179*) as well as antitumor activity (*180*) in vivo, it is not clear whether its antiproliferative activity can be attributed to its anti-PKC actions.

Recent evidence suggest that UCN-01 can cause inappropriate activation of the cyclin-dependent kinase family of cell-cycle regulatory protein kinases (*181*), perhaps by interfering with the normal regulatory phosphorylations of these entities. In the case of staurosporin, this can correlate with induction of apoptosis in some cell types (*182*).

The 2,3 bis-indolyl maleimide series of PKC antagonists, although structurally related to the staurosporin-derived compounds, are notable for the exquisite selectivity with over a 10,000-fold difference in capacity to discriminate between different kinases, and clearly have potential to be useful tools to dissect PKC-mediated pathways in living cells (*183–185*).

Recognizing the potential caveats with respect to specificity of effect in relation to inhibition of a particular kinase, the compounds discussed in this section have a number of very interesting effects at the level of the whole cell whose proper exploitation will lead to their use as modulators of response to other agents or to growth factors. For example, staurosporin has been reported to increase cell-surface expression of both forms of the TNF receptor (*186*), and bis-indolyl maleimide-based inhibitors sensitize cells to TNF-induced cytotoxicity (*187*).

Inappropriate activation of PKC, for example, by phorbol esters has been associated with delay to cell-cycle progression in such systems as melanoma cells (*188*). These findings raise the possibility that manipulation of the phorbol or other tumor-promoter pharmacophore may produce a PKC agonist with antiproliferative activity (*189*). The natural product bryostatin has some of these features, and inappropriate activation of PKC may be a partial explanation for its antiproliferative effect, especially observed in hematopoietic systems (*190,191*).

6.4. Cyclin-Dependent Kinases

Recent studies have defined that progression through the cell cycle requires the coordinated activity of a family of protein kinases that consist of a catalytic subunit that in most cases has invariant expression throughout the cell cycle and a regulatory subunit, or cyclin, whose expression oscillates. The prototype for this family of regulatory molecules is $p34^{cdc2}$ (also known as *c*yclin *d*ependent *k*inase-1, or cdk-1)/cyclin B, as the *cdc2* mutation in yeast was complemented by the catalytic subunit of 34-kD-mol wt. This complex governs entry into M phase. Other cdks with their cognate cyclins have been defined, such as cdk2/cyclin E, which governs entry into and transit through S phase, and cdk4/cyclin D, which mediates transit through G1. For a complete review of the cdks, *see* refs. (*192,193*). Of significance, in addition to the presence of a cyclin partner, the proper sequence of regulatory phosphorylations is critical for maximal activity.

Although endogenous cellular inhibitors of cdks have been defined such as $p21^{WAF}$, p16, and p27, small molecules that can accomplish this result are of interest. Three molecules with relative specificity for cdks have been defined. Olomoucine (an isopentenyl purine) has been defined as a selective inhibitor of cdks 1, 2, and 6. The inhibition is competitive with ATP (*194*) and relatively highly selective in comparison to other kinases. Butyrolactone likewise has specificity for cdks, and both compounds can inhibit the normal oscillation of DNA synthesis in extracts of amphibian eggs (*195*). However, their respective potency for inhibition of mammalian cell growth is relatively modest, with IC_{50}s in the ~50-μM range. In contrast, flavopiridol (L86-8275) (Fig. 6) was originally discerned as a potent inhibitor of cell growth (IC_{50}s 25–300 nM)

that potently blocked cell-cycle progression in either G2 or G1, and retarded cell progression through S (*196*). Potent direct inhibition of cdk1 (*197*), competitive with respect to ATP (K_i = 45 n*M*), as well as interference with the normal regulatory phosphorylations of cdk1 and cdk2 (*198,199*) appear to correlate closely with the concentrations effective in inhibiting cell growth. Flavopiridol has reproducible antitumor activity in animals (*200*), and will be of interest to consider for development whether alone, in conjucntion, or as a modulator of susceptibility to cell-cycle-specific agents.

6.5. PI-3′-Kinase

This enzyme contains a 110-kDa catalytic subunit and an 85-kDa regulatory subunit, and it phosphorylates inositol lipids on the D-3 hydroxyl position (*201*). Activation of PI-3′-kinase has been shown to occur following receptor ligation in many different systems, including growth factor receptors with intrinsic tyrosine kinase activity (*202–205*) and those associated with src-like kinases (*206–209*). Mutation of residues in the PDGF receptor critical for p85 binding has demonstrated that PI-3′-kinase plays a critical role in mitogenesis and other cell functions (*210*).

Wortmannin is a fungal metabolite that inhibits the activity of PI-3′-kinase by interacting with the p110 regulatory subunit (*211,212*). It has been shown to be very effective in vitro at micromolar concentrations and is beginning to be tested in vivo. The crucial role of PI-3′-kinase in the activation of T-cells and B-cells makes it likely that wortmannin and other agents that target this enzyme will be highly immunosuppressive. Indeed, in one model system, wortmannin has been exploited to treat graft-vs-host disease (Taub and Longo, unpublished observations). Mechanisms to target such inhibitors to specific cell types may make PI-3′-kinase inhibitors more selective in their effects.

6.6. Phosphatases

As might not be surprising, agents that inhibit the normal removal of phosphates generated during signaling can have antiproliferative effects. Okadaic acid is actually a tumor promoter that increases intracellular phosphates by inhibition of protein phosphatases 1 and 2. Recently, okadaic acid, the structurally unrelated phosphatase inhibitor calyculin-A, and the analog dinophysistoxin-1 were shown to be capable of inducing an apoptosis-like pathway in certain breast carcinoma cells (*213*), and in B-lymphocytes, to activate MAP kinase, but without inducing DNA synthesis (*214*). It has been suggested that antiproliferative effects of phosphatase antagonists may relate to their abrogation of modification of normal cell-cycle checkpoints, allowing unscheduled entry into mitosis. It has recently been suggested that fostriecin, a known low potency (IC_{50} = ~40 μM) topoisomerase II inhibitor, can inhibit phosphatase-2A with 1000-fold higher potency and appears to induce premature mitosis (*215*). Thus, protein phosphatase inhibitors may emerge as useful modulators in conjunction with agents that induce DNA damage and, therefore, require for DNA repair the induction of a pause in cell-cycle progression in G2. In phosphatase-inhibitor-treated cells, this pause may not exist or may be abrogated in a way leading to "mitotic catastrophe."

6.7. Phospholipase C and Calcium-Based Signaling

Engagement of receptors that couple through heterotrimeric G proteins to intracellular effectors can increase intracellular Ca^{2+} by stimulation of a phosphatidylinositol-

4,5-bis-phosphate (PIP_2)-specific phospholipase C, releasing inositol-1,4,5-tris-phosphate (IP3) and diacylglycerol. IP3 releases calcium from internal stores (*216*), and can be metabolized to higher-order inositol phosphates (*217*), which may play a role in opening plasma membrane Ca^{2+} channels, and lead to sustained maintenance of increased Ca^{2+} or to replenishment of internal stores.

This process can be interrupted by a variety of routes. Direct inhibitors of phospholipase-C, including some 3-nitrocoumarin derivatives, have been described (*218*), but the cell growth-inhibitory activity of this compound has not yet been unequivocally linked to inhibition of the phospholipase. Carboxy amido imitazole (CAI) (*219*) was found to inhibit tumor cell motility in a way that correlates with the compound's ability to block entry of Ca^{2+} through an as yet not clearly defined Ca^{2+} channel, and this compound is currently in clinical trial. Exposure of certain cells bearing the GM1 ganglioside receptor for cholera toxin to cholera toxin show decreased release of Ca^{2+} in response to various stimuli (*220*) after exposure to cholera toxin. Peptide antagonists and calcium channel blockers have also inhibited growth of certain tumor types in vitro and in vivo (*221*). However, the direct relationship of altered calcium flux as the unique growth-inhibitory mechanism for these agents has not been unequivocally established. Nonetheless, they remain lead agents in modulating Ca^{2+}-based signaling.

6.8. Activation-Induced Cell Death and Synergy Between Chemical and Biologic Agents

There is evidence that certain neoplastic cells respond paradoxically to signals that are associated with growth and proliferation of their normal counterparts. Thus, B-cell and T-cell lymphomas have been shown to respond to activation signals either through their antigen recognition structures (*222–224*) or through costimulation receptors, such as CD40 and class II MHC on B-cells or CD2 and CD28 on T-cells (*225–227*), by undergoing irreversible growth arrest or apoptosis. The antitumor effects of pharmacologic doses of estrogen in patients with breast cancer may reflect a similar phenomenon. Although the precise mechanism of these effects is unclear, an irreversible block in cell-cycle progression usually in G1, but occasionally in G2, is often observed. These results imply that the autonomous proliferation of cancer cells may be sensitive to superimposed normal signal transduction.

An independent set of observations lends credence to this notion. A number of investigators have noted that antibodies directed at cell-surface tyrosine kinase receptors, such as her-2/neu and the EGF receptor, appear to sensitize tumor cells to killing by DNA-damaging chemotherapeutic agents, such as cisplatin and doxorubicin (*91,92,228,229*). The mechanisms for this interaction may be diverse; however, at least one study documented that the antibody to the growth factor receptor inhibited the ability of the tumor cell to repair the cisplatin-induced DNA damage and blocked cisplatin-induced unscheduled DNA synthesis (*230*). The responses of cells to metabolic and genotoxic stress often permit them to repair damage or bypass obstructions. The use of complementary approaches that prohibit the cell's repair and evasion mechanisms may paralyze the cell. Thus, manipulations of cell-cycle progression and signal transduction may intersect with the action of conventional cytotoxic agents to enhance the cell killing (*231*).

7. SUMMARY

It is apparent that the immediate future for development of agents that modulate growth factor-mediated signal transduction is bright, with many novel approaches possible predicated on this emerging understanding of cellular pharmacology. However, our understanding remains somewhat primitive, a fact that serves to limit the degree to which our drug development efforts can be said to be clever. An important consequence of our understanding of mechanisms of action of these agents is that the boundary between classical drug development of small molecules and induction of biological responses by antibodies, growth factors, and cytokines is rather blurred by the realization that depending on what growth-promoting influence is paramount, the drug molecules may be expected to have vastly different effects. Thus, development of these agents cannot rely on empiricism, but must incorporate an understanding of the physiology of the intended target in neoplastic as compared to normal tissues if optimal development is to proceed.

REFERENCES

1. Powis G. Signaling pathways as targets for anticancer drug development. *Pharmacol Ther* 1994; 62:57-95.
2. Kerr DJ, Workman P. *New Molecular Targets for Cancer Chemotherapy*. Boca Raton, FL: CRC, 1994.
3. Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltoff S. Oncogenes and signal transduction. *Cell* 1991; 64:281-302.
4. Sporn MB, Roberts AB. Autocrine secretion—10 years later. *Ann Intern Med* 1992; 117: 408-414.
5. Levinson AD, Oppermann H, Levintow L, Varmus HE, Bishop JM. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with phosphoprotein. *Cell* 1978; 15:561-572.
6. Bolen JB. Nonreceptor tyrosine protein kinases. *Oncogene* 1993; 8:2025-2031.
7. Witte ON, Kelliher M, Muller AJ, Pendergast AM, Gishizky M, McLaughlin J, Sawyers C, Maru Y, Shah N, Denny C, Rosenberg N. Role of BCR-ABL oncogene in the pathogenesis of Philadelphia chromosome positive leukemias. In: Brugge J, Curran T, Harlow E, McCormick F, eds. *Origins of Human Cancer*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1991:521-526.
8. Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of *abl* and *bcr* genes in chronic myelogenous leukaemia. *Nature* 1985; 315:550-554.
9. Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of *bcr-abl* oncogene products. *Science* 1990; 247:1079-1082.
10. Bolen JB, Veillette A, Schwartz AM, DeSeau V, Rosen N. Activation of $pp60^{c\text{-}src}$ protein kinase activity in human colon carcinoma. *Proc Natl Acad Sci USA* 1987; 84:2251-2255.
11. Veillette A, Foss FM, Sausville EA, Bolen JB, Rosen N. Expression of the *lck* tyrosine kinase gene in human colon carcinoma and other non-lymphoid human tumor cell lines. *Oncogene Res* 1987; 1:357-374.
12. Veillette A, Bookman MA, Horak EM, Bolen JB. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. *Cell* 1988; 55:301-308.
13. Sporn M, Roberts A. *Growth Factors: Handbook of Experimental Pharmacology,* vol. 95. Berlin: Springer-Verlag, 1990.
14. Frykberg L, Palmieri S, Beug H, Graf T, Hayman MJ, Vennstrom B. Transforming capacities of avian erythroblastosis virus mutants deleted in the *erbA* or *erbB* oncogenes. *Cell* 1983; 32:227-238.

15. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD. Close similarity of epidermal growth factor receptor and v-*erb*B oncogene protein sequences. *Nature* 1984; 307:521-527.
16. Fantl WJ, Johnson DE, Williams LT. Signalling by receptor tyrosine kinases. *Annu Rev Biochem* 1993; 62:453-481.
17. Semba K, Kamata N, Toyoshima K, Yamamoto T. A v-*erbB*-related protooncogene, c-*erbB*-2, is distinct from the c-*erbB*-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. *Proc Natl Acad Sci USA* 1985; 82:6497-6501.
18. Hendler FJ, Ozanne BW. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J Clin Invest* 1984; 74:647-651.
19. Merlino GT, Xu YH, Richert N, Clark AJL, Ishii S, Banks-Schlegel S, Pastan I. Elevated epidermal growth factor receptor gene copy number and expression in a squamous carcinoma cell line. *J Clin Invest* 1985; 75:1077-1079.
20. Yamamoto T, Kamata N, Kawano H, Shimizu S, Kuroki T, Toyoshima K, Rikimaru K, Nomura N, Ishizaki R, Pastan I, Gamou S, Shimizu N. High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. *Cancer Res* 1986; 46:414-416.
21. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF. Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. *Science* 1989; 244:707-712.
22. Han M, Sternberg PW. *let*-60, a gene that specifies cell fates during *C. elegans* vulval induction, encodes a *ras* protein. *Cell* 1990; 63:921-931.
23. Morrison DK, Kaplan DR, Rapp U, Roberts TM. Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. *Proc Natl Acad Sci USA* 1988; 85:8855-8859.
24. Mayer BJ, Hamaguchi M, Hanafusa H. A novel viral oncogene with structural similarity to phospholipase C. *Nature* 1988; 332:272-275.
25. Courtneidge SA, Heber A. An 81 kd protein complexed with middle T antigen and $pp60^{c\text{-}src}$: a possible phosphatidylinositol kinase. *Cell* 1987; 50:1031-1037.
26. Otsu M, Hiles I, Gout I, Fry MJ, Ruiz-Larrea F, Panayotou G, Thompson A, Dhand R, Hsuan J, Totty N, Smith AD, Morgan SJ, Courtneidge SA, Parker PJ, Waterfield MD. Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/$pp60^{c\text{-}src}$ complexes, and P13-kinase. *Cell* 1991; 65:91-104.
27. Shoelson SE, Sivaraja M, Williams KP, Hu P, Schlessinger J, Weiss MA. Specific phosphopeptide binding regulates a conformational change in the PI 3-kinase SH2 domain associated with enzyme activation. *EMBO J* 1993; 12:795-802.
28. Margolis B, Rhee SG, Felder S, Mervic M, Lyall R, Levitski A, Ullrich A, Zilberstein A, Schlessinger J. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell* 1989; 57:1101-1117.
29. Margolis B, Bellot F, Honegger A, Ullrich A, Schlessinger J. Zilberstein A. Tyrosine kinase activity is essential for the association of phospholipase C-γ with the epidermal growth factor receptor. *Mol Cell Biol* 1990; 10:435-441.
30. Margolis B, Li N, Koch A, Mohammadi M, Hurwitz DR, Zilberstein A, Ullrich A, Pawson T, Schlessinger J. The tyrosine-phosphorylated carboxy terminus of the EGF receptor is a binding site for GAP and PLC-γ. *EMBO J* 1990; 9:4375-4380.
31. Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar-Sagi D, Schlessinger J. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* 1992; 70:431-442.
32. Downward J. The GRB2/Sem-5 adaptor protein. *FEBS Lett* 1994; 338:113-117.
33. Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA. Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* 1993; 363:45-51.
34. Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* 1993; 363:83-85.
35. Warne PH, Viciana PR, Downward J. Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. Activation of serine pepetide kinase activity by myelin basic protein kinases *in vitro*. *Nature* 1993; 364:352-355.

36. Zhang XF, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall MS, Bruder JT, Rapp UR, Avruch J. Normal and oncogenic p21^{ras} proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature* 1993; 364:308–313.
37. Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF. Activation of Raf as a result of recruitment to the plasma membrane. *Science* 1994; 264:1463–1467.
38. Wartmann M, Davis RJ. The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. *J Biol Chem* 1994; 269:6695–6701.
39. Ahn NG, Weiel JE, Chan CP, Krebs EG. Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. *J Biol Chem* 1990; 265: 11,487–11,494.
40. Ahn NG, Krebs EG. Evidence for an epidermal growth factor-stimulated protein kinase cascade in Swiss 3T3 cells. *J Biol Chem* 1990; 265:11,495–11,501.
41. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem* 1993; 268:14,553–14,556.
42. Rossomando A, Wu J, Weber MJ, Sturgill TW. The phorbol ester-dependent activator of the mitogen-activated protein kinase p42^{mapk} is a kinase with specificity for the threonine and tyrosine regulatory sites. *Proc Natl Acad Sci USA* 1992; 89:5221–5225.
43. Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR, Avruch J. Raf-1 activates MAP kinase-kinase. *Nature* 1992; 358:417–421.
44. Taylor SS, Buechler JA, Yonemoto W. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu Rev Biochem* 1990; 59:971–1005.
45. Strader CD, Fong TM, Tota MR, Underwood D, Dixon RAF. Structure and function of G protein-coupled receptors. *Annu Rev Biochem* 1994; 63:101–132.
46. Allende JE. GTP-mediated macromolecular interactions: the common features of different systems. *FASEB J* 1988; 2:2356–2367.
47. Corjay MH, Dobrzanski DJ, Way JM, Viallet J, Shapira H, Worland P, Sausville EA, Battey JF. Two distinct bombesin receptor subtypes are expressed and functional in human lung carcinoma cells. *J Biol Chem* 1991; 266:18,771–18,779.
48. Sharoni Y, Viallet J, Trepel JB, Sausville EA. Effect of guanine and adenine nucleotides on bombesin-stimulated phospholipase C activity in membranes from Swiss 3T3 and small cell lung carcinoma cells. *Cancer Res* 1990; 50:5257–5262.
49. Landis CA, Masters SB, Spada A, Pace AM, Bourne HR, Vallar L. GTPase inhibiting mutations activate the α chain of G_s and stimulate adenyl cyclase in human pituitary tumours. *Nature* 1989; 340:692–696.
50. Allen LF, Lefkowitz RJ, Caron MG, Cotecchia S. G-protein-coupled receptor genes as protooncogenes: constitutively activating mutation of the α_{1B}-adrenergic receptor enhances mitogenesis and tumorigenicity. *Proc Natl Acad Sci USA* 1991; 88:11,354–11,358.
51. Murata J, Lee HY, Clair T, Krutzsch HC, Årestad AA, Sobel ME, Liotta LA, Stracke ML. cDNA cloning of the human tumor motility-stimulating protein, autotaxin, reveals a homology with phosphodiesterase. *J Biol Chem* 1994; 269:30,479–30,484.
52. Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. Mechanism of activation of the TGF-β receptor. *Nature* 1994; 370:341–347.
53. Koff A, Ohtsuki M, Polyak K, Roberts JM, Massagué J. Negative regulation of G1 in mammalian cells: inhibition of cyclin E-dependent kinase by TGF-β. *Science* 1993; 260:536–539.
54. Heller RA, Krönke M. Tumor necrosis factor receptor-mediated signaling pathways. *J Cell Biol* 1994; 126:5–9.
55. Kolesnick R, Golde DW. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell* 1994; 77:325–328.
56. Weller M, Frei K, Groscurth P, Krammer PH, Yonekawa Y, Fontana A. Anti-Fas/APO-1 antibody-mediated apoptosis of cultured human glioma cells. Induction and modulation of sensitivity by cytokines. *J Clin Invest* 1994; 94:954–964.
57. Fu XY, Schindler C, Improta T, Aebersold R, Darnell JE Jr. The proteins of ISGF-3, the interferon α-induced transcriptional activator, define a gene family involved in signal transduction. *Proc Natl Acad Sci USA* 1992; 89:7840–7843.
58. Larner AC, David M, Feldman GM, Igarashi K, Hackett RH, Webb DSA, Sweitzer SM, Petricoin EF III, Finbloom DS. Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. *Science* 1993; 261:1730–1733.

59. Sadowski HB, Shuai K, Darnell JE Jr, Gilman MZ. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science* 1993; 261:1739–1744.
60. Shuai K, Stark GR, Kerr IM, Darnell JE Jr. A single phosphotyrosine residue of STAT91 required for gene activation by interferon-γ. *Science* 1993; 261:1744–1746.
61. Trowbridge IS. CD45. A prototype for transmembrane protein tyrosine phosphatases. *J Biol Chem* 1991; 266:23,517–23,520.
62. Molina TJ, Kishihara K, Siderovski DP, van Ewijk W, Narendran A, Timms E, Wakeham A, Paige CJ, Hartmann KU, Veillette A, Davidson D, Mak TW. Profound block in thymocyte development in mice lacking $p56^{lck}$. *Nature* 1992; 357:161–164.
63. Fischer EH, Charbonneau H, Tonks NK. Protein tyrosine phosphatases: a diverse family of intracellular and transmembrane enzymes. *Science* 1991; 253:401–406.
64. Sigal NH, Dumont FJ. Cyclosporin A, FK-506; and rapamycin: pharmacologic probes of lymphocyte signal transduction. *Annu Rev Immunol* 1992; 10:519–560.
65. Clipstone NA, Fiorentino DF, Crabtree GR. Molecular analysis of the interaction of calcineurin with drug-immunophilin complexes. *J Biol Chem* 1994; 269:26,431–26,437.
66. Eng CP, Sehgal SN, Vezina C. Activity of rapamycin (AY-22,989) against transplanted tumors. *J Antibiot (Tokyo)* 1984; 37:1231–1237.
67. Lin CR, Chen WS, Lazar CS, Carpenter CD, Gill GN, Evans RM, Rosenfeld MG. Protein kinase C phosphorylation at Thr 654 of the unoccupied EGF receptor and EGF binding regulate functional receptor loss by independent mechanisms. *Cell* 1986; 44:839–848.
68. Betsholtz C, Johnsson A, Heldin CH, Westermark B. Efficient reversion of simian sarcoma virus-transformation and inhibition of growth factor-induced mitogenesis by suramin. *Proc Natl Acad Sci USA* 1986; 83:6440–6444.
69. Huang SS, Huang JS. Rapid turnover of the platelet-derived growth factor receptor in sis-transformed cells and reversal by suramin. Implications for the mechanism of autocrine transformation. *J Biol Chem* 1988; 263:12,608–12,618.
70. Kopp R, Pfeiffer A. Suramin alters phosphoinositide synthesis and inhibits growth factor receptor binding in HT-29 cells. *Cancer Res* 1990; 50:6490–6496.
71. Myers C, Cooper M, Stein C, LaRocca R, Walther MM, Weiss G, Choyke P, Dawson N, Steinberg S, Uhrich MM, Cassidy J, Kohler DR, Trepel J, Linehan WM. Suramin: a novel growth factor antagonist with activity in hormone-refractory metastatic prostate cancer. *J Clin Oncol* 1992; 10:881–889.
72. Mills GB, Zhang N, May C, Hill M, Chung A. Suramin prevents binding of interleukin 2 to its cell surface receptor: a possible mechanism for immunosuppression. *Cancer Res* 1990; 50: 3036–3042.
73. Cuttitta F, Carney DN, Mulshine J, Moody TW, Fedorko J, Fischler A, Minna JD. Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. *Nature* 1985; 316:823–826.
74. Heikkila R, Trepel JB, Cuttitta F, Neckers LM, Sausville EA. Bombesin-related peptides induce calcium mobilization in a subset of human small cell lung cancer cell lines. *J Biol Chem* 1987; 262:16,456–14,460.
75. Trepel JB, Moyer JD, Heikkila R, Sausville EA. Modulation of bombsin-induced phosphatidylinositol hydrolysis in a small-cell lung-cancer cell line. *Biochem J* 1988; 255:403–410.
76. Jensen RT, Mrozinski JE Jr, Coy DH. Bombesin receptor antagonists: different classes and cellular basis of action. *Recent Results in Cancer Res* 1993; 129:87–113.
77. Thomas F, Arvelo F, Antoine E, Jacrot M, Poupon MF. Antitumoral activity of bombesin analogues on small cell lung cancer xenografts: relationship with bombesin receptor expression. *Cancer Res* 1992; 52:4872–4877.
78. Radulovic S, Miller G, Schally AV. Inhibition of growth of HT-29 human colon cancer xenografts in nude mice by treatment with bombesin/gastrin releasing peptide antagonist (RC-3095). *Cancer Res* 1991; 51:6006–6009.
79. Yano T, Pinski J, Szepeshazi K, Halmos G, Radulovic S, Groot K, Schally AV. Inhibitory effect of bombesin/gastrin-releasing peptide antagonist SB-75 on the growth of MCF-7 MIII human breast cancer xenografts in athymic nude mice. *Cancer* 1994; 73:1229–1238.
80. Qin Y, Ertl T, Cai RZ, Halmos G, Schally AV. Inhibitory effect of bombesin receptor antagonist RC-3095 on the growth of human pancreatic cancer cells in vivo and in vitro. *Cancer Res* 1994; 54:1035–1041.

81. Woll PJ, Rozengurt E. Multiple neuropeptides mobilize calcium in small cell lung cancer: effects of vasopressin, brudykinin, cholecytokinin, galonin and neurostensin. *Biochem Biophys Res Commun* 1989; 164:66-73.
82. Bunn PA, Dienhart DG, Chan D, Puck TT, Tagawa M, Jewett PB, Braunschweiger. Neuropeptide stimulation of calcium flux in human lung cancer cells: delination of alternative pathways. *Proc Natl Acad Sci USA* 1990; 87:2162-2166.
83. Lamberts SWJ, Reubi JC, Krenning EP. The role of somatostatin analogs in the control of tumor growth. *Semin Oncol* 1994; 21:61-64.
84. Bogden AE, Taylor JE, Moreau JP, Coy DH, LePage DJ. Response of human lung tumor xenografts to treatment with a somatostatin analogue (Somatuline). *Cancer Res* 1990; 50: 4360-4365.
85. Hershkovitz E, Marbach M, Bosin E, Levy J, Roberts CT Jr, LeRoith D, Schally AV, Sharoni Y. Luteinizing hormone-releasing hormone antagonists interfere with autocrine and paracrine growth stimulation of MCF-7 mammary cancer cells by insulin-like growth factors. *J Clin Endocrinol Metab* 1993; 77:963-968.
86. Kleinman D, Roberts CT Jr, LeRoith D, Schally AV, Levy J, Sharoni Y. Regulation of endometrial cancer cell growth by insulin-like growth factors and the luteinizing hormone-releasing hormone antagonist SB-75. *Regul Pept* 1993; 20:91-98.
87. Coffey RJ, Goustin AS, Soderquist AM, Shipley GD, Wolfshohl J, Carpenter G, Moses HL. Transforming growth factor α and β expression in human colon cancer lines: implications for an autocrine model. *Cancer Res* 1987; 47:4590-4594.
88. Smith JJ, Derynck R, Korc M. Production of transforming growth factor α in human pancreatic cancer cells: evidence for a superagonist autocrine cycle. *Proc Natl Acad Sci USA* 1987; 84:7567-7570.
89. Masui H, Kawamoto T, Sato JD, Wolf B, Sato G, Mendelsohn J. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res* 1984; 44:1002-1007.
90. Ennis BW, Valverius EM, Bates SE, Lippman ME, Bellot F, Kris R, Schlessinger J, Masui H, Goldenberg A, Mendelsohn J, Dickson RB. Anti-epidermal growth factor receptor antibodies inhibit the autocrine-stimulated growth of MDA-468 human breast cancer cells. *Mol Endocrinol* 1989; 3:1830-1838.
91. Fan Z, Baselga J, Masui H, Mendelsohn J. Antitumor effects of anti-epidermal growth factor receptor monoclonal antibodies plus *cis*-diamminedichloroplatinum on well established A431 cell xenografts. *Cancer Res* 1993; 53:4637-4642.
92. Baselga J, Norton L, Masui H, Pandiella A, Coplan K, Miller JH Jr, Mendelsohn J. Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. *J Natl Cancer Inst* 1993; 85:1327-1333.
93. Rieman DJ, Anzano MA, Chan GW, Imburgia TJ, Chan JA, Johnson RK, Greig RG. Antagonism of TGF-α receptor binding and TGF-α induced stimulation of cell proliferation by methyl pheophorbides. *Oncol Res* 1992; 4:193-200.
94. Todo T, Adams EF, Fahlbusch R. Inhibitory effect of trapidil on human meningioma cell proliferation via interruption of autocrine growth stimulation. *J Neurosurg* 1993; 78:463-469.
95. Baserga R. The insulin-like growth factor I receptor: a key to tumor growth? *Cancer Res* 1995; 55:249-252.
96. Figueroa JA, Jackson JG, McGuire WL, Krywicki RF, Yee D. Expression of insulin-like growth factor binding proteins in human breast cancer correlates with estrogen receptor status. *J Cell Biochem* 1993; 52:196-205.
97. Arteaga CL. Interference of the IGF system as a strategy to inhibit breast cancer growth. *Breast Cancer Res Treat* 1992; 22:101-106.
98. Vassbotn FS, Östman A, Langeland N, Holmsen H, Westermark B, Heldin CH, Nistér M. Activated platelet-derived growth factor autocrine pathway drives the transformed phenotype of a human glioblastoma cell line. *J Cell Biochem* 1994; 158:381-389.
99. Engström U, Engström A, Ernlund A, Westermark B, Heldin CH. Identification of a peptide antagonist for platelet-derived growth factor. *J Biol Chem* 1992; 267:16,581-16,587.
100. Nikaido T, Shimizu A, Ishida H, Sabe K, Teshigawara M, Maeda T, Uchiyama J, Yodoi J, Honjo T. Molecular cloning of cDNA encoding human interleukin-2 receptor. *Nature* 1984; 311:631-635.

101. Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyasaka M, Taniguchi T. Interleukin-2 receptor beta chain: generation of three receptor forms by loned alpha and beta chain cDNA's. *Science* 1989; 244:551-556.
102. Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, Munakata H, Nakamura M, Sugamura K. Cloning of the γ chain of the human IL-2 receptor. *Science* 1992; 257:379-382.
103. Strauchen J, Breakstone B. IL-2 receptor expression in human lymphoid lesions. *Am J Pathol* 1987; 126:506-512.
104. Waldmann TA, Goldman C, Top L, Grant A, Burton J, Bamford R, Roessler E, Horak I, Zakorn S, Kaston-Sporks C, White J, England R, Horak E, Martinucci J, Tinubu SA, Mishra B, Junghans R, Dipre M, Carasquillo J, Reynolds J, Gansow O, Nelson D. The interleukin-2 receptor: a target for immunotherapy. *Ann NY Acad Sci* 1993; 685:603-610.
105. Kreitman RJ, Chang CN, Hudson DV, Queen C, Bailon P, Pastan I. Anti-Tac (Fab)-PE40, a recombinant double-chain immunotoxin which kills interleukin-2-receptor-bearing cells and induces complete remission in an in vivo tumor model. *Int J Cancer* 1994; 57:856-864.
106. Williams D, Parker K, Bacha P, Bishai W, Borowski M, Genbauffe F, Strom T, Murphy J. Diphtheria toxin receptor binding domain substitution with interleukin-2: genetic reconstitution and properties of a diphtheria toxin related interleukin-2 fusion protein. *Protein Eng* 1987; 1:493-498.
107. Bacha P, Williams D, Waters C, Murphy J, Strom T. Interleukin-2 receptor mediated action of a diphtheria toxin related interleukin-2 fusion protein. *J Exp Med* 1988; 167:612-622.
108. LeMaistre CF, Meneghetti C, Rosenblum M, Reuben J, Shaw J, Deisseroth A, Woodworth T, Parkinson D. Phase I trial of an interleukin-2 fusion toxin (DAB 486IL2) in hematologic malignancies expressing the IL-2 receptor. *Blood* 1992; 79:2547-2554.
109. LeMaistre CF, Craig F, Meneghetti C, McMullin B, Parker K, Reuben J, Boldt D, Rosenblum M, Woodworth T. Phase I trial of a 90-minute infusion of the fusion toxin DAB(486)IL2 in hematologic cancers. *Cancer Res* 1993; 53:3930-3934.
110. Hesketh P, Caguioa P, Koh H, Dewey H, Facada A, McCaffrey R, Parker K, Nylen P, Woodworth T. Clinical activity of a cytotoxic fusion protein in the treatment of cutaneous T-cell lymphoma. *J Clin Oncol* 1993; 11:1682-1690.
111. Foss FM, Borkowski TA, Gilliom M, Stetler-Stevenson M, Jaffe ES, Figg WD, Tompkins A, Bastian A, Nylen P, Woodworth T, Udey MC, Sausville EA. Chimeric fusion protein toxin DAB_{486}IL-2 in advanced mycosis fungoides and the Sezary syndrome: correlation of activity and interleukin-2 receptor expression in a phase II study. *Blood* 1994; 84:1765-1774.
112. Kondo M, Takeshita T, Ishii N, Nakamura M, Watanabe S, Arai K, Sugamura K. Sharing of the interleukin 2 (IL-2) receptor γ chain between receptors for IL-2 and IL-4. *Science* 1993; 262:1874-1877.
113. Noguchi M, Nakamura Y, Russell SM, Ziegler SF, Tsang M, Cao X, Leonard WJ. Interleukin-2 receptor γ chain: a functional component of the interleukin-7 receptor. *Science* 1993; 262:1877-1880.
114. Foss FM, Koc Y, Stetler-Stevenson MA, Nguyen DT, O'Brien MC, Turner R, Sausville EA. Costimulation of cutaneous T-cell lymphoma cells by interleukin-7 and interleukin-2: potential autocrine or paracrine effectors in the Sézary syndrome. *J Clin Oncol* 1994; 12:326-335.
115. Kishimoto T, Akira S, Taga T. Interleukin-6 and its receptor: a paradigm for cytokines. *Science* 1992; 258:593-597.
116. Falini B, Pileri S, Pizzolo G, Dürkop H, Flenghi L, Stirpe F, Martelli MF, Stein H. CD30 (Ki-1) molecule: a new cytokine receptor of the tumor necrosis factor receptor superfamily as a tool for diagnosis and immunotherapy. *Blood* 1995; 85:1-14.
117. Kilian PL, Kaffka KL, Biondi DA, Lipman JM, Benjamin WR, Feldman D, Campen CA. Antiproliferative effect of interleukin-1 on human ovarian carcinoma cell line (NHI:OVCAR-3). *Cancer Res* 1991; 51:1823-1828.
118. Lewis MP, Sullivan MH, Elder MG. Regulation by interleukin-1 beta of growth and collagenase production by choriocarcinoma cells. *Placenta* 1994; 15:13-20.
119. Powell CB, Manning K, Collins JL. Interferon-alpha (IFN alpha) induces a cytolytic mechanism in ovarian carcinoma cells through a protein kinase C-dependent pathway. *Gynecol Oncol* 1993; 50:208-214.
120. Boente MP, Berchuck A, Rodriguez GC, Davidoff A, Whitaker R, Xu FJ, Marks J, Clarke-Pearson DL, Bast RC Jr. The effect of interferon gamma on epidermal growth factor receptor

expression in normal and malignant ovarian epithelial cells. *Am J Obstet Gynecol* 1992; 167: 1877–1882.
121. Hamburger AW, Pinnamaneni GD. Increased epidermal growth factor receptor gene expression by gamma-interferon in human breast carcinoma cell line. *Br J Cancer* 1991; 4:64–68.
122. Chakravarthy A, Chen LC, Mehta D, Hamburger AW. Modulation of epidermal growth factor receptors by gamma interferon in a breast cancer cell line. *Anticancer Res* 1991; 11:347–351.
123. Wu S, Boyer CM, Whitaker RS, Berchuck A, Wiener JR, Weinberg JB, Bast RC Jr. Tumor necrosis factor alpha as an autocrine and paracrine growth factor for ovarian cancer: monokine induction of tumor cell proliferation and tumor necrosis factor alpha expression. *Cancer Res* 1993; 53:1939–1944.
124. Kalthoff H, Roeder C, Gieseking J, Humburg I, Schmiegel W. Inverse regulation of human ERBB2 and epidermal growth factor receptors by tumor necrosis factor alpha. *Proc Natl Acad Sci USA* 1993; 90:8972–8976.
125. Bates S, McManaway ME, Lippman ME, Dickson RB. Characterization of estrogen responsive transforming activity in human breast cancer cell lines. *Cancer Res* 1986; 46:1707–1713.
126. Dickson RB, McManaway ME, Lippman ME. Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. *Science* 1986; 232:1540–1543.
127. Knabbe C, Zugmaier G, Schmahl M, Dietel M, Lippman ME, Dickson RB. Induction of transforming growth factor beta by the antiestrogens droloxifene, tamoxifen, and toremifene in MCF-7 cells. *Am J Clin Oncol* 1991; 14:S15–S20.
128. Kawamura I, Lacey E, Mizota T, Tsujimoto S, Nishigaki F, Manda T, Shimomura K. The effect of droloxifene on the insulin-like growth factor-I stimulated growth of breast cancer cells. *Anticancer Res* 1994; 14:427–431.
129. Le Roy X, Escot C, Brouillet JP, Theillet C, Maudelonde T, Simony-Lafontaine J, Pujol H, Rochefort H. Decrease of c-*erbB*-2 and c-*myc* RNA levels in tamoxifen-treated breast cancer. *Oncogene* 1991; 6:431–437.
130. Zheng ZS, Polakowska R, Johnson A, Goldsmith LA. Transcriptional control of epidermal growth factor receptor by retinoic acid. *Cell Growth Differ* 1992; 3:225–232.
131. Halter SA, Winnier AR, Arteaga CL. Pretreatment with vitamin A inhibits transforming growth factor alpha stimulation of human mammary carcinoma cells. *J Cell Physiol* 1993; 156: 80–87.
132. Fontana JA, Nervi C, Shao ZM, Jetten AM. Retinoid antagonism of estrogen-responsive transforming growth factor alpha and pS2 gene expression in breast carcinoma cells. *Cancer Res* 1992; 52:3938–3945.
133. Hsu HC, Yang K, Kharbanda S, Clinton S, Datta R, Stone RM. All-trans retinoic acid induces monocyte growth factor receptor (c-*fms*) gene expression in HL-60 leukemia cells. *Leukemia* 1993; 7:458–462.
134. de Gentile A, Toubert ME, Dubois C, Krawice I, Schlageter MH, Balitrand N, Castaigne S, Degos L, Rain JD, Najean Y, Chomienne C. Induction of high-affinity GM-CSF receptors during all-trans retinoic acid treatment of acute promyelocytic leukemia. *Leukemia* 1994; 8: 1758–1762.
135. Hunter T. A thousand and one protein kinases. *Cell* 1987; 50:823–829.
136. Burke TR Jr. Protein-tyrosine kinase inhibitors. *Drugs of the Future* 1992; 17:119–131.
137. Fry DW. Protein tyrosine kinases as therapeutic targets in cancer chemotherapy and recent advances in the development of new inhibitors. *Exp Opinion Invest Drugs* 1994; 3:577–595.
138. Graziani Y, Erikson E, Erikson RL. The effect of quercetin on the phosphorylation activity of the Rous sarcoma virus transforming gene product *in vitro* and *in vivo*. *Eur J Biochem* 1983; 135:583–589.
139. Cushman M, Nagarathnam D, Burg DL, Geahlen RL. Synthesis and protein-tyrosine kinase inhibitory activities of flavonoid analogues. *J Med Chem* 1991; 34:798–806.
140. Akiyama T, Ishida J, Nakagawa S, Ogawa H, Watanabe S, Itou N, Shibata M, Fukami Y. Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J Biol Chem* 1987; 262: 5592–5595.
141. Imoto M, Umezawa K, Isshiki K, Kanimoto S, Takeuchi T, Umezawa H. Kinetic studies of tyrosine kinase inhibition by erbstatin. *J Antibiot* 1987; 40:1471–1473.
142. Simizu S, Imoto M, Umezawa K. Induction of apoptosis by erbstatin in mouse leukemia L1210 cells. *Biosci Biotech Biochem* 1994; 58:1549–1552.

143. Daya-Makin M, Pelech SL, Levitzki A, Hudson AT. Erbstatin and tyrphostins block protein-serine kinase activation and meiotic maturation of sea star oocytes. *Biochim Biophys Acta* 1991; 1093:87-94.
144. Smyth MS, Stefanova I, Hartmann F, Horak ID, Osherov N, Levitzki A, Burke TR Jr. Non-amine based analogues of lavendustin A as protein-tyrosine kinase inhibitors. *J Med Chem* 1993; 36:3010-3014.
145. Nussbaumer P, Winiski AP, Cammisuli S, Hiestand P, Weckbecker G, Stütz A. Novel anti-proliferative agents derived from lavendustin A. *J Med Chem* 1994; 37:4079-4084.
146. Slate DL, Lee RH, Rodriguez J, Crews P. The marine natural product, halistanol trisulfate, inhibits pp60$^{v\text{-}src}$ protein tyrosine kinase activity. *Biochem Biophys Res Commun* 1994; 203: 260-264.
147. Ōmura S, Iwai Y, Takahashi Y, Sadakane N, Nakagawa A, Ōiwa H, Hasegawa Y, Ikai T. Herbimycin, a new antibiotic produced by a strain of *Streptomyces*. *J Antibiot* 1979; 32:255-261.
148. Uehara Y, Hori M, Takeuchi T, Umezawa H. Phenotypic change from transformed to normal induced by benzoquinonoid ansamycins accompanies inactivation of p60^{src} in rat kidney cells infected with Rous sarcoma virus. *Mol Cell Biol* 1986; 6:2198-2206.
149. Murakami Y, Mizuno S, Hori M, Uehara Y. Reversal of transformed phenotypes by herbimycin A in *src* oncogene expressed rat fibroblasts. *Cancer Res* 1988; 48:1587-1590.
150. Oikawa T, Hirotani K, Shimamura M, Ashino-Fuse H, Iwaguchi T. Powerful antiangiogenic activity of herbimycin A (named angiostatic antibiotic). *J Antibiot* 1989; 42:1202-1204.
151. Uehara Y, Murakami Y, Sugimoto Y, Mizuno S. Mechanism of reversion of Rous sarcoma virus transformation by Herbimycin A: reduction of total phosphotyrosine levels due to reduced kinase activity and increased turnover of p60$^{v\text{-}src}$. *Cancer Res* 1989; 49:780-785.
152. Murakami Y, Mizuno S, Uehara Y. Accelerated degradation of 160 kDa epidermal growth factor (EGF) receptor precursor by the tyrosine kinase inhibitor herbimycin A in the endoplasmic reticulum of A431 human epidermoid carcinoma cells. *Biochem J* 1995; 301:63-68.
153. Miller P, DiOrio C, Moyer M, Schnur RC, Bruskin A, Cullen W, Moyer JD. Depletion of the *erbB*-2 gene product p185 by benzoquinoid ansamycins. *Cancer Res* 1994; 54:2724-2730.
154. Whitesell L, Shifrin SD, Schwab G, Neckers LM. Benzoquinonoid ansamycins possess selective tumoricidal activity unrelated to *src* kinase inhibition. *Cancer Res* 1992; 52:1721-1728.
155. Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM. Inhibition of heat shock protein HSP90-pp60$^{v\text{-}src}$ heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proc Natl Acad Sci USA* 1994; 91:8324-8328.
156. Preis PN, Saya H, Nádasdi L, Hochhaus G, Levin V, Sadée W. Neuronal cell differentiation of human neuroblastoma cells by retinoic acid plus herbimycin A. *Cancer Res* 1988; 48: 6530-6534.
157. Honma Y, Okabe-Kado J, Hozumi M, Uehara Y, Mizuno S. Induction of erythroid differentiation of K562 human leukemic cells by herbimycin A, an inhibitor of tyrosine kinase activity. *Cancer Res* 1989; 49:331-334.
158. Yaish P, Gazit A, Gilon C, Levitzki A. Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. *Science* 1988; 242:933-935.
159. Gazit A, Yaish P, Gilon C, Levitzki A. Tyrphostins I: synthesis and biological activity of protein tyrosine kinase inhibitors. *J Med Chem* 1989; 32:2344-2352.
160. Gazit A, Osherov N, Posner I, Yaish P, Poradosu E, Gilon C, Levitzki A. Tyrphostins. Heterocyclic and α-substituted benzylidenemalononitrile tyrphostins as potent inhibitors of EGF receptor and ErbB2/neu tyrosine kinases. *J Med Chem* 1991; 34:1896-1907.
161. Levitzki A. Tyrphostins: tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction. *FASEB J* 1992; 6:3275-3282.
162. Levitzki A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science* 1995; 267:1782-1788.
163. Aflalo E, Iftach S, Segal S, Gazit A, Priel E. Inhibition of topoisomerase I activity by tyrphostin derivatives, protein tyrosine kinase blockers: mechanism of action. *Cancer Res* 1994; 54:5138-5142.
164. Lyall RM, Zilberstein A, Gazit A, Gilon C, Levitzki A, Schlessinger J. Tyrphostins inhibit epidermal growth factor (EGF)-receptor tyrosine kinase activity in living cells and EGF-stimulated cell proliferation. *J Biol Chem* 1989; 264:14,503-14,509.

165. Yoneda T, Lyall RM, Alsina MM, Persons PE, Spada AP, Levitzki A, Zilberstein A, Mundy GR. The antiproliferative effects of tyrosine kinase inhibitors tyrphostins on a human squamous cell carcinoma in vitro and in nude mice. *Cancer Res* 1991; 51:4430–4435.
166. Anafi M, Gazit A, Gilon C, Ben-Neriah Y, Levitzki A. Selective interactions of transforming and normal *abl* proteins with ATP, tyrosine-copolymer substrates, and tyrphostins. *J Biol Chem* 1992; 267:4518–4523.
167. Kaur G, Gazit A, Levitzki A, Stowe E, Cooney DA, Sausville EA. Tyrphostin induced growth inhibition: correlation with effect on $p210^{bcr\text{-}abl}$ autokinase activity in K562 chronic myelogenous leukemia. *Anti-Cancer Drugs* 1994; 5:213–222.
168. Anafi M, Gazit A, Zehavi A, Ben-Neriah Y, Levitski A. Tyrphostin-induced inhibition of $p210^{bcr\text{-}abl}$ tyrosine kinase activity induces K562 to differentiate. *Blood* 1993; 82:3524–3529.
169. Bryckaert MC, Eldor A, Fontenay M, Gazit A, Osherov N, Gilon C, Levitzki A, Tobelem G. Inhibition of platelet-derived growth factor-induced mitogenesis and tyrosine kinase activity in cultured bone marrow fibroblasts by tyrphostins. *Exp Cell Res* 1992; 199:255–261.
170. Kovalenko M, Gazit A, Böhmer A, Rorsman C, Rönnstrand L, Heldin CH, Waltenberger J, Böhmer FD, Levitzki A. Selective platelet-derived growth factor receptor kinase blockers reverse *sis*-transformation. *Cancer Res* 1994; 54:6106–6114.
171. Fry DW, Kraker AJ, McMichael A, Ambroso LA, Nelson JM, Leopold WR, Connors RW, Bridges AJ. A specific inhibitor of the epidermal growth factor receptor tyrosine kinase. *Science* 1994; 265:1093–5.
172. Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ, Neel BG, Birge RB, Fajardo JE, Chou MM, Hanafusa H, Schaffhausen B, Cantley LC. SH2 domains recognize specific phosphopeptide sequences. *Cell* 1993; 72:767–778.
173. Waksman G, Kominos D, Robertson SC, Pant N, Baltimore D, Birge RB, Cowburn D, Hanafusa H, Mayer BJ, Overduin M, Resh MD, Rios CB, Silverman L, Kuriyan J. Crystal structure of the phosphotyrosine recognition domain SH2 of v-*src* complexed with tyrosine-phosphorylated peptides. *Nature* 1992; 358:646–653.
174. Burke TR Jr, Smyth MS, Otaka A, Nomizu M, Roller PP, Wolf G, Case R, Shoelson SE. Nonhydrolyzable phosphotyrosyl mimetics for the preparation of phosphatase-resistant SH2 domain inhibitors. *Biochemistry* 1994; 33:6490–6494.
175. Harris W, Hill CH, Lewis EJ, Nixon JS, Wilkinson SE. Protein kinase C inhibitors. *Drugs of the Future* 1993; 18:727–735.
176. Tamaoki T, Nakano H. Potent and specific inhibitors of protein kinase C of microbial origin. *Bio/Technology* 1990; 8:732–735.
177. Ruegg UT, Burgess GM. Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends Pharmacol Sci* 1989; 10:218–220.
178. Seynaeve CM, Kazanietz MG, Blumberg PM, Sausville EA, Worland PJ. Differential inhibition of protein kinase C isozymes by UCN-01, a staurosporine analogue. *Mol Pharmacol* 1994; 45:1207–1214.
179. Seynaeve CM, Stetler-Stevenson M, Sebers S, Kaur G, Sausville EA, Worland PJ. Cell cycle arrest and growth inhibition by the protein kinase antagonist UCN-01 in human breast carcinoma cells. *Cancer Res* 1993; 53:2081–2086.
180. Akinaga S, Gomi K, Morimoto M, Tamaoki T, Okabe M. Antitumor activity of UCN-01, a selective inhibitor of protein kinase C, in murine and human tumor models. *Cancer Res* 1991; 51:4888–4892.
181. Wang Q, Worland PJ, Clark JL, Carlson BA, Sausville EA. Apoptosis in 7-hydroxystaurosporine-treated T lymphoblasts correlates with activation of cyclin-dependent kinases 1 and 2. *Cell Growth Differ* 1995; 6:927–936.
182. Shi L, Nishioka WK, Thing J, Bradburg EM, Litchfield DW, Greenbury AM. Premature $p34^{cdc2}$ activation required for apoptosis. *Science* 1994; 263:1143–1145.
183. Davis PD, Elliott LH, Harris W, Hill CH, Hurst SA, Keech E, Kumar MKH, Lawton G, Nixon JS, Wilkinson SE. Inhibitors of protein kinase C. Substituted bisindolylmaleimides with improved potency and selectivity. *J Med Chem* 1992; 35:994–1001.
184. Bit RA, Davis PD, Elliott LH, Harris W, Hill CH, Keech E, Kumar H, Lawton G, Maw A, Nixon JS, Vesey DR, Wadsworth J, Wilkinson SE. Inhibitors of protein kinase C. Potent and highly selective bisindolylmaleimides by conformational restriction. *J Med Chem* 1993; 36:21–29.

185. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* 1991; 266: 15,771-15,781.
186. Zhang L, Higuchi M, Totpal K, Chaturvedi MM, Aggarwal BB. Staurosporine induces the cell surface expression of both forms of human tumor necrosis factor receptors on myeloid and epithelial cells and modulates ligand-induced cellular response. *J Biol Chem* 1994; 269:10,270-10,279.
187. Sampson LE, Mire-Sluis A, Meager A. Protein kinase C-dependent phosphorylation is involved in resistance to tumour necrosis factor-alpha-induced cytotoxicity in a human monocytoid cell line. *Biochem J* 1993; 292:289-294.
188. Coppock DL, Tansey JB, Nathanson L. 12-*O*-tetradecanoylphorbol-13-acetate induces transient cell cycle arrest in G_1 and G_2 in metastatic melanoma cells: inhibition of phosphorylation of $p34^{cdc2}$. *Cell Growth Differ* 1992; 1:485-1194.
189. Berkow RL, Kraft AS. Byrostatin, a non-phorbol macrocyclic lactone, activates intact human polymorphonuclear leukocytes and binds to the phorbol ester receptor. *Biochem Biophys Commun* 1985; 131:1109-1116.
190. Kraft AS, Smith JB, Berkow RL. Byrostatin, an activator of the calcium phospholipid-dependent protein kinase, blocks phorbol ester-induced differentiation of human promyelocytic leukemia cells, HL-60. *Proc Natl Acad Sci USA* 1986; 83:1334-1338.
191. Gebbia V, Citarrella P, Miserendino V, Valennza R, Borsellino N. The effects of the macrocytic lactone bryostatin-1 on leukemic cells in vitro. *Tumori* 1992; 78:167-171.
192. Nurse P. Ordering S phase and M phase in the cell cycle. *Cell* 1994; 79:547-550.
193. Sherr CJ. G1 phase progression: cycling on cue. *Cell* 1994; 79:551-555.
194. Vesely J, Havlicek L, Strnad M, Blow J, Donella-Deanna A, Pinna L, Letham DS, Kato J, Detivaud L, Leclerc S, Meijer L. Inhibition of cyclin-dependent kinases by purine analogues. *Eur J Biochem* 1994; 224:771-786.
195. Kitagawa M, Okabe T, Ogino H, Matsumoto H, Suzuki-Takahashi I, Kokubo T, Higashi M, Saitoh S, Taya Y, Yasuda H, Ohba Y, Nishimura S, Tanaka N, Okuyama A, Butyrolactone I. A selective inhibitor of cdk2 and cdc2 kinase. *Oncogene* 1993; 8:2425-2432.
196. Kaur G, Stetler-Stevenson M, Sebers S, Worland P, Sedlacek H, Myers C, Czech J, Naik R, Sausville E. Growth inhibition with reversible cell cycle arrest of carcinoma cells by flavone L86-8275. *J Natl Cancer Inst* 1992; 84:1736-1740.
197. Losiewicz MD, Carlson BA, Kaur G, Sausville EA, Worland PJ. Potent inhibition of Cdc2 kinase activity by the flavonoid L86-8275. *Biochem Biophys Res Commun* 1994; 201:589-595.
198. Worland PJ, Kaur G, Stetler-Stevenson M, Sebers S, Sartor O, Sausville EA. Alteration of the phosphorylation state of $p34^{cdc2}$ kinase by the flavone L86-8275 in breast carcinoma cells. Correlation with decreased H1 kinase activity. *Biochem Pharmacol* 1993; 46:1831-1840.
199. Carlson BA, Sausville EA, Worland PJ. Flavopiridol induced G1-S phase block in MDA-MB-468 breast carcinoma cells with deregulation of CDK2. *Proc Am Assoc Cancer Res* 1995; 36:35.
200. Czech J, Hoffman D, Naik R, Sedlacek HH. Antitumoral activity of flavone L 86-8275. *Int J Oncol* 1995; 6:31-36.
201. Fry MJ. Structure, regulation and function of phosphoinositide 3-kinases. *Biochim Biophys Acta* 1994; 1226:237-268.
202. Escobedo JA, Kaplan DR, Kavanaugh WM, Turck CW, Williams LT. A phosphatidylinositol-3 kinase binds to platelet-derived growth factor receptors through a specific receptor sequency containing phosphotyrosine. *Mol Cell Biol* 1991; 11:1125-1132.
203. Lev S, Givol D, Yarden Y. Interkinase domain of kit contains the binding site for phosphatidylinositol 3′ kinase. *Proc Natl Acad Sci USA* 1992; 89:678-682.
204. Backer JM, Myers MG Jr, Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, Hu P, Margolis B, Skolnik EY, Schlessinger J, White MF. Phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J* 1992; 11:3469-3479.
205. Raffioni S, Bradshaw RA. Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basis fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells. *Proc Natl Acad Sci USA* 1992; 89:9121-9125.

206. Yamanashi Y, Fukui Y, Wongsasant B, Kinoshita Y, Ichimori Y, Toyoshima K, Yamamoto T. Activation of src-like protein-tyrosine kinase Lyn and its association with phosphatidylinositol 3-kinase upon B-cell antigen receptor-mediated signaling. *Proc Natl Acad Sci USA* 1992; 89: 1118–1122.
207. Gold MR, Chan VW-F, Turck CW, DeFranco AL. Membrane Ig cross-linking regulates phosphatidylinositol 3-kinase in B lymphocytes. *J Immunol* 1992; 148:2012–2022.
208. Thompson PA, Gutkind JS, Robbins KC, Ledbetter JA, Bolen JB. Identification of distinct populations of PI-3 kinase activity following T-cell activation. *Oncogene* 1992; 7:719–725.
209. Prasad KVS, Janssen O, Kapeller R. Raab M, Cantley LC, Rudd CE. Src-homology 3 domain of protein kinase $p59^{fyn}$ mediates binding to phosphatidylinositol 3-kinase in T cells. *Proc Natl Acad Sci USA* 1993; 90:7366–7370.
210. Fantl WJ, Escobedo JA, Martin GA, Turck CW, del Rosario M, McCormick F, Williams LT. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell* 1992; 69:413–423.
211. Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomura Y, Matsuda Y. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. *J Biol Chem* 1993; 268:25,846–25,856.
212. Powis G, Bonjouklian R, Berggren MM, Gallegos A, Abraham R, Ashendel C, Zalkow L, Matter WF, Dodge J, Grindley G, Vlahos CJ. Wortmannin, a potent a selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res* 1994; 54:2419–2423.
213. Kiguchi K, Glesne D, Chubb CH, Fujiki H, Huberman E. Differential induction of apoptosis in human breast tumor cells by okadaic acid and related inhibitors of protein phosphatases 1 and 2A. *Cell Growth Differ* 1994; 5:995–1004.
214. Casillas AM, Amaral K, Chegini-Farahani S, Nel AE. Okadaic acid activates p42 mitogen-activated protein kinase (MAP kinase; ERK-2) in B-lymphocytes but inhibits rather than augments cellular proliferation: contrast with phorbol 12-myristate 13-acetate. *Biochem J* 1993; 290:545–550.
215. Roberg M, Tudan C, Hung SMF, Harder KW, Jirik FR, Anderson H. Antitumor drug fostriecin inhibits the mitotic entry checkpoint and protein phosphatases 1 and 2A. *Cancer Res* 1994; 54:6115–6121.
216. Crooke ST, Bennett CF. Mammalian phosphoinositide-specific phospholipase C isoenzymes. *Cell Calcium* 1989; 10:309–323.
217. Merritt JE, Rink TJ. The effects of substance P and carbachol on inositol tris- and tetrakisphosphate formation and cytosolic free calcium in rat parotid acinar cells. *J Biol Chem* 1987; 262:14,912–14,916.
218. Perrella FW, Chen SF, Behrens DL, Kaltenbach RF III, Seitz SP. Phospholipase C inhibitors: a new class of cytotoxic agents. *J Med Chem* 1994; 37:2232–2237.
219. Kohn EC, Sandeen MA, Liotta LA. In vivo efficacy of a novel inhibitor of selected signal transduction pathways including calcium, arachidonate, and inositol phosphates. *Cancer Res* 1992; 52:3208–3212.
220. Viallet J, Sharoni Y, Frucht H, Jensen RT, Minna JD, Sausville EA. Cholera toxin inhibits signal transduction by several mitogens and the *in vitro* growth of small cell lung cancer. *J Clin Invest* 1990; 86:1904–1912.
221. Bunn PA Jr, Chan D, Stewart J, Gera L, Tolley R, Jewett R, Tagawa M, Alford C, Mochzuki T, Yanaihara N. Effects of neuropeptide analogues on calcium flux and proliferation in lung cancer cell lines. *Cancer Res* 1994; 54:3602–3610.
222. Gordon J, Melamed MD, Ley SC, Hughes-Jones NC. Anti-immunoglobulin inhibits DNA synthesis in Epstein Barr virus-transformed lymphoblastoid cell lines. *Immunology* 1984; 52:79–85.
223. Ashwell JD, Longo DL, Bridges SH. T-cell tumor elimination as a result of T-cell receptor-mediated activation. *Science* 1987; 237:61–64.
224. Beckwith M, Urba WJ, Ferris DK, Freter CE, Kuhns DB, Moratz CM, Longo DL. Anti-IgM-mediated growth inhibition of a human B lymphoma cell line is independent of phosphatidylinositol turnover and protein kinase C activation and involves tyrosine phosphorylation. *J Immunol* 1991; 147:2411–2418.
225. Bridges SH, Kruisbeek AM, Longo DL. Selective in vivo antitumor effects of monoclonal anti-I-A antibody on B cell lymphoma. *J Immunol* 1987; 139:4242–4249.

226. Sussman JJ, Saito T, Shevach EM, Germain RN, Ashwell JD. Thy-1 and Ly-6-mediated lymphokine production and growth inhibition of a T cell hybridoma require coexpression of the T cell antigen receptor complex. *J Immunol* 1988; 140:2520–2526.
227. Funakoshi S, Longo DL, Beckwith M, Conley DK, Tsarfaty G, Tsarfaty I, Armitage RJ, Fanslow WC, Spriggs MK, Murphy WJ. Inhibition of human B-cell lymphoma growth by CD40 stimulation. *Blood* 1994; 83:2787–2789.
228. Aboud-Pirak E, Hurwitz E, Pirak ME, Bellot F, Schlessinger J, Sela M. Efficacy of antibodies to epidermal growth factor receptor against KB carcinoma in vitro and in nude mice. *J Natl Cancer Inst* 1988; 80:1605–1611.
229. Hancock MC, Langton BC, Chan T, Toy P, Monahan JJ, Mischak RP, Shawver LK. A monoclonal antibody against the c-erB-2 protein enhances the cytotoxicity of cis-diamminedichloroplatinum against human breast and ovarian tumor cell lines. *Cancer Res* 1991; 51:4575–4580.
230. Pietras RJ, Fendly BM, Chazin VR, Pegram MD, Howell SB, Slamon DJ. Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. *Oncogene* 1994; 9:1829–1838.
231. Kohn KW, Jackman J, O'Connor PM. Cell cycle control and cancer chemotherapy. *J Cell Biochem* 1994; 54:440–452.

17 Immunoconjugates

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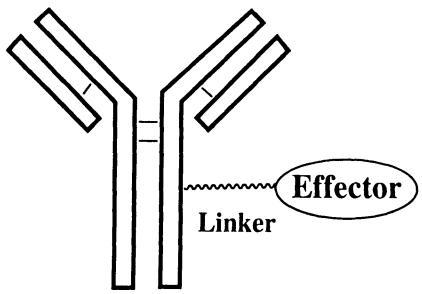
CONTENTS

1. INTRODUCTION

Successful anticancer drugs must exploit known or unknown, gross or ever so subtle, differences between normal and malignant cells. The development of immunotoxins is one of the first attempts to develop rationally anticancer drugs that are based on known cellular differences associated with cancer cells. Much immunological evidence had accumulated that transformed cells express tumor-specific antigens. However, it was difficult to generate heterosera with well-defined antitumor reactivity. The isolation in 1967 of an agglutinin from wheat germ that identified a tumor-specific determinant on neoplastic cell surfaces (*1*) marked the first time that a pure molecular species was available for targeting of tumors.

Further probing of cell surfaces with lectins and agglutinins, however, was hampered by the availability of only a small number of lectins with an even smaller number of different binding specificities. This situation changed dramatically with the advent of the monoclonal antibody (MAb) technology (*2*). The potential for generating a nearly unlimited reservoir of reagents each with its own binding specificity for an antigen was rapidly exploited in creating MAbs that bound to novel tumor cell-specific antigens. Although some naked antibodies were used in clinical tests for the treatment of cancer, many immunologists doubted that the humoral part of the immune system would have sufficient cytotoxic potential to eliminate millions of tumor cells. MAb were, therefore, armed with extraneous cytotoxic effector functions and became delivery vehicles that imparted tumor specificity to otherwise nonselective cytotoxic effector molecules.

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ



Effector	Conjugate
Radioisotope	Radioimmunoconjugate
Toxin	Immunotoxin
Cytotoxic Drug	Antibody-Drug Conjugate

Fig. 1. Schematic representation of immunoconjugates.

The covalent binding of an effector molecule to an MAb yields an immunoconjugate (Fig. 1), which is called an immunotoxin, when the effector molecule is a toxin, an antibody–drug conjugate when cytotoxic drugs are used as effectors, and a radioimmunoconjugate in the case of linked radioisotopes. Common to all three methodologies is their reliance on the tumor-specific binding of their MAb component. Therefore, we shall first discuss the generation of "tumor-specific" MAbs and then describe the development and testing of radioimmunopharmaceuticals, of immunotoxins, and of antibody–drug conjugates.

2. TUMOR-SPECIFIC ANTIBODIES

The ideal MAb for the generation of immunoconjugates would bind to an antigen exclusively present on the surface of tumor cells, and would further be expressed homogeneously on all tumor cells or at least on all tumor stem cells (the latter, however, is difficult to assay). In addition, the antigen should not be shed from cells, should not be present in the serum of patients, and ideally, for practical medical and commercial reasons, should be present on the tumors of all patients with the same type of cancer.

In the infancy of immunotoxin development, several MAb were claimed to be tumor-specific. However, the development and use of more thorough analytical methods, such as analysis with a fluorescence activated cell sorter (FACS), sensitive immunohistochemical staining techniques using large panels of fresh-frozen tissue sections, and modern biochemical and molecular biological techniques, contributed to today's generally accepted view that most antibodies recognize tumor-associated antigens that are expressed only preferentially on tumors. Some antigens may be found on only a limited number of tissues, whereas others are on only one specific tissue type and are, therefore, tissue-specific. In the best case, some tumor-associated antigens may be expressed only during a particular developmental stage of a certain cell type. Some degree of tumor specificity often presents itself by the overexpression of certain surface antigens on transformed cells, such as *erb*B-2/*HER*-2 on breast tumor cells of a subgroup of patients (*3*), or certain carbohydrate antigens on epidermoid carcinomas (*4*). The only surface antigens that are absolutely tumor-specific are the surface immunoglobulin or idiotype present on the cells of B-cell leukemia and lymphomas, and the clonotypic T-cell receptor on T-cell leukemia and lymphoma cells. Not only are these structures tumor-specific, but individualized, patient-specific MAbs have been created (*5*).

To generate MAb with antihuman tumor reactivity, typically mice or rats were immunized with whole cells or cell membrane preparations from tumor cell lines or from tumor biopsies. The spleens of the immunized animals were then used to generate and select antibody-producing hybridomas. However, many MAbs used in immunoconjugates, in particular, antibodies reactive with hematopoietic cells, were originally developed as research tools to differentiate between various normal cell types and were, therefore, generated by injecting animals with normal human cells, such as the various cell types from blood.

MAb that have the potential to be used in anticancer immunoconjugates are conveniently grouped into those that react with hematopoietic tumors and those that bind to antigens on solid tumors. Because of the rapid renewal of hematopoietic cells and the experience of regeneration of blood cells after bone marrow transplantation, tissue-specific antibodies were widely used in immunoconjugates against leukemias and lymphomas. T-cell malignancies were treated, for example, with conjugates binding to the T-cell markers CD5, CD7, or the IL-2 receptor β-chain (CD 25); B-cell malignancies with antibody conjugates against the B-cell differentiation antigens CD19, CD20, and CD22; and analogously, myeloid malignancies with conjugates against the myeloid marker CD33 (*6*). Most of these antigens are differentiation antigens that are expressed throughout the ontogeny of a particular cell type starting at the earliest lineage restricted stage to ensure that the conjugates were able to treat the yet unidentified clonogenic tumor cells.

It has been much more difficult to identify cell-surface markers useful for immunoconjugates against solid tumors. The principle of tissue specificity is not as easily applied as in the hematopoietic area, except possibly for tumors of nonessential tissues, where the temporary removal of certain cell populations may be tolerated. In the absence of tumor specificity and tissue specificity, the selection of antigens was largely based on their overexpression on tumor cells relative to normal tissues. For lists of possible candidate surface antigens for immunoconjugate targeting, the reader is referred to two comprehensive reviews (*7,8*).

For the development of highly cytotoxic immunoconjugates that bind to antigens also expressed on some normal tissues, although hopefully at lower levels, it was essential to find animal models for toxicity studies, where similar crossreactivity was observed. Fortunately, many of the antigenic determinants were found to be preserved in nonhuman primates where they were expressed with a similar tissue distribution as in humans. A good example is the data presented for the anti-Le^y antibody in ref. (*4*).

A problem commonly encountered in solid tumors is the heterogeneous expression of an antigen on cells of a given tumor. Although some cells may express large numbers of an antigen on their surface, other cells in the same biopsy sample, equally having a transformed phenotype, may be antigen-negative. If transformation is a clonogenic event, then these different cell populations may represent differentiation stages that are not necessarily all tumorigenic. Heterogeneous expression of an antigen may, therefore, not necessarily disqualify it from being a target for therapeutic immunoconjugates.

If one surveys the known antigenic cell-surface markers for human solid tumors, (*see*, e.g., *7,8*), one is struck by the paucity of such known markers. Also, when antibodies were generated with different tumor tissues or tumor cell lines, often antibodies to the same antigens were generated. For example, when mice were immunized with the breast tumor line MCF-7, MAb B1 and B3 were obtained that reacted with the Le^y carbohydrate chain (*4*), and immunization with cell line H3396 derived from a

metastatic breast adenocarcinoma yielded antibodies BR64 and BR96, both of which also react with the Le^y carbohydrate chain (*9*). These results are a reflection of the limitations of the immunological methodology used to identify these antigens. They probably represent the most immunodominant markers recognized by the murine immune system, and only the screening of much larger panels of hybridomas, a work-intensive and time-consuming undertaking, might allow the discovery of further novel antigens with this technology. This realization, far from being discouraging, predicts that we have barely scratched the surface for the discovery of tumor cell-surface markers for therapeutic targeting, and it has spawned the development of several new methodologies. The most promising techniques might be the phage display of the entire murine or human immunological repertoire and its use in the probing of cell surfaces (*10*), or the searching for interactions on cell surfaces with combinatorial libraries of peptides that carry their genetic information in the form of amplifiable DNA sequences (*11*).

In most patients treated with murine MAb, a prompt human antimurine antibody (HAMA) response was observed, which led to the development of several "humanization" technologies. Humanization is the attempt to give murine antibodies an appearance that is not recognized as foreign by the human immune system while preserving their specificity and binding avidity.

It was well known that heterosera against xenogeneic immunoglobulins largely reacted with the constant region or Fc portion of the molecule, and the first approach at "humanization" was therefore the genetic construction of chimeric antibodies, comprising the murine variable region and the human constant region of IgG (*12*). Most chimeric antibodies displayed much reduced immunogenecity, but a response to the murine Fv portion could ultimately be observed. In reshaped or CDR-grafted antibodies, the murine content was further reduced by grafting the murine complementary determining regions (CDRs) or hypervariable region onto a human variable region framework (*13*). These antibodies were generally found not to be immunogeneic, but it was often difficult to maintain the binding affinity of the parent murine antibodies. Further amino acid changes in the framework region are generally necessary to maintain the original conformations of the CDRs. These changes need to be deduced for each antibody through computer model building, and the ultimate success—preservation of full binding—is often difficult to achieve even with extensive changes that potentially negate the advantage of CDR grafting over chimerization. In the newest approach, called variable domain resurfacing (*14*), the affinity is maintained by retaining the CDRs and the core of the murine variable region framework. Only the surface residues of the murine variable region framework are replaced by those from a human variable region. A simple algorithm predicts the necessary changes in the framework region, and when this method was applied to two murine antibodies, their affinities were unaffected (*14*). This approach assumes that the immunogenecity of murine antibody variable regions is determined by the accessible surface residues only, an assumption not yet tested with globulins, but generally accepted for the antigenecity of proteins (*15,16*).

3. RADIOIMMUNOCONJUGATES

Ever since the appreciation of the cytocidal effects of high doses of radiation, oncologists have attempted to harness the energy of radioactivity to eradicate tumors in

patients afflicted with cancer. The goal of radiotherapy is to deliver a sufficiently high dose of radiation locally to the tumor in order to sterilize the tumor without causing lethal damage to the surrounding tissues. Successful killing of all tumor cells requires radiation doses of at least 60 gy to be concentrated at the tumor site, which is at the limit of the dose that can be delivered by external beam radiation while sparing normal tissue. Unfortunately, the wide application of external beam radiotherapy, while improving survival, has rarely resulted in cure. The notion that the ability of oncologists to eradicate tumors could be improved by in vivo administration of a radionuclide was first developed using iodine-131 to treat thyroid carcinomas, which concentrate radioiodine from blood resulting in delivery of local tumoricidal doses of 80–300 gy (*17*).

Radioimmunoconjugate therapy, which exploits the availability of specific antibodies that can localize to tumor cells, has been under investigation for a number of years as one way of improving radiotherapy. The hope of radioimmunoconjugate therapy is that targeting of radioactivity by antibodies could overcome two drawbacks of external beam radiotherapy: (1) specific targeting by radiolabeled antibodies should allow more precise delivery of the radiation dose to the tumor with concomitant sparing of a greater amount of the surrounding normal tissue; and (2) radiolabeled antibody will deliver a radiation dose to small undetected areas of tumor or micrometastases.

Radionuclides that are useful for radioimmunoconjugate therapy must emit particles whose energy can be deposited locally, ideally within a radius that encompasses one or a few cells. Furthermore, such radionuclides should have relatively short half-lives, so that radioactivity incorporated into the patient decays within a reasonable period of time, and in addition, they should be isotopes of elements whose chemistry allows them to be readily conjugated to antibodies. Several radioisotopes that may meet these criteria and that have been used in trials of radioimmunoconjugate therapy are shown in Table 1.

Chemically, the radioisotopes shown in Table 1 comprise two groups, the radiometals and radioactive isotopes of iodine. Iodine (and astatine) is generally conjugated directly to tyrosine residues in antibodies simply by mixing the protein with sodium iodide in the presence of an oxidizing agent, such as Chloramine T or related compounds (*20*). The reaction is extremely rapid, even at 0 °C, although one must take care to avoid damage to the antibody by excessive oxidation. Alternatively, radioiodine can be conjugated to antibodies using iodinated compounds that allow labeling without exposing the protein to oxidative conditions, and furthermore, allow the possibility of utilizing iodinated compounds that are not subject to enzymatically catalyzed dehalogenation (*21–23*).

The radioactive metals are conjugated to antibodies by the use of chelating agents that are in turn chemically linked to the protein. Although the early chelates have high stability constants, they are kinetically labile, and in vivo, the radiometal readily exchanges into metal-transport proteins, such as transferrin, thereby losing any target specificity. Once lost from a conjugated chelate, a radiometal, such a yttrium-90, can ultimately be deposited in bone, resulting in prolonged irradiation of bone marrow. Recently, chelating agents that "cage" the metal and are far more stable have been developed for diagnostic and therapeutic applications with antibodies (*24,25*). Figure 2 illustrates the structure of two such antibody-conjugated macrocyclic chelators, which are ideal reagents for binding copper-67 and yttrium-90. In vivo studies show that radiometals targeted by antibodies linked to caged chelating agents have greatly

Table 1
Radionuclides with Potential for Radioimmunotherapy[a]

Radioisotope	*Half-life*	*Decay particle*	*Particle energy, maximum energy, MeV*	*Path length, mm[b]*	*Comments*
Astatine-211	7.2 h	α	5.9	0.04–0.08	Iodine chemistry
Bismuth-212	1 h	α	6.1	0.04–0.08	
Copper-67	2.4 d	β	0.57	0.6	γ-Emission for imaging
Iodine-125	60.1 d	Auger electron (electron capture)	7.5	0.001–0.02	Requires internalization for cytocidal effect
Iodine-131	8.1 d	β	0.81	0.8	High-energy γ-emission for imaging
Rhenium-186	3.5 d 3.7 d	β	1.1 and 0.93	1.8	γ-Emission for imaging
Rhenium-188	17 h	β	2.1	4.4	γ-Emission for imaging
Yttrium-90	2.5 d	β	2.7	5.3	

[a] Compiled from published data (*6,18,19*).
[b] The path length is defined as the radius of a sphere within which 90% of the energy emitted by a radionuclide is absorbed (*19*).

improved tumor localization of the radioactivity, with less deposition into bone and less marrow toxicity (*26*).

The β-emitters, yttrium-90 and iodine-131, have been the radioisotopes used most extensively in therapeutic studies to date, more because of their ready availability than because they have the most ideal characteristics for therapy (*27,28*). Iodine-131 is a medium-range β-emitter whose energy is absorbed within one or two cell diameters, whereas the more energetic β-particle of yttrium-90 can penetrate several cell diameters. This is the basis for the theoretical benefit of using radionuclides as the effector killing moiety for antibody-directed therapy, namely that the antigen targeted by the antibody need not be expressed on all of the tumor cells in a tumor mass. Thus, antigen-negative tumor cells may also be killed by the radiation concentrated at the tumor by antigen-positive tumor cells (a "bystander" killing effect). The α-emitters may not share this potential advantage because of the extremely short range of α-particles. However, this property could be an advantage when targeting an antigen expressed homogeneously on all tumor cells and that is internalized by the cells, in that a higher proportion of the energy of the radiation is deposited in the target cell. Unfortunately, the two α-emitters with appropriate chemical properties for conjugation, bismuth-212 (*29*) and astatine-211 (*30*), have very short half-lives, which may reduce their effectiveness in vivo (*27*), and which presents logistical difficulties in their use.

The fate of the antigen/antibody complex on the surface of the tumor cell will influence the best choice of radioisotope or method of linking it to the antibody. Radioiodine is retained better in tumor tissues if it is targeted by an antibody that is not internalized. Otherwise, on internalization, radioimmunoconjugates are enzymatically degraded and dehalogenated with the consequence that the radioactivity

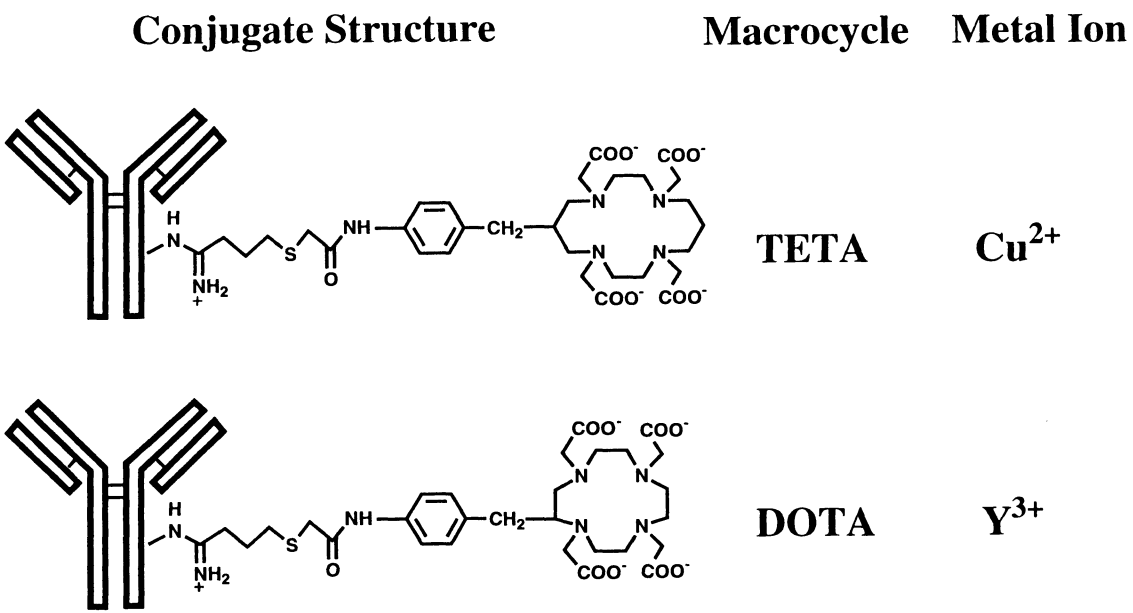


Fig. 2. Structural formula of conjugated macrocyclic chelators for copper and yttrium ions.

rapidly diffuses away and is cleared (*6,18*). Internalizing antibodies are better suited for targeting radiometals, such as yttrium-90 and copper-67, which are retained by the target cells on internalization and eventual degradation of the radioimmunoconjugate (*6*), since cellular proteins are generally good chelators of transition metals. Antibodies that target antigens that readily internalize are necessary for targeting iodine-125 whose decay produces Auger electrons of extremely short path length requiring proximity to the nucleus to elicit cell killing. Iodine-125 is therefore best conjugated via nonmetabolizable adducts (*21–23*).

There has been some debate about what are the most desirable properties for the antibody component of a radioimmunoconjugate, given a high specificity for an antigen selectively expressed on tumor tissue. In contrast to diagnostic uses of radioimmunoconjugates, where the most important parameters are (1) a high ratio of radioisotope delivered to the tumor compared with that delivered to normal tissue and (2) rapid clearance of radioisotope from the blood pool, which otherwise masks the radioactivity concentrated at the tumor (*31*), the most important factor for the radiotherapeutic is the total amount of radioisotope delivered to the tumor and its residence time in the tumor (i.e., dose deposited at the tumor), provided toxicity to normal tissues is tolerated. Although intact IgG penetrates from blood vessels more slowly than Fab or genetically engineered antibody fragments, most studies show that a greater dose of radioactivity is deposited at tumor sites when using radioimmunoconjugates containing intact IgG, suggesting that its slower clearance from blood, and the possibility for bivalent binding to target cells, are the most important parameters for a therapeutic application. Most clinical experience to date has been with mouse IgG in radioimmunoconjugates, which means that the generation of HAMA has been a factor that may limit the ability of patients to receive multiple doses of conjugate. The advent of humanized antibodies may overcome this limitation. Genetic engineering can also be used to make small fragments of humanized antibodies where the single binding domain can have very high affinity, and may, therefore, both penetrate into tumor tissue quickly and be well retained by the tumor, thereby increasing the dose delivered to the tumor.

What is the clinical experience in the evaluation of radioimmunoconjugate therapy in clinical trials? Can a sufficiently high dose of radiation be delivered to tumor in vivo to kill enough tumor cells to effect a therapeutic response? The clinical studies to date can be divided into two general groups, those treating tumors that are particularly radiosensitive, such as lymphomas and leukemias (*6*), and those treating solid tumors (*18*).

Clinical evaluation of radioimmunoconjugate therapy for non-Hodgkin's lymphoma (NHL) has been facilitated by the availability of a variety of B-cell-specific MAb, such as anti-idiotype antibodies, LYM-1 (anti-HLA-DR), anti-B1 (anti-CD20), MB-1 (anti-CD37), and OKB7 (anti-CD21) (*6,32–34*). These antibodies have been coupled to iodine-131 and have been used in cumulative doses of up to 750 mCi/patient. These large doses are well tolerated with the important exception of severe myelosuppression. Even though this severe side effect can be ameliorated by fractionating the dose of radiotherapeutic into multiple smaller doses given over several weeks, it would appear that the best clinical results are obtained in those trials that employ massive myeloablative doses of the radioimmunotherapeutic (*6*). In the studies from the Fred Hutchinson Cancer Center in Seattle, Washington, 16 of 19 patients who

were administered therapeutic doses achieved a complete remission (*34*). However, the cost of this therapeutic benefit is that 15 of the patients required an autologous bone marrow transplant (ABMT). These investigators suggest that the only possibility for complete eradication of the tumor is to use massive doses of radioimmunotherapy that are so high as to require bone marrow transplant support. It remains to be determined whether the therapeutic benefit of systemic delivery of massive doses of radiation with ABMT support is superior to other protocols utilizing chemotherapy and total body irradiation (external beam) as ablative regimens for ABMT protocols in the treatment of patients with relapsed lymphoma.

Other leukemias and lymphomas that have been targeted in trials of radioimmunotherapy are acute myelogenous leukemia (AML), T-cell malignancies, and Hodgkin's disease (*6*, and references therein). Iodine-131-labeled anti-CD33 and anti-CD45 antibodies have been used to target AML (*35*), whereas patients with chronic lymphocytic leukemia (CLL) or cutaneous T-cell lymphoma have been treated with anti-CD5 labeled with iodine-131 or yttrium-90, and those with adult T-cell leukemia have been treated with anti-CD25 (IL-2 receptor) conjugated with yttrium-90 (*6*). The most promising responses in these studies were also achieved at dose levels that caused severe myelosuppression as the major side effect (*6*).

In studies where Hodgkin's disease was treated with antiferritin antibodies coupled to yttrium-90 (the tumor cells are rich in ferritin), impressive response rates have been reported (*36*), although again at doses that were also myeloablative so that 17 of 37 patients required ABMT rescue (3 patients died of bone marrow aplasia). The yttrium-90 was conjugated to antibody using diethylenetriamine penta-acetic acid as the chelator, from which yttrium-90 is known to escape in vivo to be taken up by bone, which thus contributes to hematopoietic toxicity (*26*). It may be that the ferritin-rich tumor can also take up the released radiometal by chelation, which may contribute to the therapeutic efficacy of this conjugate.

The clinical experience with the treatment of solid tumors by radioimmunoconjugate therapy has generally been disappointing (*18*). Indeed, if optimal therapeutic effects in relatively radiosensitive neoplasms, such as NHL, can only be achieved at doses of radioimmunoconjugate that are myeloablative, then it is unlikely that therapeutic efficacy in solid tumors can be achieved at doses that are not also myeloablative. Furthermore, the **highest** doses delivered via radioimmunoconjugates are usually estimated to be in the range of 10–20 gy, although it is generally accepted that doses of at least 60 gy are needed to eradicate solid tumors (*27,37*). A Phase II clinical trial of radioimmunotherapy with iodine-131-labeled CC49 antibody in colorectal cancer exemplifies the lack of therapeutic efficacy in the treatment of solid tumors. Despite an antibody of relatively high affinity for the target tumor-associated glycoprotein 72, no tumor responses were observed, and the doses delivered to the tumor were only in the range of 0.2–6.7 gy (*38*). Recent Phase I studies with iodine-131-labeled A33 antibody were similarly disappointing (*39*). A Phase II trial of the CC49 radioimmunoconjugate in metastatic prostate cancer also failed to demonstrate any efficacy with maximal tumor doses estimated in the range of only 2–10 gy (*40*).

One approach to increasing the dose delivered to the tumor, while maintaining the total body dose at tolerable levels, is to treat locally tumors that are confined to particular body cavities. Intralesional radioimmunotherapy of malignant glioma may offer one compartmentalized setting where cytocidal doses of radiation may be delivered to

the tumor without significant toxicity to bone marrow, liver, or kidney. Treatment of 17 patients with iodine-131-labeled antitenascin antibodies resulted in 3 partial responses and 3 complete remissions (*41*). Intraperitoneal infusion of yttrium-90-labeled HMFG1 antibody was given to 52 patients with ovarian cancer (*42*). The results were encouraging, with 19 of 21 patients that were regarded as receiving treatment in an adjuvant setting still alive (median followup, 35 mo). However, even in this intracompartment setting, the authors doubt that the therapeutic effect was due to a cytocidal effect of the radiation dose, and suspect that the HMFG1 murine antibody induced immunological reactivity against the tumor, an observation also noted by others when treating breast cancer with iodine-131-labeled L6 antibody (*43*).

What are the future prospects for radioimmunotherapy? Several investigators are beginning to think of this modality as a complement to conventional external beam radiotherapy. For example, several patients with AML achieved complete remission when given iodine-131-labeled antibody together with 12 gy of external beam irradiation and cytoxan (*6,44*). A similar approach may be appropriate in treatment of certain solid tumors in order to achieve a sterilizing total dose of radiation at the tumor (*45*). The early results in the use of radioimmunotherapy for treating relapsed leukemias and lymphomas have been encouraging, although the therapy is far from optimized and may generally require concomitant ABMT. In particular, the optimal radionuclide and method of linkage to antibody still need to be defined (*27,28*), and humanized antibodies need to be tested in the clinic to overcome the limitations on multiple-course therapy imposed by the generation of HAMA (*35*). Nevertheless, even with these improvements, it may be that the long-term prospects for radioimmunotherapy may be confined to treating radiosensitive tumors utilizing myeloablative doses together with bone marrow rescue, or as an adjunct to external beam irradiation, owing to the intrinsic limitations of radiolabeled antibodies to deliver a sterilizing dose of radiation to tumor (*46*). Radioimmunotherapy will likely remain confined to specialized clinical centers with facilities for performing ABMT and for coping with issues, such as radiation exposure of medical staff and handling radioactive waste, which are problematic with systemic administration of radioactivity.

4. IMMUNOTOXINS

The limited expression of antigens suitable as targets for immunoconjugates on the surface of tumor cells (in general 10^4–10^5 and very rarely more than 10^6 antigens/cell) coupled with the pharmacodynamics of large molecules, such as γ-immunoglobulins, compelled scientists to search for the most potent cytotoxic agents to be used as effectors in immunoconjugates. Known protein toxins from plants, such as ricin, abrin, volkensin, and viscumin, and from bacteria, such as diphtheria toxin and pseudomonas exotoxin A, fit into this category. This spurred research into a better understanding of the mechanism by which these toxins destroy cells, so as to be able to harness their deadly power for the selective killing of tumor cells.

The above-listed toxins kill cells by catalytically inactivating cellular protein synthesis. The plant toxins, also called ribosome-inactivating proteins (RIPs) are *N*-glycosidases that remove the adenine base of residue 4324 of the 28S ribosomal RNA of the 60S subunit of eukaryotic cells (*47*). The bacterial toxins use NAD^+ to ADP-ribosylate elongation factor 2 (*48*). Because the final targets for the toxic action are cytoplasmic, the process of intoxication involves, therefore, at least three functions:

1. A binding function to localize the toxin to a cell surface;
2. A translocation function to transfer the toxin or its catalytic subunit to the cytoplasm, which needs a mechanism to translocate the protein through the lipid bilayer of a membrane, and;
3. An enzymatic function that inactivates the essential cellular process of protein synthesis.

Plant toxins are composed of two protein subunits of approximately equal size, called A-chain and B-chain, that are linked via a disulfide bond. The A-chain contains the enzymatic activity, and the B-chain provides the binding and translocation function. B-chains are lectins with specificity for galactosides and interact tightly with terminal galactose moieties of oligosaccharides found on the surface of eukaryotic cells (*49*). For ricin, it has been demonstrated that the translocation function is inseparably linked with the binding to galactose residues (*50*).

Diphtheria toxin (DT) binds to a protein receptor present on human cells and is internalized by endocytosis into acidified vesicles, where it is proteolytically cleaved into disulfide-linked fragments A and B. Cleavage establishes the full enzymatic activity in fragment A and fragment B retains the binding and membrane translocation function (*48*). (The similarity between the bacterial DT and the plant toxin ricin is remarkable, especially when one considers that ricin is transcribed initially as a single-chain preproricin, where the A and B chains are connected via a 12 amino acid fragment that is proteolytically removed, leaving the disulfide-linked A and B chains.) In pseudomonas exotoxin A (PE), the three functions are located in three molecularly and structurally separated domains of the single-chain protein. The N-terminal domain Ia binds to a protein receptor on human cells, the middle domain II encodes the membrane translocation function, and the C-terminal domain III contains the enzymatic activity. As with DT, only the catalytic domain III reaches the cytosol of the cell after PE is proteolytically cleaved in acidified vesicles following endocytosis (*48,51*). Interestingly, domain III contains at the C-terminus the amino acid sequence REDLK (ArgGluAspLeuLys) that is required for its cytotoxicity. It may act as an intracellular localization signal, since replacement with the known endoplasmic retention signal KDEL (LysAspGluLys) preserves its toxicity (*52*).

In all toxins, the binding and enzymatic toxicity functions are physically separated, either into separate subunits or into separate domains. When the first immunotoxins were prepared, the membrane translocation function was little appreciated or understood, and the easily separated toxic subunits ricin A chain or DT fragment A were simply linked to more specific binding moieties, the newly developed MAbs. More elegantly even, it was found that most plants produce single-chain RIPs that have a similar molecular size and the same catalytic function as ricin A chain (*53*), and immunotoxins could be rapidly produced without fear of contamination by nonspecifically binding B chain. Indeed, it was only the disappointing results with these early immunotoxins—their in vitro cytotoxicities were generally much lower than that of their respective native toxins—that led to the general recognition of the third function of native toxins, the membrane translocation function.

The different structural and functional arrangements found in the above toxins necessitated different approaches for each toxin to eliminate their native, nonselective binding function, while maintaining their membrane translocation and enzymatic activities. For ricin, the binding affinity to its natural cell-surface ligands was lowered by > 1000-fold by covalently linking affinity ligands into the two high-affinity galac-

tose binding sites of its B chain (*54*). This so-called blocked ricin, when incorporated into immunotoxins, killed the antibody target cells with a potency and efficiency similar to native ricin (*55*). Thus, it was concluded that the membrane translocation function in B chain had been preserved. Mechanistic studies demonstrated that the residual low-affinity binding of blocked ricin to galactoside residues, although not interfering with the antibody-directed selectivity and specificity on the cell surface, was necessary for the potency of the immunotoxin (*56*).

The single-chain bacterial toxins PE and DT were cloned, and genetic mutational methods were applied to change their binding characteristics. In PE, where the three domains are each associated with a single function, binding domain Ia was simply deleted to create PE40. When PE40 was then linked chemically to an antibody, the cytotoxicity and specificity of the resulting immunotoxin demonstrated that PE40 contributed the translocation and toxicity function of PE, but not the nonselective binding (*57*). The genetic manipulation not only allowed the removal of the coding region for domain Ia of the PE gene, but allowed its replacement by a gene of another binding protein, therefore creating fusion toxins. Fusion toxins with growth factors, hormones, lymphokines, and single-chain antibodies (Fv) were created (fusion toxins have been reviewed in [*58,59*]), and this chapter only discusses antibody-based toxins, i.e., immunotoxins). Similar genetic approaches have been tried with DT. Indeed, fusion toxins were first created between α-melanocyte-stimulating hormone or interleukin-2 and a truncated form of DT lacking its C-terminal binding domain (*60*). However, the relatively low cytotoxicity of these constructs indicated that efficient membrane penetration was linked to the specific binding of DT to its receptor. Indeed, the most potent specific immunotoxin with DT incorporated the whole toxin with point mutations in the B fragment that lowered the binding activity without affecting the translocation. The promising mutant CRM107 has two point mutations and a 8000-fold lower binding affinity (*61*).

The first immunotoxins evaluated clinically in cancer patients were conjugates of antilymphocyte antibodies linked to ricin A chain. As discussed above, it is the authors' opinion that most of these conjugates lacked adequate cytotoxicity, and not surprisingly, most of these conjugates were not pursued beyond Phase I clinical trials in cancer patients. One lesson learned, however, was that relatively large amounts of foreign proteins—murine IgG and ricin A chain from a plant—could be administered without significant allergic reactions.

The first published report on the clinical use of an immunotoxin that incorporated the translocation domain of a bacterial toxin was the ip administration of OVB3-PE to patients with refractory ovarian cancer (*62*). Dose-limiting toxic encephalopathy, likely owing to crossreactivity of OVB3 with normal human brain tissue, was observed early at the low dose of 5 μg/kg/d $\times$ 3 d that was not able to induce any clinical antitumor responses. Intact PE with its binding domain Ia was used, and no PE-directed binding is reported. Conjugation of the antibody via the noncleavable linker might have occurred in the binding domain, and, therefore, diminished its activity. The same authors proceeded then to prepare chemical conjugates or fusion toxins between truncated forms of PE that lacked the binding domain and antibodies or fragments of antibodies. Some of the promising conjugates that might enter clinical evaluation are anti-Tac(Fv)-PE40 for leukemias and transplant rejection, one of the different anti-HER2/*erb*B2-PE40 or anti-HER2/*erb*B2-PE38KDEL conjugates against breast

cancer, and B3(Fv)-PE38KDEL against Le^y antigen-positive carcinomas (reviewed in ref. *59*).

The clinical evaluation of conjugates containing mutant whole DT is made more difficult by the immunity of most patients against DT. It was, therefore, proposed to use immunoconjugates with the DT mutant CRM107 for regional therapy at immunologically privileged sites, such as tumors in the central nervous system (*63,64*).

The altered whole ricin toxin, called blocked ricin (bR), was incorporated into three immunoconjugates that were clinically tested. Anti-My9-bR, which binds to CD33 found on cells of myeloid lineage, was used to deplete *ex vivo* AML cells from autologous bone marrow from AML patients (*65,66*). Treatment with Anti-My9-bR eliminated more than 4 logs of leukemic cells, and fewer normal hematopoetic progenitor cells were affected than with a treatment using anti-My9 antibody and complement, leading to a more rapid engraftment (*67*). N901-bR reactive with a neural cell adhesion molecule (NCAM, CD56) uniformly expressed on small-cell lung carcinomas (SCLC) was tested in a Phase I clinical trial on 19 patients with relapsed and/or refractory SCLC at doses ranging from 5 to 40 μg/kg/d given as a continuous infusion over 7 d (*68*). Specific in vivo binding of N901-bR to tumor in the lung, bone marrow, and liver was demonstrated on biopsies. All patients developed human anti-mouse Ig antibodies (HAMA) and antiricin antibodies (HARA). One patient at the maximal tolerated dose (MTD) of 30 μg/kg/d × 7 d achieved a partial remission. The investigator proposed to use this conjugate when patients are in a state of minimal residual disease (MRD) and plans to initiate a Phase II trial studying patients with SCLC following aggressive induction chemotherapy (*68*).

Anti-B4-bR binds to the B-cell lineage-restricted antigen CD19, which is expressed uniformally on normal and malignant B-cells. The immunoconjugate was first evaluated clinically in a Phase I trial, where 25 patients with refractory B-cell malignancies were treated with daily 1-h infusions on five successive days (*69*). Despite the clinical responses observed—one complete, two partial, and eight mixed or transient responses—it was believed that the chosen schedule of administration was not ideal. The pharmacokinetic results indicated that significant serum levels were only maintained for about 4 h. The same authors undertook, therefore, a second Phase I study, where a 7-d continuous infusion schedule was used to treat 34 patients with NHL, CLL, or acute lymphocytic leukemia (ALL) (*70*). Significant serum levels were now maintained for the duration of drug administration. Two complete responses lasting more than 32 and 15 mo and five partial responses of short duration were reported. Both complete responses were observed in patients with lower tumor burden, which suggested that Anti-B4-bR might be most successfully applied after reduction of the tumor burden by chemotherapy and radiation. Because of the different mechanism of action, Anti-B4-bR would still be capable of eradicating the remaining chemo- and radioresistant tumor cells. This adjuvant immunotoxin therapy was tested in the setting of autologous bone marrow transplantation for NHL patients. After patients had successfully engrafted and were in complete remission, they were treated with the conjugate in the hope of eliminating MRD and to prolong their disease-free survival (*71*). Again, the unique mechanism of killing makes immunotoxins ideal drug candidates for this setting, where the residual tumor cells are often resistant to further chemotherapy.

The above clinical trials evaluate immunotoxins as single agents in different clinical settings. However, as with other anticancer agents, their full potential may ultimately

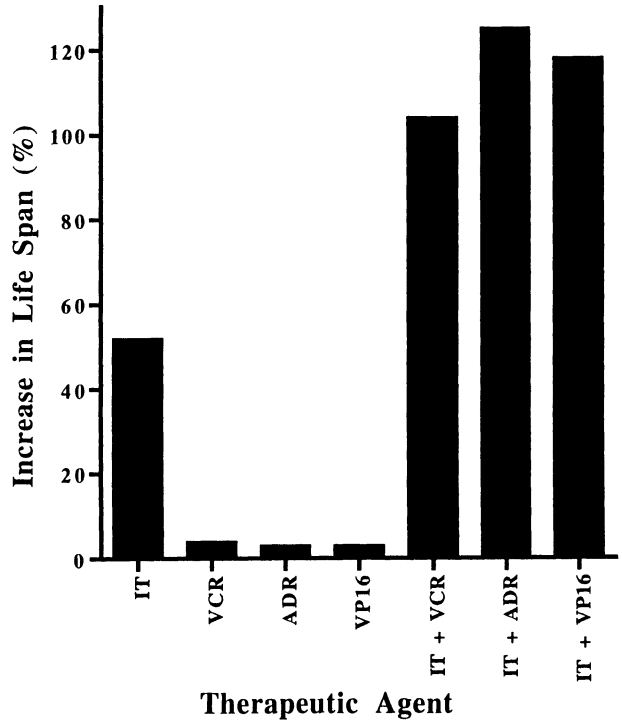


Fig. 3. Synergism between Anti-B4-bR and cytotoxic drugs for the treatment of disseminated mdr-1-expressing Burkitt's lymphoma tumors in SCID mice. Animals bearing 7-d-old tumors administered iv (*73*) were treated either with Anti-B4-bR alone (IT, 50 μg/kg/d × 5 d) or a chemotherapeutic drug alone at its MTD (VCR, vincristine, 3 × 1 mg/kg qod; ADR, adriamycin, 3 × 3 mg/kg q4d; VP16, etoposide, 3 × 15 mg/kg qod), or with a combination of Anti-B4-bR first (50 μg/kg/d × 5 d on days 7–11) followed by a chemotherapeutic drug (IT + VCR at 3 × 400 μg/kg q5d, on days 12, 17, and 22; IT + ADR at 3 × 3 mg/kg q5d, on d 12, 17, and 22; IT + VP16 at 3 × 15 mg/kg q5d, on days 12, 17, and 22). Untreated control animals died with a mean survival time of 24 d. Chemotherapeutic drugs alone showed no antitumor efficacy against the mdr-1 tumors, since they could not increase the life-span of animals. Immunoconjugate alone increased the life-span by 50%. All three combination treatments increased the life-span by more than 100%, demonstrating a synergistic effect between the immunoconjugate and the chemotherapeutic drugs.

come to fruition in combination treatments with other chemotherapeutic agents. It has already been demonstrated that Anti-B4-bR synergizes with chemotherapeutic agents in the treatment of human disseminated lymphomas in SCID mice (*72,73*), and more dramatically in the same model for the treatment of disseminated lymphomas expressing the multidrug resistance P-glycoprotein (*74*) (Fig. 3). These exciting findings have led to the design of several human clinical trials exploring this potential.

Immunotoxins are novel chemotherapeutic agents for the treatment of cancer. However, no drug of this category has yet been approved by health authorities. The most advanced in testing, Anti-B4-bR, is currently undergoing evaluation in a randomized multicenter Phase III trial. The potential of immunotoxins is lodged in their potency, tumor selectivity and, foremost, in their unique killing mechanism. This renders them drug candidates for treatment in disease settings refractory to further standard treatment and for incorporation into multidrug regimens.

5. ANTIBODY–DRUG CONJUGATES

Anticancer drugs in clinical use, such as doxorubicin, methotrexate, and the Vinca alkaloids, have limited selectivity for tumors, and hence, their cytotoxic potency cannot be fully exploited for the destruction of cancer cells. Their high toxicity toward actively proliferating nonmalignant cells, such as cells of the gastrointestinal tract and bone marrow, becomes dose-limiting. The linkage of cytotoxic drugs to MAb binding to specific cell-surface tumor-associated antigens should enhance the selectivity of these drugs by targeted delivery. It was expected that this approach would improve the therapeutic index of these drugs by lowering their systemic toxicity to sensitive normal tissues, while enhancing the local concentration of the cytotoxic agent at the tumor site. Several reports summarize the early development of MAb–drug conjugates (*75–79*), where, in general, clinically used cytotoxic anticancer drugs, such as daunorubicin, doxorubicin, methotrexate, Vinca alkaloids, mitomycin C, idarubicin, and melphalan, were conjugated to a multitude of murine MAbs. Initially, these drugs were linked directly to antibodies via noncleavable bonds and, in most cases, an average of 4–8 drug molecules were linked/molecule of antibody. Most conjugates lacked cytotoxic potency—they often were less cytotoxic than the free drugs—and attempts were made to improve them by linking to the antibody a larger number of drug molecules via macromolecular carriers, such as dextran, polyglutamic acid, polylysine or human serum albumin. However, the unfavorable pharmacokinetics and pharmacodynamics observed in animals with such conjugates discouraged further development (*80*).

In the next development phase, the emphasis shifted toward the development of conjugates, where the antibody molecules were linked to drugs via linkers that would be cleaved inside the cell to release active drug. One of the cleavable linkers that has been widely employed is an acid-labile linker that takes advantage of the acidic environment of the endosomes that might be encountered after receptor-mediated endocytosis. Acid-labile linkers based on cis-aconitic acid have been used for the preparation of conjugates of daunorubicin with macromolecular carriers (*81*), with an antimelanoma antibody (*82*) and an anti-T-cell antibody (*83*). Vinblastine and its analogs were linked to antibodies using an acid-labile hydrazide linker (*84,85*), which was also used for doxorubicin conjugates (*86*). In an alternative approach, daunorubicin was linked to an antibody via a peptide spacer arm under the premise that free drug would be released from the conjugate by the action of lysosomal peptidases (*87*).

Evaluation of their in vitro cytotoxicity revealed that these early conjugates were usually less potent than the parent unconjugated drugs, indicating the therapeutic levels of the drugs were not achieved inside target cells. Also, only marginal antigen-specific killing of the target cancer cells in vitro was observed. Typically, a target-specific and an analogous nontargeted conjugate differed in their effective concentrations against a given cell line only by factors of 2–10. Nevertheless, when evaluated in vivo, several of these conjugates showed greater therapeutic efficacy in human tumor xenograft models in immunodeficient mice than the corresponding unconjugated drugs or isotype-matched nonbinding antibody–drug conjugates. For example, it has been recently reported that immunoconjugates prepared with the MAb BR96 and doxorubicin completely cured athymic mice that had been implanted sc with human tumor xenografts (*88*). This result maybe explained by the high antigen expression ($> 10^6$ antigens/cell for BR96) and the large doses of immunoconjugate administered.

The latter is a common theme in successful treatment of tumor xenografts in animal models.

The preclinical results obtained with antibody–drug conjugates were sufficiently encouraging to warrant evaluation of these agents in humans. An immunoconjugate of methotrexate and the MAb KS1/4, which binds to a 40-kDa glycoprotein antigen that is expressed in human lung, colorectal, pancreatic, and ovarian adenocarcinomas, was evaluated in two different Phase I human clinical trials in nonsmall-cell lung cancer patients (*89,90*). A majority of patients elicited a HAMA response. Immunoperoxidase staining of carcinoma samples provided evidence of posttreatment localization of the conjugate. However, there were no clinical responses in either study. The same KS1/4 antibody was also linked to vinblastine via either an acid-labile hydrazide linker or an ester link and then evaluated in clinical trials in patients with adenocarcinomas (*91,92*). Again, a majority of the patients elicited a HAMA response, and again, no clinical responses were observed in these studies (*89*). Although conjugates of KS1/4 with methotrexate and vinblastine showed antitumor efficacy in human xenograft tumor models in mice, these results did not translate into clinically useful products and, to the best of our knowledge, human studies with these immunoconjugates have been discontinued. Recently, a human clinical trial has been initiated in colon cancer patients with the MAb BR96 linked to the anti-cancer drug doxorubicin via acid-labile hydrazide bonds (*93*).

Lack of clinical success with these early antibody–drug conjugates suggests that it was not possible to achieve intracellular concentrations of the drug required to kill sufficient numbers of cancer cells. Possible reasons for these outcomes may be:

1. Lack of cytotoxic potency: a majority of commonly used anticancer drugs are only moderately cytotoxic and a large number of drug molecules, too large to be achievable by antibody delivery, have to be internalized to cause cell death;
2. Dearth of antigens on cell surfaces;
3. Inefficient internalization of antigen–antibody complexes; and
4. Inefficient release of the drug from the antibody and often release of a modified, less active form of the drug.

New and very promising antibody–drug conjugates seek to overcome these shortcomings through the use of 100- to 1000-fold more potent cytotoxic drugs and the use of disulfide-containing linkers to ensure rapid release inside target cells. Maytansinoids, CC-1065 analogs, calicheamicin derivatives, and morpholinodoxorubicin were incorporated into new conjugates. Linkage of a highly cytotoxic maytansinoid drug to MAb produced immunoconjugates that exhibited high, antigen-specific cytotoxicity in vitro (*94*). Thus, a disulfide-linked maytansinoid immunoconjugate (C242-May) prepared with the MAb C242, which recognizes the CanAg antigen (*95*) expressed on the surface of human colon cancer cells, kills antigen-positive COLO 205 cells with an IC_{50} value of $1.2 \times 10^{-11} M$, with >99.999% of the cells killed at a conjugate concentration of $5 \times 10^{-9} M$. In contrast, the conjugate is at least 600-fold less cytotoxic toward antigen-negative A-375 cells ($IC_{50} = 7.6 \times 10^{-9} M$), demonstrating the antigen specificity of the cytotoxic effect (Fig. 4A). The high antigen-specific cytotoxicity of these conjugates observed in vitro encouraged further evaluation of this conjugate for therapeutic efficacy in vivo in a human tumor xenograft model in SCID mice. Animals were implanted sc with COLO 205 cells, which were allowed to grow for

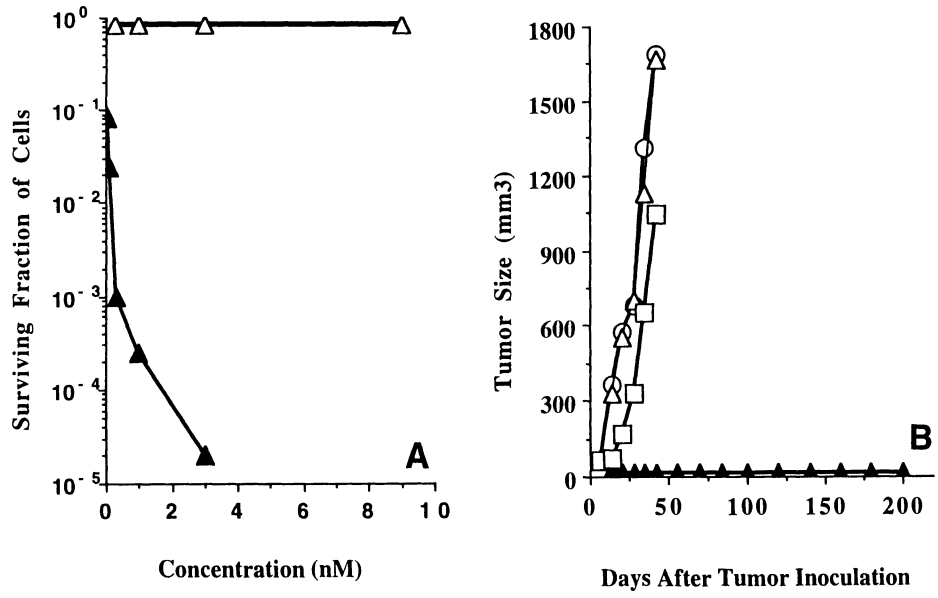


Fig. 4. (**A**) In vitro cytotoxicity and specificity of C242-maytansinoid conjugate. The in vitro cytotoxicity of C242-maytansinoid conjugate was measured on antigen-positive human colon carcinoma COLO 205 (ATCC CCL 222) cells (closed triangles) and antigen-negative human melanoma A-375 (ATCC CRL 1619) cells (open triangles), using a clonogenic assay. Cells were plated in varying numbers in 96-well plates in 0.2 mL growth media containing varying concentrations of immunoconjugate. The cells were maintained in a humidified atmosphere at 37 °C and 6% CO_2 for 18–21 d. Colonies were then counted, and the plating efficiency and surviving fractions determined. Surviving fractions of cells are plotted as a function of immunoconjugate concentration. (**B**) Antitumor efficacy of C242-May conjugate. The therapeutic efficacy of C242-May was determined in a established human tumor xenograft model in SCID mice that had been implanted sc with COLO 205 colon carcinoma cells. Mice (8–10 animals/group, 4 groups) were inoculated sc with 1×10^7 COLO 205 cells. The tumor was allowed to establish for 7–10 d, and treatment was begun when the average tumor size was between 65 and 100 mm^3. One group of mice was left untreated, and a second group was treated iv with C242-May at a maytansinoid dose of 380 μg/kg/d administered every other day for 5 d. The remaining two groups of animals served as negative controls and were treated, using the same schedule, with an equivalent amount of unconjugated C242 antibody (15 mg/kg/d) plus free maytansinoid (380 μg/kg/d), or with the isotype matched yet nonbinding maytansinoid conjugate N901-May (maytansinoid dose 380 μg/kg/d). Tumor size is plotted as a function of time. Control mice: open circles; treated mice: C242-May, closed triangles; C242 + May, open triangles; N901-May, open squares.

7–10 d before treatment started when the tumor size was between 65 and 100 mm^3. C242-May, at a maytansinoid dose of 380 μg/kg/d × 5 (conjugate dose 15 mg/kg/d), completely cured all mice of the tumor and animals were disease-free for > 140 d. In contrast, treatment with a mixture of unconjugated maytansinoid and antibody or with the isotype-matched yet nonbinding conjugate N901-May had little effect on the rate of tumor growth when compared with the untreated control mice (Fig. 4B). The results are very encouraging especially in view of the fact that cures with C242-May were also seen at doses (225 μg/kg/d × 5 maytansinoid dose), well below the maximum tolerated dose of the immunoconjugate.

This approach was extended to another potent cytotoxic drug, DC1 (*96*), which is a synthetic analog of the potent natural product CC-1065. An immunoconjugate called

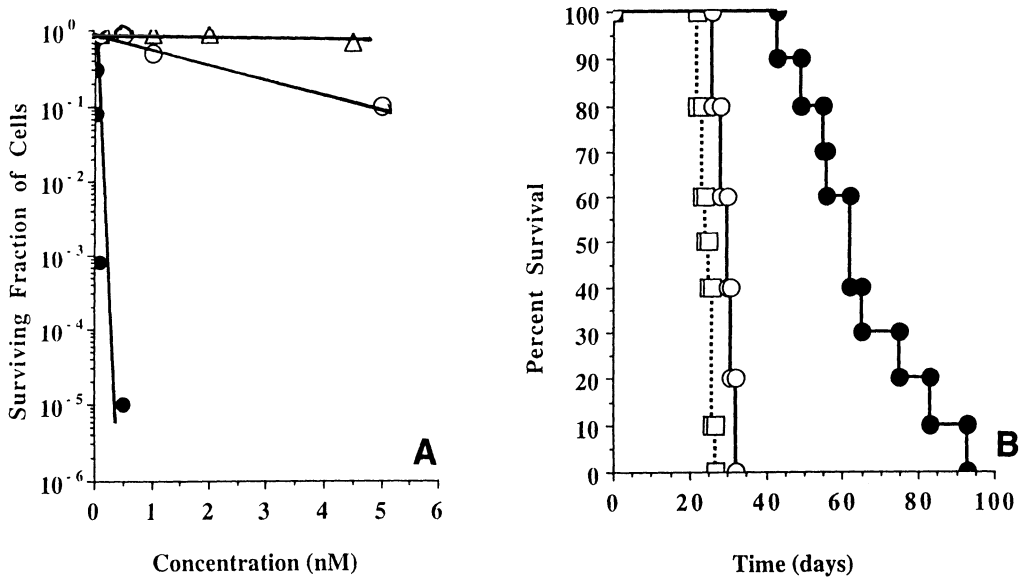


Fig. 5. (A) In vitro cytotoxicity and specificity of Anti-B4-DC1. In vitro cytotoxicity was measured using a growth-back extrapolation assay. Namalwa cells (ATCC CRL 1432) and MOLT-4 cells (ATCC CRL 1582) (4×10^5 cells, 2 mL) were exposed for 24 h to different concentrations of conjugate at 37 °C. Cells were washed, centrifuged, resuspended in fresh growth media, and counted. Cells were then incubated at 37 °C and counted daily using a Coulter counter. Surviving fractions of cells were determined and plotted as a function of conjugate concentration. Namalwa cells: closed circles; Namalwa cells in the presence of $5 \times 10^{-7}M$ unconjugated anti-B4 antibody: open circles; MOLT-4 cells: open triangles. **(B)** Therapeutic efficacy of Anti-B4-DC1. The therapeutic efficacy of Anti-B4-DC1 was determined using an established iv human tumor xenograft survival model in SCID mice. Mice (10 animals/group) were inoculated iv with 4×10^6 Namalwa (Burkitt's lymphoma) cells. Treatment was begun iv after tumor was established (day 7) with either Anti-B4-DC1 (5 mg/kg/d $\times$ 5, corresponding to 82 μg/kg/d DC1 content) or with an equivalent mixture of unconjugated anti-B4 antibody plus free DC1 drug. A control group of animals was left untreated. Control animals: open squares; treated animals: Anti-B4-DC1, closed circles; anti-B4 plus free DC1, open circles.

Anti-B4-DC1 prepared with DC1 and the MAb anti-B4, which recognizes the CD19 antigen found on cells in B-cell malignancies, kills antigen-positive Namalwa cells efficiently with an IC_{50} value of $1.3 \times 10^{-11}M$ after 24 h of exposure to the conjugate. The killing curve is very steep with >99.999% of cells eliminated (surviving fraction of cells $< 1 \times 10^{-5}$) at an immunoconjugate concentration of $5 \times 10^{-10}M$. Again the cytotoxic effect was antigen-specific, since the conjugate was about 400-fold less cytotoxic toward antigen-negative MOLT-4 cells. The addition of a 100-fold excess of unconjugated anti-B4 antibody abolished the cytotoxic effect of anti-B4-DC1 toward antigen-positive Namalwa cells, further demonstrating the antigen specificity of the cytotoxic effect (Fig. 5A). Anti-B4-DC1 also showed significant therapeutic efficacy against an established human tumor survival model in SCID mice (*73*). Mice (10 animals/group) were inoculated intravenously with Namalwa cells (4×10^6 cells/animal) and iv treatment was begun on day 7, when the tumor had grown to 7×10^7 cells. The group of untreated control mice had a median survival time (MST) of 24 d, whereas the group treated with a mixture of antibody and unconjugated drug showed a slight therapeutic effect with an MST of 30 d. Anti-B4-DC1 (80 μg/kg/d DC1 dose)

administered once every day for 5 d showed a pronounced antitumor effect with a 2.6-fold increase in life-span (MST = 62 d) (Fig. 5B).

Similar antitumor efficacy in an animal model was described for an immunoconjugate comprising a hydrazide-linked calicheamicin derivative (*97*). The preparation and in vitro evaluation of an immunoconjugate with the fourth drug mentioned above, morpholinodoxorubicin, which is about 40-fold more potent than doxorubicin itself, have also been described recently (*98*).

The new antibody–drug conjugates were built with the experience and insight gained during the last 15 years of immunoconjugate research, and they incorporate features that make them promising improved chemotherapeutic agents whose clinical evaluation is eagerly awaited. All the clinical drugs will include humanized antibodies allowing their use during several cycles of remission induction and consolidation treatment. Whether the size will hamper their efficacy against solid tumors is not at all clear from the preclinical models. However, molecular biological methods allow for the easy production of antibody fragments, such as Fab, F(ab'), and Fv fragments, and it is possible to incorporate them into drug conjugates. Coupled with their smaller size, however, is a much faster clearance rate and often an inability to retain their binding affinity on drug conjugation. Only careful preclinical and clinical evaluation will ultimately allow the determination of the most efficacious type and forms of antibody–drug conjugates.

6. CONCLUSIONS

The use of immunoconjugates for the treatment of cancer, i.e., the selective delivery of cytotoxic agents to tumor cells, seemed to be a simple, straightforward idea whose time had come with the arrival of the MAb technology in 1975. Today, 20 years later, no such agent is yet an approved drug. Every element contributing to the success (antitumor efficacy in patients) of an immunoconjugate had to be newly developed. Specific target antigens had to be discovered, and appropriate effector molecules had to be found and had to be linked via suitable linkers. Killing mechanisms needed to be explored to preserve the potency of toxins. Novel, more potent chemotherapeutic drugs had to be evaluated and had to be modified for covalent linkage to antibodies, and new chelators for radioisotopes had to be synthesized. Pharmacokinetic and pharmacodynamic problems with the large immunoconjugates as well as the patients' immune responses to foreign proteins had to be considered. Today, immunoconjugates are at the stage of evaluation in Phase II and Phase III clinical trials, giving testimony to success in finding solutions to these challenging questions. Important new technologies were also developed, such as the humanization of murine antibodies, the phage display of the antibody repertoire, and combinatorial chemistry, that allowed the identification of novel tumor-selective antibodies at a more rapid pace and their efficient incorporation into effective, nonimmunogenic immunoconjugates. The need for selective anticancer agents has not changed, and immunoconjugate development is positioned well to contribute its part in filling this need.

REFERENCES

1. Burger MM, Goldberg AR. Identification of a tumor specific determinant on neoplastic cell surfaces. *Proc Natl Acad Sci USA* 1967; 57:359–366.

2. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 1975; 256:495–497.
3. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; 235:177–182.
4. Pastan I, Lovelace ET, Gallo MG, Rutherford AV, Magnani JL, Willingham MC. Characterization of monoclonal antibodies B1 and B3 that react with mucinous adenocarcinomas. *Cancer Res* 1991; 51:3781–3787.
5. Kwak LW, Campbell MJ, Czerwinski DK, Hart S, Miller RA, Levy R. Induction of immune response in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. *N Engl J Med* 1992; 327:1209–1215.
6. Grossbard ML, Press OW, Appelbaum FR, Bernstein ID, Nadler LM. Monoclonal antibody-based therapies of leukemia and lymphoma. *Blood* 1992; 80:863–868.
7. Boyer CM, Lidor Y, Lottich SC, Bast RC Jr. Antigenic cell surface markers in human solid tumors. *Antibody, Immunoconjugates, Radiopharmaceuticals* 1988; 1:105–116.
8. Carroll AM, Greene MI. Tumor cell biology: tumor specific and associated antigens. In: Zalutsky MR, ed. *Antibodies in Radiodiagnostics and Therapy.* Boca Raton, FL: CRC. 1989:13–43.
9. Hellstöm I, Garrigues HJ, Garrigues U, Hellström KE. Highly tumor-reactive internalizing mouse monoclonal antibodies to Le^y-related cell surface antigens. *Cancer Res* 1990; 50:2183–2190.
10. Winter G, Milstein C. Man-made antibodies. *Nature* 1991; 349:293–299.
11. Moos WH, Green GD, Pavia MR. Recent advances in the generation of molecular diversity. In: Bristol JA, ed. *Annual Reports in Medicinal Chemistry*, vol. 28. San Diego, CA: Academic. 1993:315–324.
12. Morrison SL, Johnson MJ, Herzenberg LA, Oi VT. Chimeric human tumor antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc Natl Acad Sci USA* 1984; 81:6851–6855.
13. Reichman L, Clark M, Waldmann H, Winter G. Reshaping human antibodies for therapy. *Nature* 1988; 332:323–327.
14. Roguska MA, Pedersen JT, Keddy CA, Henry AH, Searle SJ, Lambert JM, Goldmacher VS, Blättler WA, Rees AR, Guild BC. Humanization of murine antibodies through variable domain resurfacing. *Proc Natl Acad Sci USA* 1994; 91:969–973.
15. Thornton JM, Edwards MS, Taylor WR, Barlow DJ. Location of "continuous" antigenic determinants in the protruding regions of proteins. *EMBO J* 1986; 5:409–413.
16. Novatny J, Handschumacher M, Haber E, Bruccoleri RE, Carlson WB, Smith DW, Rose GD. Antigenic determinants in protein coincide with surface regions accessible to large probes (antibody domains). *Proc Natl Acad Sci USA* 1986; 83:226–230.
17. Maxon HR, Thomas SR, Hertzberg VS, Kereiakes JG, Chen IW, Sperling MI, Saenger EL. Relation between effective radiation dose and outcome of radioiodine therapy for thyroid cancer. *N Engl J Med* 1983; 309:937–941.
18. Mello AM, Pauwels EKJ, Cleton FJ. Radioimmunotherapy: no news from the newcomer. *J Cancer Res Clin Oncol* 1994; 120:121–130.
19. Simpkin DJ, Mackie TR. EGS4 Monte Carlo determination of the beta dose kernel in water. *Med Phys* 1990; 17:179–186.
20. Fraker PJ, Speck JC. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,5-tetrachloro-3α,6α-diphenyl-glycoluril. *Biochem Biophys Res Commun* 1978; 80:849–857.
21. Ali SA, Warren SD, Richter KY, Badger CC, Eary JF, Press OW, Krohn KA, Bernstein ID, Nelp WB. Improving the tumor retention of radioiodinated antibody: aryl carbohydrate adducts. *Cancer Res* 1990; 50:783s–788s.
22. Zalutsky MR, Narula AS. Radiohalogenation of a monoclonal antibody using an *N*-succinimidyl 2-(tri-*n*-butylstannyl) benzoate intermediate. *Cancer Res* 1988; 48:1446–1450.
23. Ram S, Buchsbaum DJ. Radioiodination of monoclonal antibodies D612 and 17-1A with 3-iodophenylisothiocyanate and their biodistribution in tumor-bearing nude mice. *Cancer* 1994; 73:808–815.
24. Moi MK, DeNardo SJ, Meares CF. Stable bifunctional chelates of metals used in radiotherapy. *Cancer Res* 1990; 50:789s–793s.

25. Kukis DL, Diril H, Grenier DP, DeNardo SJ, DeNardo GL, Salako QA, Meares CF. A comparative study of copper-67 radiolabeling and kinetic stabilities of antibody-macrocycle chelate conjugates. *Cancer* 1994; 73:779–786.
26. DeNardo GL, Kroger LA, DeNardo SJ, Miers LA, Salako Q, Kukis DL, Fand I, Shen S, Renn O, Meares CF. Comparative toxicity studies of yttrium-90 MX-DTPA and 2-IT-BAD conjugated monoclonal antibody (BrE-3). *Cancer* 1994; 73:1012–1022.
27. Rao DV, Howell RW. Time-dose-fractionation in radioimmunotherapy: implications for selecting radionuclides. *J Nucl Med* 1993; 34:1801–1810.
28. Smith A, Alberto R, Blaeuenstein P, Novak-Hofer I, Maecke HR, Schubiger PA. Preclinical evaluation of ^{67}Cu-labeled intact and fragmented anti-colon carcinoma monoclonal antibody MAb35. *Cancer Res* 1993; 53:5727–5733.
29. Junghans RP, Dobbs D, Brechbiel MW, Mirzadeh S, Raubitschek AA, Gansow OA, Waldmann TA. Pharmacokinetics and bioactivity of 1,4,7,10-tetra-azacylododecane *N,N',N'',N'''*-tetraacetic acid (DOTA)-bismuth-conjugated anti-Tac antibody for α-emitter (^{212}Bi) therapy. *Cancer Res* 1993; 53:5683–5689.
30. Larsen RH, Bruland OS, Hoff P, Alstad J, Rofstad EK. Analysis of the therapeutic gain in the treatment of human osteosarcoma microcolonies *in vitro* with ^{211}At-labelled monoclonal antibody. *Br J Cancer* 1994; 69:1000–1005.
31. Goldenberg DM. New developments in monoclonal antibodies for cancer detection and therapy. *CA Cancer J Clin* 1994; 44:43–64.
32. Kaminski MS, Zasadny KR, Francis IR, Milik AW, Ross CW, Moon SD, Crawford SM, Burgess JM, Petry NA, Butchko GM, Glenn SD, Wahl RL. Radioimmunotherapy of B-cell lymphoma with [^{131}I]anti-B1 (anti-CD20) antibody. *N Engl J Med* 1993; 329:459–465.
33. Czuczman MS, Straus DJ, Divgi CR, Graham M, Garin-Chesa P, Finn R, Myers J, Old LJ, Larson SM, Scheinberg DA. Phase I dose-escalation trial of iodine-131-labeled monoclonal antibody OKB7 in patients with non-Hodgkin's lymphoma. *J Clin Oncol* 1993; 11:2021–2029.
34. Press OW, Eary JF, Appelbaum FR, Martin PJ, Badger CC, Nelp WB, Glenn S, Butchko G, Fisher D, Porter B, Matthews DC, Fisher LD, Bernstein ID. Radiolabeled-antibody therapy of B-cell lymphoma with autologous bone marrow support. *N Engl J Med* 1993; 329:1219–1224.
35. Caron PC, Schwartz MA, Man Sung Co, Queen C, Finn RD, Graham MC, Divgi CR, Larson SM, Scheinberg DA. Murine and humanized constructs of monoclonal antibody M195 (anti-CD33) for therapy of acute myelogenous leukemia. *Cancer* 1994; 73:1049–1056.
36. Vriesendorp HM, Herpst JM, Germack MA, Klein JL, Leichner PK, Loudenslager DM, Order SE. Phase I-II studies of yttrium-labeled antiferritin treatment for end-stage Hodgkin's disease including radiation therapy: oncology group 87-01. *J Clin Oncol* 1991; 9:918–928.
37. Vaughan ATM, Anderson P, Dykes PW, Chapman CE, Bradwell AR. Limitations to the killing of tumors using radiolabelled antibodies. *Br J Radiol* 1987; 60:567–572.
38. Murray J, Macey DJ, Kasi LP, Rieger P, Cunningham J, Bhadkamkar V, Zhang H-Z, Schlom J, Rosenblum MG, Podoloff DA. Phase II radioimmunotherapy trial with ^{131}I-CC49 in colorectal cancer. *Cancer* 1994; 73:1057–1066.
39. Welt S, Divgi CR, Kemeny N, Finn RD, Scott AM, Graham M, St Germain J, Richards EC, Larson SM, Oettgen HF, Old LJ. Phase I/II study of iodine 131-labeled monoclonal antibody A33 in patients with advanced colon cancer. *J Clin Oncol* 1994; 12:1561–1571.
40. Meredith RF, Bueschen AJ, Khazaeli MB, Plott WE, Grizzle WE, Wheeler RH, Schlom J, Russell CD, Liu T, LoBuglio AF. Treatment of metastatic prostate carcinoma with radiolabeled antibody CC49. *J Nucl Med* 1994; 35:1017–1022.
41. Riva P, Arista A, Tison V, Sturiale C, Franceschi G, Spinelli A, Riva N, Casi M, Moscatelli G, Frattarelli M. Intralesional radioimmunotherapy of malignant gliomas. *Cancer* 1994; 73:1076–1082.
42. Hird V, Maraveyas A, Snook D, Dhokia B, Soutter WP, Meares C, Stewart JSW, Mason P, Lambert HE, Epenetos AA. Adjacent therapy of ovarian cancer with radioactive monoclonal antibody. *Br J Cancer* 1993; 68:403–406.
43. DeNardo SJ, Mirick GR, Kroger LA, O'Grady LF, Erickson KL, Yuan A, Lamborn KR, Hellström I, Hellström KE, DeNardo GL. The biological window for chimeric L6 radioimmunotherapy. *Cancer* 1994; 73:1023–1032.
44. Applebaum FR, Matthews DC, Eary JF, Badger CC, Kellogg M, Press OW, Martin PJ, Fisher DR, Nelp WB, Thomas ED, Bernstein ID. The use of radiolabeled anti-CD33 antibody to aug-

ment marrow irradiation prior to marrow transplantation for acute myelogenous leukemia. *Transplantation* 1992; 54:829–833.
45. O'Donoghue J. The impact of tumor cell proliferation in radioimmunotherapy. *Cancer* 1994; 73:974–980.
46. Vaughan ATM, Bradwell AR, Dykes PW, Anderson P. Illusions of tumor killing using radiolabeled antibodies. *Lancet I* 1986:1492,1493.
47. Endo Y. Mechanism of action of ricin and related toxins on the inactivation of eukaryotic ribosomes. In: Frankel AE, ed. *Immunotoxins*. Boston: Kluwer Academic Publishers. 1988:75–89.
48. Collier RJ. Structure-activity relationships in diphtheria toxin and pseudomonas exotoxin A. In: Frankel AE, ed. *Immunotoxins*. Boston: Kluwer Academic Publishers. 1988:25–35.
49. Olsnes S, Sandvig K. How protein toxins enter and kill cells. In: Frankel AE, ed. *Immunotoxins*. Boston: Kluwer Academic Publishers. 1988:39–73.
50. Newton DL, Wales R, Richardson PT, Walbridge S, Saxena SK, Ackerman EJ, Roberts LM, Lord MJ, Youle RJ. Cell surface and intracellular functions for ricin galactose binding. *J Biol Chem* 1992; 267:11,917–11,922.
51. Ogata M, Chaudhary VK, Pastan I, FitzGerald DJ. Processing of pseudomonas exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. *J Biol Chem* 1990; 265:20,678–20,685.
52. Chaudhary VK, Jinno Y, FitzGerald D, Pastan I. Pseudomonas exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc Natl Acad Sci USA* 1990; 87:308–312.
53. Barbieri L, Battelli MG, Stirpe F. Ribosome-inactivating proteins from plants. *Biochim Biophys Acta* 1993; 1154:237–282.
54. Lambert JM, McIntyre G, Gauthier MN, Zullo D, Rao V, Steeves RM, Goldmacher VS, Blättler WA. The galactose-binding sites of the cytotoxic lectin ricin can be chemically blocked in high yield with reactive ligands prepared by chemical modification of glycopeptides containing triantennary N-linked oligosaccharides. *Biochemistry* 1991; 30:3234–3247.
55. Lambert JM, Goldmacher VS, Collinson AR, Nadler LM, Blättler WA. An immunotoxin prepared with blocked ricin: a natural plant toxin adapted for therapeutic use. *Cancer Res* 1991; 51:6326–6342.
56. Goldmacher VS, Lambert JM, Blättler WA. The specific cytotoxicity of immunoconjugate containing blocked ricin is dependent on the residual binding capacity of blocked ricin: evidence that the membrane binding and A-chain translocation activities of ricin cannot be separated. *Biochem Biophys Res Commun* 1992; 183:758–766.
57. Pastan I, FitzGerald D. Recombinant toxins for cancer treatment. *Science* 1991; 254:1173–1177.
58. Frankel AE, ed. *Genetically Engineered Toxins*. New York: Marcel Dekker, 1992.
59. Siegall CB. Targeted toxins as anticancer agents. *Cancer (suppl)* 1994; 74:1006–1012.
60. Murphy JR, Lakkis FG, VanderSpek JC, Anderson P. Protein engineering of diphtheria toxin. Development of receptor-specific cytotoxic agents for the treatment of human disease. In: Frankel AE, ed. *Genetically Engineered Toxins*. New York: Marcel Dekker. 1992:365–393.
61. Greenfield L, Johnson VG, Youle RJ. Mutations in diphtheria toxin separate binding from entry and amplify immunotoxin selectivity. *Science* 1987; 238:536–539.
62. Paul LH, Bookman MA, Ozols RF, Young RC, Smith II JW, Longo DL, Gould B, Frankel A, McClay ET, Howell S, Reed E, Willingham MA, FitzGerald DJ, Pastan I. Clinical evaluation of intraperitoneal pseudomonas exotoxin immunoconjugate OVB3-PE in patients with ovarian cancer. *J Clin Oncol* 1991; 12:2095–2103.
63. Johnson VG, Woo C, Wilson D, Zovickian J, Greenfield L, Oldfield EH, Youle R. Improved tumor-specific immunotoxins in the treatment of CNS and ceptomeningeal neoplasia. J Neurosurg 1989; 70:240–248.
64. Lashe DW, Ibercil O, Akbasak A, Youle RJ, Oldfield EH. Efficacy of direct intratumoral therapy with targeted protein toxins for solid human gliomas in nude mice. *J Neurosurg* 1994; 80:520–526.
65. Roy DC, Robertson MJ, Belanger R, Gyger M, Perreault C, Bonny Y, Soiffer R, Epstein C, Ritz J. Engraftment following Anti-My9-bR depleted autologous marrow transplantation for patients with acute myeloid leukemia. *Blood (suppl)* 1992; 80:376a.

66. Roy DC, Griffin JD, Belvin M, Blättler WA, Lambert JM, Ritz J. Anti-My-9-blocked ricin: an immunotoxin for selective targeting of acute myeloid leukemia cells. *Blood* 1991; 77:2404–2412.
67. Robertson MJ, Roy DC, Soiffer R, Belanger R, Gyger M, Perreault C, Anderson K, Freedman A, Nadler LM, Ritz J. More rapid engraftment after infusion of autologous bone marrow treated with Anti-My-9 and complement. *Blood (suppl)* 1993; 82:640a.
68. Lynch TJ, Jr. Immunotoxin therapy of small-cell lung cancer. N901-blocked ricin for relapsed small-cell lung cancer. *Chest* 1993; 103:436s–439s.
69. Grossbard ML, Freedman AS, Ritz J, Coral F, Goldmacher VS, Eliseo L, Spector NK, Lambert JM, Blättler WA, Taylor JA, Nadler LM. Serotherapy of B-cell neoplasms with Anti-B4-blocked ricin: a phase I trial of daily bolus infusion. *Blood* 1992; 79:576–585.
70. Grossbard ML, Lambert JM, Goldmacher VS, Spector NL, Kinsella J, Eliseo L, Coral F, Taylor JA, Blättler WA, Epstein CL, Nadler LM. Anti-B4-blocked ricin: a phase I trial of 7-day continuous infusion in patients with B cell neoplasms. *J Clin Oncol* 1993; 11:726–737.
71. Grossbard ML, Gribben JG, Freedman AS, Lambert JM, Kinsella J, Rabinowe SN, Eliseo L, Taylor JA, Blättler WA, Epstein CL, Nadler LM. Adjuvant immunotoxin therapy with Anti-B4-blocked ricin after autologous bone marrow transplantation for patients with B-cell non-Hodgkin's lymphoma. *Blood* 1993; 81:2263–2271.
72. O'Connor R, Liu C, Ferris CA, Guild BC, Teicher BA, Corvi C, Liu Y, Arceci RJ, Goldmacher VS, Lambert JM, Blättler WA. Anti-B4-blocked recin synergizes with doxorubicin and etoposide on multidrug-resistant and drug-sensitive tumors. *Blood* 1995; 86:4286–4294.
73. Shah SA, Halloran PM, Ferris CA, Levine BA, Bourret LA, Goldmacher VS, Blättler WA. Anti-B4-blocked ricin immunotoxin shows therapeutic efficacy in four different SCID mouse models. *Cancer Res* 1993; 53:1360–1367.
74. Liu C, Lambert JM, Teicher BA, Blättler WA, O'Connor R. Cure of multidrug-resistant human B-cell lymphoma xenografts by combinations of Anti-B4-blocked ricin and chemotherapeutic drugs. *Blood* 1996; 87:3892–3898.
75. Sela M, Hurwitz E. Conjugates of antibodies with cytotoxic drugs. In: Vogel CW, ed. *Immunoconjugates.* New York: Oxford University Press. 1987:189–216.
76. Ghose T, Blair AH, Vaughan K, Kulkarni P. Antibody-directed drug targeting in cancer therapy. In: Goldberg EP, ed. *Targeted Drugs.* New York: John Wiley. 1993:1–22.
77. Rodwell, JD. *Antibody-Mediated Delivery Systems.* New York: Marcel Dekker, 1988.
78. Pietersz GA. The linkage of cytotoxic drugs to monoclonal antibodies for the treatment of cancer. *BioConjugate Chem* 1990; 1:89–95.
79. Garnett MC, Baldwin RW. An improved synthesis of a methotrexate-albumin-791T/36 monoclonal antibody conjugate cytotoxic to human osteogenic sarcoma cell lines. *Cancer Res* 1986; 46:2407–2412.
80. Endo N, Takeda Y, Umemoto N, Kishida K, Watanabe K, Saito M, Kato Y, Hara T. Nature of linkage and mode of action of methotrexate conjugated with antitumor antibodies: implications for future preparation of conjugates. *Cancer Res* 1988; 48:3330–3355.
81. Shen EC, Ryser J-PH. Cis-aconityl spacer between daunomycin and macromolecular carriers: a model of pH sensitive linkage releasing drug from a lysosomotropic conjugate. *Biochem Biophys Res Commun* 1981; 102:1048–1054.
82. Yang HM, Reisfeld RA. Doxorubicin conjugated with a monoclonal antibody directed to a human melanoma-associated proteoglycan suppresses the growth of established tumor xenografts in nude mice. *Proc Natl Acad Sci USA* 1988; 85:1189–1193.
83. Dillman RO, Johnson DE, Shawler DL, Koziol JA. Superiority of an acid-labile daunorubicin-monoclonal antibody immunoconjugate compared to free drug. *Cancer Res* 1988; 48:6097–6102.
84. Laguzza BC, Nichols CL, Briggs SL, Cullinan GJ, Johnson DJ, Starling JJ, Baker AL, Bumol TF, Corvalan JRF. New antitumor monoclonal antibody–vinca conjugate LY203725 and related compounds: design, preparation, and representative in vivo activity. *J Med Chem* 1989; 32: 548–555.
85. Apelgren LD, Zimmerman DL, Briggs SL, Bumol TF. Antitumor activity of the monoclonal antibody-Vinca alkaloid immunoconjugate LY203725 (KS1/4-4 desacetylvinblastine-3-carboxhydrazide) in a nude mouse model of ovarian cancer. *Cancer Res* 1990; 50:3540–3544.
86. Greenfield RS, Kaneko T, Daues A, Edson MA, Fitzgerald KA, Olech LJ, Grattan JA, Spitalny

GL, Braslawsky GR. Evaluation in vitro of adriamycin immunoconjugates synthesized using an acid-sensitive hydrazone linker. *Cancer Res* 1990; 50:6600–6607.

87. Trouet A, Masquelier M, Baurain R, de Campenère DD. A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier: *in vitro* and *in vivo* studies. *Proc Natl Acad Sci USA* 1982; 79:626–629.
88. Trail PA, Willner D, Lasch SJ, Henderson AJ, Hofstead S, Casazza AM, Firestone RA, Hellström I, Hellström KE. Cure of xenografted human carcinoma by BR96-doxorubicin immunoconjugates. *Science* 1993; 261:212–215.
89. Elias DJ, Hirshowitz L, Kline LE, Kroener JF, Dillman RO, Walker LE, Robb JA, Timms RM. Phase I clinical comparative study of monoclonal antibody KS1/4 and KS1/4-methotrexate immunoconjugate in patients with non-small cell lung cancer. *Cancer Res* 1990; 50:4154–4159.
90. Elias DJ, Kline LE, Robbins BA, Johnson HCL, Pekny K, Benz M, Robb JA, Walker LE, Kosty M, Dillman RO. Monoclonal antibody KS1/4-methotrexate immunoconjugate in non-small cell lung carcinoma. *Am J Respir Crit Care Med* 1994; 150:1114–1122.
91. Petersen BF, DeHerdt SV, Schneck DW, Bumol TF. The human immune response to KS1/4-desacetylvinblastine (LY256787) and KS1/4-desacetylvinblastine hydrazide (LY203728) in single and multiple dose clinical studies. *Cancer Res* 1991; 51:2286–2290.
92. Schneck D, Butler F, Dugan W, Littrel D, Dorrbecker S, Petersen B, Browsher R, DeLong A, Zimmerman J. Phase I studies with a murine monoclonal antibody-Vinca conjugate (KS1/4-DAVLB) in patients with adenocarcinoma. *Antibody Immunoconjugate Radiopharmaceuticals* 1989; 2:93–100.
93. Trail PA, Slichenmyer WJ, Birkhofer MJ, Warner G, Knipe J, Willner D, Firestone RA, Sikkema D, Onetto N, Canetta R, Saleh MN, Murray JL, Gilewski TA, Bookman MA, Hellström I, Hellström KE. BR96-doxorubicin immunoconjugate for treatment of patients with carcinoma. *Proc Amer Assoc Cancer Res* 1996; 37:626.
94. Chari RVJ, Martell BA, Gross JL, Cook SB, Shah SA, Blättler WA, McKenzie SJ, Goldmacher VS. Immunoconjugates containing novel maytansinoids: promising anticancer drugs. *Cancer Res* 1992; 52:127–131.
95. Baeckstrom D, Hansson GC, Nilsson O, Johansson C, Gendler SJ, Lindholm L. Purification and characterization of a membrane-bound and secreted mucin-type glycoprotein carrying the carcinoma-associated sialyl-Le^a epitope on distinct core proteins. *J Biol Chem* 1991; 266: 21,537–21,547.
96. Bourret LA, Derr SM, Tadayoni M, Mattocks KM, Shah SA, Liu C, Blättler WA, Goldmacher VS. Enhancement of the selectivity and antitumor efficacy of a CC-1065 analogue through immunoconjugate formation. *Cancer Res* 1995; 55:4079–4084.
97. Hillman LM, Hamann PR, Wallace R, Menendez AT, Durr FE, Upeslacis J. Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. *Cancer Res* 1993; 53:3336–3342.
98. Mueller BM, Wrasidlo WA, Reisfeld RA. Antibody conjugates with morpholino-doxorubicin and acid-cleavable linkers. *BioConjugate Chem* 1990; 1:325–330.

18 A Case for *ras* Targeted Agents as Antineoplastics

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CONTENTS

1. INTRODUCTION

Conventional cancer treatment generally employs cytotoxic agents that, by inhibiting DNA replication or mitosis, are most effective against rapidly growing tumors. Clearly we have witnessed successes against various leukemias, lymphomas, and some solid tumors, such as testicular cancer. With some exceptions, there generally exists a close correlation between tumor proliferation rate and sensitivity to cytotoxic drugs (*1*). Therefore, a critical need still exists for the development of agents that will target the more refractory tumors that are distinguished by a low growth fraction, such as colon adenocarcinoma, nonsmall-cell lung cancers, and pancreatic carcinomas. Despite a roughly 40-year search for more efficaceous antitumor drugs, very few new agents have shown sufficient broad-spectrum activity for entering mainstream chemotherapy.

During the last decade, our understanding of the molecular basis of neoplasia has been significantly expanded, consequently opening up a number of previously unexplored targets for anticancer drug design. One such target is the *ras* oncogene and its 21-kDa protein product (p21 Ras), which plays a pivotal role in signal transduction pathways involved in growth control and, when mutated, has been linked to transformation. In light of the fact that cancer is a disease of uncontrolled proliferation, *ras* has emerged as an attractive candidate for the design of therapies suppressing its expression or function.

This chapter will describe the normal cellular function of Ras and discuss how *ras* mutations can have dire consequences both with regard to development and subsequent treatment of human malignancies. The rationale behind several diverse strategies for *ras*-directed therapeutic intervention will then be explored along with a review of the progress reported to date.

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

2. CELLULAR DEPENDENCE ON *ras* FUNCTION

The regulation of normal cell growth involves the interplay of a number of proto-oncogene products that ensure the efficient transmission of extracellular signals to the nucleus. Central to the complex circuitry within the cell are a family of 21-kDa proteins encoded by the *ras* proto-oncogenes, which act as molecular switches by virtue of their guanine nucleotide binding capacity. More than 50 proteins have been identified to date as belonging to the *ras* superfamily and collectively act as signaling molecules for a broad range of cellular functions ranging from cellular proliferation and differentiation to cytoskeleton assembly and vesicular trafficking. The universal importance of Ras proteins is evidenced by their ubiquitous and highly conserved nature among eukaryotic organisms.

Microinjection studies provided perhaps the earliest compelling evidence for the critical role played by Ras in signal transduction. The stimulation of DNA synthesis that normally occurs on the addition of serum to quiescent fibroblasts did not occur when fibroblasts were microinjected with a neutralizing anti-*ras* antibody (*2*). Conversely, microinjection of oncogenic Ras protein stimulated DNA synthesis in the absence of exogenous growth factors (*3*). The universal role of Ras in cell growth and differentiation is also supported by microinjection studies showing that anti-Ras antibodies inhibit the neuronal differentiation of PC12 pheochromocytoma cells and block insulin-induced maturation of *Xenopus* oocytes (*4,5*).

A single base mutation in one of the three mammalian *ras* genes, H(arvey)-, K(irsten)-, and N(euroblastoma)-*ras*, is sufficient to transform cells to malignancy. Although the relevance of *ras* mutations in neoplastic disease has been known for over 20 years, the design of Ras inhibitors has historically been hampered by a lack of clear understanding of the precise role of Ras and its regulatory interactions in signal transduction. This is no longer the case.

Signal transduction begins with an external signal, e.g., a growth factor, hormone, or antigen, binding to its cell-surface receptor. As illustrated in Fig. 1, once the extracellular ligand, e.g., epidermal growth factor (EGF), binds to its receptor, dimerization of the receptor occurs. Intermolecular mechanisms are then responsible for the activation of intrinsic tyrosine kinase activity followed by autophosphorylation of specific tyrosine residues (*6*).

Subsequent to receptor activation, a number of protein–protein interactions come into play by switching Ras "on" as opposed to its normal state when it is tightly bound to GDP and inactive. One feature shared by these regulatory proteins is that they contain Src-homology domains, responsible for the recognition of specific amino acid sequences containing either phosphotyrosine (SH2) or proline and hydrophobic residues (SH3) (*7*). The adapter protein Grb-2 contains both SH2 and SH3 domains; Grb-2 first binds to the activated receptor by virtue of its SH2 region and then associates with the Ras activator protein Sos1, the latter event being facilitated by the presence of two SH3 regions within Grb-2 (*8*). Complex formation between Grb-2 and Sos1 serves to recruit Sos1 into close proximity to Ras, thereby promoting activation of this G protein. Sos1 functions as a guanine nucleotide exchange factor and, on binding to Ras, disrupts its conformation, thereby causing the dissociation of GDP, which leaves the Ras protein free to bind GTP (*9,10*). In this scenario, Ras must be present at the plasma membrane. Since Ras does not contain a transmembrane domain, membrane

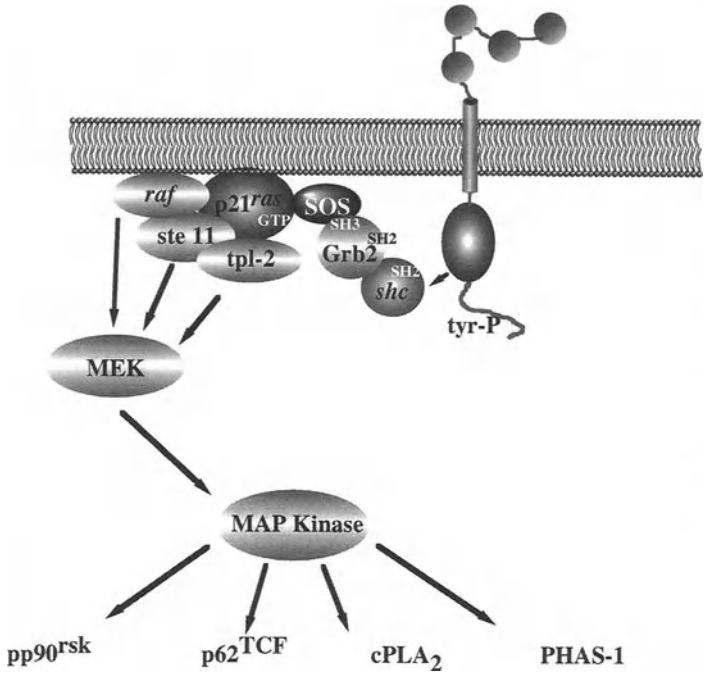

Fig. 1. Key players in the *ras* signaling pathway (*see text* for details).

localization is accomplished by the posttranslational addition of a lipid moiety (farnesyl group) to the carboxyl-terminus of p21, thereby facilitating insertion of the Ras protein into the plasma membrane. This modification will be covered more extensively in Section 4.

Under normal conditions, the cell tightly regulates the proportion of active Ras such that quiescent cells in G_0 generally have $< 5\%$ of their total Ras pool in the active state compared to nearly 50% on mitogenic stimulation (*11*). Although Ras, as a G protein, has intrinsic GTPase activity and will eventually hydrolyze bound GTP to GDP and inorganic phosphate, the negative regulatory protein GAP (GTPase-activating protein) acts to accelerate the deactivation of Ras, so that the rate of proliferation is kept under control. GAP was first identified by Trahey and McCormick as an activity that stimulated GTP hydrolysis of microinjected oncogenic Ras in *Xenopus* oocytes (*12*). A dual functionality has been ascribed to GAP, which serves as a downstream target of Ras function in addition to playing the role of negative regulator. Its effector properties were suggested when it was discovered that oncogenic *ras* mutants, despite their unresponsiveness to GAP stimulation, still required interaction with GAP for transforming activity (*13*). GAP, which has a mol wt of 120 kDa, contains two SH2 domains and one SH3 domain at its N-terminus, allowing it to interact with the cellular proteins p62 and p190, a feature that is apparently critical for its signal transducing activity (*14,15*). The cellular functions of p62 and p190 remain unclear, although it is believed that a complex between GAP120 and p190 could serve to couple activated growth factor receptor signals to Ras and Rho/Rac proteins, which are involved in regulation of cytoskeletal actin assembly (*16*). In this manner, cell-cycle progression could be coordinated with cytoskeletal reorganization (*17*).

Once Ras is switched "on," activation of the mitogen-activated protein kinases, i.e., the MAP kinase cascade, is the final series of events leading to transmission of growth signals to the nucleus. The MAP kinases, also referred to as extracellular signal-regulated kinases (ERKs), are serine/threonine kinases. The activity of the first enzyme in this cascade, MAP kinase, depends on a dual specificity kinase capable of phosphorylating both a threonine and a tyrosine residue. Such dual functionality has been ascribed to the c-*raf*-1 proto-oncogene product. Raf, which acts as an MAP kinase kinase, has been found to complex with Ras, only when the latter protein is activated, i.e., in its GTP-bound state (*18*). Recent studies have elegantly shown that if *raf* is genetically manipulated to contain the membrane localization signal of k-Ras conferred by its carboxy-terminus, then it becomes constitutively localized at the plasma membrane and completely bypasses a need for Ras (*19,20*). They conclude that Ras is able to transmit signals downstream by virtue of its ability to recruit Raf to the plasma membrane where a distinct, Ras-independent, Raf activation event occurs. Once Raf is activated, the enzymatic activity of Raf, namely MAP kinase kinase kinase, phosphorylates MEK (MAP kinase kinase), which in turn phosphorylates MAP kinase (ERK). Like Ras, MAP kinase appears to be tightly regulated, becoming activated on phosphorylation and deactivated by various tyrosine phosphatases. The substrates for MAP kinase are diverse; on its own phosphorylation, MAP kinase directs the phosphorylation of transcription factors, including c-*jun* and p62TCF (*21*), and also phosphorylates other kinases, e.g., pp90rsk, promoting their entry into the nucleus to carry out activation of transcription factors (*22*).

The preceding overview highlights only the key interactions of Ras and its many upstream and downstream partners in mitogenic signaling, and points to obvious potential targets for the design of Ras inhibitors. However, it should be kept in mind that the whole picture is considerably more complex, in part because of the existence of multiple Ras partner proteins that sometimes appear to have identical functionality (*see* comprehensive review by Khosravi-Far and Der, ref. *23*). For example, there exist multiple adapter proteins for Ras (Grb-2 and shc) as well as two GAP regulators (p120GAP and neurofibromin-1). Divergent roles for seemingly similar signaling molecules are most likely a reflection of the evolution of the *ras* superfamily, with its large number of structurally homologous, yet functionally unique members. Because of their involvement in distinctly different physiological processes, ensuing crossregulation is likely to occur. For example, the Ras-related protein rap-1 was recently found to compete with Ras for binding to Raf (*24*). Furthermore, p120-GAP is thought to act on all Ras family members. The reader is referred to an excellent review by Boguski and McCormick devoted to the array of proteins identified to date that are known to regulate members of the Ras superfamily (*25*). As we will see in Section 5, the existence of highly homologous proteins within the Ras superfamily is likely to pose special challenges in the design and development of a tumor-specific Ras inhibitor.

3. IMPACT OF *RAS* MUTATIONS IN HUMAN CANCER

ras genes have been intensely studied since 1982, when their ability to induce malignant transformation was first demonstrated (*26*). The critical role played by *ras* mutations in the development of human tumors can be inferred from three separate lines

Table 1
Incidence of *ras* Mutations in Human Malignancies

Tumor	*Activated ras gene*	*Frequency, %*[a]
Pancreatic adenocarcinoma	K	75–93
Colon adenocarcinoma	K	40–47
Lung adenocarcinoma	K	22–33
Thyroid carcinoma	H, K, N	53–60
Melanoma	N	8–19
Bladder carcinoma	H	7–17
Hepatocarcinoma	N	0–30
Renal carcinoma	H	7–13
Glioblastoma	—	0
Neuroblastoma	—	0
Prostate	—	5[b]
Ovary	—	0
Cervix	—	0
Sarcoma	—	0
Breast	—	0–8
Lymphoid		
ALL	N	0–18
NHL	—	0
Hodgkins's	N	0–67
Myeloid		
AML	N	11–70
CML	N	0–50

[a]Data represent the range of values reported in individual studies as reported by Bos (*31*).
[b]Data of Gumerlock et al. (*32*).
Abbreviations: ALL (acute lymphocytic leukemia), NHL (non-Hodgkin's lymphoma), AML (acute myelogenous leukemia), CML (chronic myelogenous leukemia).

of evidence. Foremost are the observations made by many groups that oncogenic Ras, but not normal Ras, induces increased cellular proliferation of rodent fibroblasts and also renders them tumorigenic (*27–29*). Second, transgenic mice harboring oncogenic *ras* mutations show an increased incidence of tumor formation (*30*). Finally comes the strong circumstantial evidence from epidemiological studies showing a high frequency of *ras* mutations in a wide variety of tumor types.

In this section, we will see that *ras* mutations occur at a high rate in colon and pancreatic cancers, two tumor types notoriously refractory to standard chemotherapy. Treatment outcome may be further compromised by the ability of oncogenic Ras to enhance a tumor's metastatic potential. Finally, the potential negative impact of *ras* mutations on the outcome of alternative treatment modalities, specifically radiotherapy, will be explored.

3.1. Incidence and Tumor Type

The use of specific recombinant DNA-based assays and the polymerase chain reaction have afforded a ready and accurate means for quantitating the prevalence of *ras* mutations in human tumors. *ras* mutations have been detected in a wide array of human tumors, and are especially prevalent in tumors of the pancreas (90%), colon (50%), and thyroid (50%) (Table 1). These mutations are relatively infrequent in

tumors of the breast and prostate. Overall, roughly 30% of all tumors contain an activated *ras* allele. Mutations generally occur in either codon 12, 13, or 61 of one of the three *ras* genes (H-, K, or N-), most frequently occurring in K-*ras* and least often in H-*ras*.

Evidence in support of a multistep nature of carcinogenesis is compelling and has been most clearly demonstrated for colorectal cancer, where stepwise and cumulative genetic changes are thought to occur (*33*). Among the early events is hypomethylation of DNA, followed by a mutation in the APC gene and subsequent allelic loss on three different chromosomes. It is thought that these genetic events set the stage for subsequent activating mutations in the *ras* genes.

There is increasing evidence that K-*ras* gene mutations may actually serve as reliable pathogenetic and diagnostic markers of neoplastic disease (*34*). Mutations in codon 12 of K-*ras* are prevalent in aberrant crypt foci in the colon, which represent lesions that may be preneoplastic (*35*). Furthermore, mucous cell hyperplasias of the pancreas, which arise in association with chronic pancreatitis, have also been found to harbor K-*ras* mutations with high frequency (*36*). At the present time, the clinical significance of these findings is unknown. However, should the evidence become compelling for *ras* mutations being confined to neoplastic and preneoplastic tissue, then they may serve as useful markers for earlier diagnosis.

3.2. *Role of Oncogenic* Ras *in Metastasis*

The final stage in tumor progression is acquisition by transformed cells of the ability to metastasize, i.e., they invade the bloodstream and lymphatic system, stimulate angiogenesis, and subsequently proliferate in tissues distant to the primary site. When metastatic and nonmetastatic disease has been compared for the incidence of *ras* mutations, a higher frequency has been reported for the invasive tumors (*37,38*). In view of the selective growth advantage conferred on a cell when Ras activation occurs, one might argue that metastatic potential is an indirect consequence. However, there is compelling evidence suggesting that this is not the case. Expression of oncogenic Ras appears to in effect reprogram the expression of critical genes associated with the metastatic phenotype. Specifically, Ras transformation results in increased expression of key metalloproteinases, i.e., stromelysin and gelatinase, whereas transcription and mRNA levels of TIMP-1 are decreased (*39*). Along these same lines, expression of the extracellular matrix molecule thrombospondin, which is decreased in metastatic tumors, is also reduced on transfection with viral K-*ras* (*40*).

The mechanisms whereby oncogenes, such as *ras*, act to control stromelysin expression are just now beginning to be characterized. Recent work has shown that protein kinase C may play a critical role in PDGF-induced activation of the stromelysin promoter by virtue of a novel palindromic element (*41*). These data suggest the existence of a bifurcation downstream of *ras*, which leads to stromelysin expression.

3.3. *ras Expression and Radiation Resistance*

Roughly half of all cancer patients receive radiotherapy at some point during treatment of their disease. Since 30% of all tumors contain oncogenic *ras* mutations, then it stands to reason that a negative impact of activated *ras* on radiosensitivity of tumor cells would be a concern. Sklar first reported in 1988 that increased radiation resistance accompanied Ras transformation (*42*). Since that time, however, there have

been conflicting reports regarding the generality of this phenomenon. Although some researchers have confirmed a correlation between *ras* expression and radioresistance (*43,44*), many groups have reported that *ras* does not confer a change in radiosensitivity (*45–49*). The genetic heterogeneity of the various cell lines studied by these groups may be contributing to the conflicting data, especially in view of the fact that other oncogenes, e.g., *raf* and *myc*, have also been reported to affect radiosensitivity adversely (*50*). Furthermore, simultaneous transformation by two oncogenes, *ras* and *myc*, confers radioresistance to a much greater extent than either oncogene alone (*51*). It has also been suggested that full-length proto-oncogene may influence cellular radiosensitivity in a different manner from that of the activated oncogene (*52*). Data indicating that *ras*-conferred radioresistance is cell-cycle-phase-dependent suggests yet another variable among the various studies (*53*).

4. EMERGENCE OF NEW THERAPEUTIC APPROACHES: RATIONALE AND PROGRESS

4.1. Therapies Targeting ras Expression

Although the concept of repairing a single base mutation by genetic therapy is theoretically appealing, clinically this approach remains in the distance. However, researchers have made excellent progress toward reducing expression of the Ras protein at the translational level as discussed below.

4.1.1. Antisense DNA

One approach that has demonstrated utility in decreasing Ras protein expression involves the use of antisense oligonucleotides complementary to RNA transcripts of the activated *ras* oncogene. Binding of the antisense DNA presumably inhibits expression of mutant *ras* by several mechanisms, including blocking ribosomal translation of the RNA transcript as well as triggering RNase H degradation of the target RNA (*54,55*). Chemical improvements have focused in part on decreasing the general susceptibility of antisense reagents to nuclease degradation. Therefore oligomer analogs containing phosphorothioate linkages in place of the naturally occurring phosphodiester linkage have been employed to increase resistance to nucleases and therefore prolong biological half-life. Oligonucleoside methylphosphonates and phosphoramidates, which are quite hydrophobic and taken up readily by cells, have subsequently been tested with favorable in vitro results reported (*56,57*). Intercalating agents and/or hydrophobic tails have also been employed as linkers to increase affinity for the mRNA target and to promote tumor uptake of the antisense oligomers (*58*).

The short-term nature of the above studies calls into question the degree to which sustained antisense treatment can suppress *ras* expression. Gray and colleagues addressed this issue by treating NIH-3T3 cells transformed with H-*ras* (from the T24 human bladder carcinoma) for 72 h with an appropriate antisense DNA pentadecamer for targeting H-*ras* and then measuring tumorigenicity in athymic nude mice posttreatment (*59*). These investigators were thereby able to demonstrate that tumor growth was significantly reduced for up to 14 d following treatment.

Although these studies demonstrated proof of concept, their clinical significance remained unclear, since treatment of tumor cells with antisense DNA had occurred *ex vivo* prior to inplantation. The critical and logical next step was to address whether

antisense DNA reagents could be successfully delivered intact to the tumor site and show efficacy against established tumors without unacceptable toxicity. One approach to delivery, favored over transfection, was to develop a retroviral antisense K-*ras* expression vector; subsequently, this antisense vector was shown to eliminate expression of the mutant Ras protein in human lung cancer cells (*60*). Using an orthotopic human lung cancer model in nude mice, these researchers showed that their antisense K-*ras* retroviral construct, administered by intratracheal instillation on the third day after implantation of the tumor, showed significant activity; 87% of the mice receiving the antisense construct were tumor-free vs only 10% of the control group (*61*).

In general, the concept of antisense DNA therapy to treat mutant *ras*-expressing tumors has shown much promise. The progress of Roth and his colleagues has led to what may be one of the first gene therapy trials for lung cancer (*62*). It remains too early to report clinical results. However, a Phase I trial employing a phosphorothioate oligonucleotide with a sequence complementary to the p53 suppressor gene has been initiated in patients with acute lymphocytic leukemia (AML) or myelodysplastic syndrome; early reports indicate no major toxicity after 10 d of continuous infusion with approx 10–20% of the administered dose recovered in the urine as intact oligonucleotide (*63*).

4.1.2. Ribozymes

The production of catalytic RNA that will specifically cleave within the mutated sequence of mRNA unique to oncogenic *ras* represents another approach for altering Ras protein expression. This approach involves the design of synthetic DNA that encodes hammerhead ribozymes containing thermodynamically stable loop structures to prevent aggregation of ribozymes while also containing the requisite recognition sequence for mutated *ras* mRNA. NIH 3T3 cells have successfully been cotransfected with a plasmid expressing such a ribozyme along with an activated *ras* gene, resulting in abrogation of the transformed phenotype (*64,65*). Furthermore, it has also been reported that transfection of EJ human bladder carcinoma cells with hammerhead ribozyme-encoded DNA results in reduced tumorigenicity in nude mice (*66,67*). These data provide proof of concept that ribozymes can in fact affect Ras protein expression and also support the role of Ras in both tumorigenicity and invasion (*67*). However, it should be kept in mind that the in vivo experiments reported to date with anti-*ras*-directed ribozymes have involved ribozyme-treatment of cells prior to their introduction into animals. Again, as with antisense DNA, successful delivery to the tumor site becomes critical. The exogenous delivery of synthetic ribozymes as well as vector-based approaches to ribozyme therapy are currently being pursued; although great strides have been made chemically to improve catalytic activity and decrease nuclease susceptibility, the major challenge of increasing uptake in order to facilitate localization of RNA remains. It is encouraging in this regard that successful in vivo delivery of ribozymes have been accomplished for noncancer indications (*68*).

4.2. Inhibitors of Ras *Function*

As we have seen, a single base change mutation is responsible for the conversion of a *ras* proto-oncogene to its activated oncogenic counterpart. Once this molecular switch consequently becomes stuck in the "on" position, uncontrolled proliferation ensues. Because of its complex and critical role in mitogenic signaling, a number of

events that involve direct participation of the Ras protein can be envisioned as possibilities for therapeutic intervention.

4.2.1. Antagonists of Protein-Protein Interactions

A review on the subject of the amenability of protein–protein interactions as anticancer targets appears elsewhere (*69*). With regard to the Ras signaling pathway, one could envision that disruption of the interaction of Ras with either Sos, Raf, or GAP could result in favorable antiproliferative effects. Because technical advances have greatly expedited the synthesis of multiple peptides and also because of the generation of combinatorial peptide libraries, peptidic antagonists of these protein complexes are actively being sought.

Peptide inhibitors that inhibit the interaction of Ras with GAP corresponding to amino acids 19–32 of the Ras protein have been reported (*70*). These peptide inhibitors should serve as a useful starting point for the design of smaller peptide fragments that will retain activity and not be subject to impermeability and the lability limitations of their predecessors. Inhibitors of Ras/Sos have not yet been reported, but it is noteworthy that peptide inhibitors of Src SH3/SH2 phosphoprotein interactions have recently been reported (*71*); however, they were found to lack antiproliferative effects, presumably because of poor cell penetration and/or lability of the phosphate group on the tyrosine residue.

Although small molecule inhibitors would be desirable, the likelihood of identifying a specific inhibitor with sufficient affinity may be compromised by virtue of the large surface over which the targeted protein–protein interaction occurs. For instance, the minimal region of Raf required for interaction with Ras appears to be approx 70 amino acids (*72*). Thus, antagonists of any of the various Ras–protein interactions must be sufficiently large to disrupt Ras binding in a biologically meaningful way, but at the same time be small enough to enter cells readily while not being degraded.

In addition, the existence of multiple SH2 and SH3 domains will most likely make the issue of selectivity a formidable challenge to rational drug design. Encouraging in this regard were the results of a study where *Xenopus* oocytes were microinjected with either a monoclonal antibody (MAb) recognizing the SH3 domain of p120GAP or SH3 domain peptides as short as nine residues; Ras-dependent, but not Ras-independent, maturation of the oocytes was blocked with no adverse effect on GAP activity (*73*). In general, effective antagonists, whether peptidic or nonpeptidic, should be smaller and demonstrate an improved profile with regard to potency, efficacy, bioavailability, stability, and selectivity compared to the natural ligand (*69*).

4.2.2. Inhibitors of Membrane Localization of RAS

Not only must Ras bind GTP to be active, but it must also be associated with the inner face of the plasma membrane. Initially synthesized as a soluble, cytosolic protein, Ras undergoes a series of posttranslational modifications that provide it with the lipophilicity that is required for membrane association (*74,75*). To compensate for lack of a transmembrane domain, the Ras protein is prenylated, specifically undergoing addition of a farnesyl group at its carboxy-terminus. All Ras proteins are prenylated by virtue of a common consensus sequence, termed the CAAX motif, at their carboxyl-termini; this CAAX box consists of a cysteine residue (C), followed by two aliphatic amino acids (A), and ending in any one of several amino acids (X). The nature of X

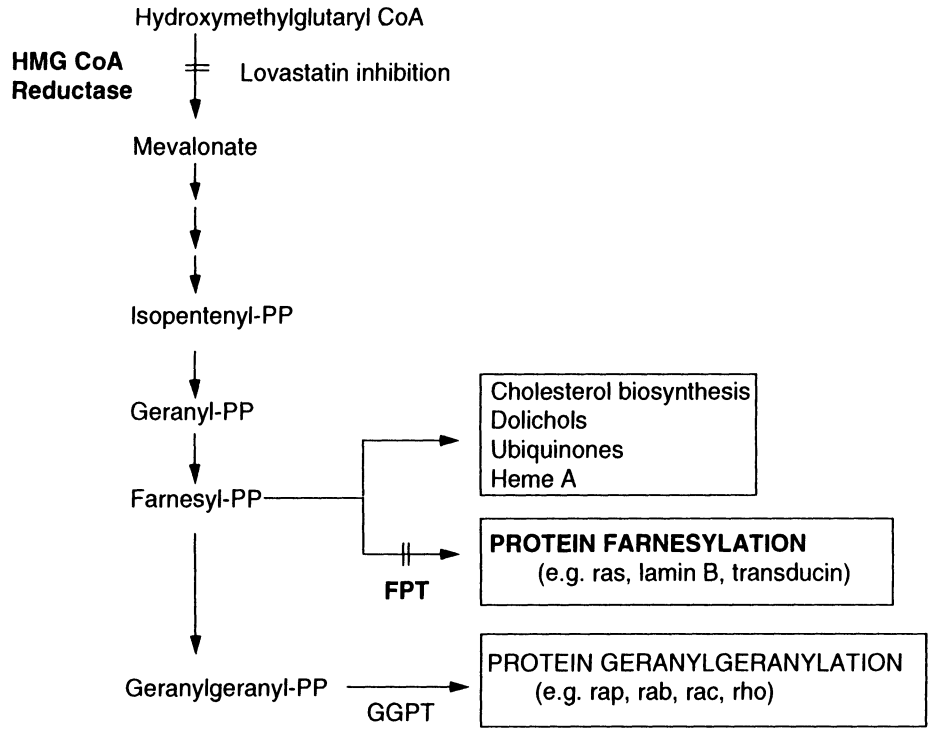

Fig. 2 Biosynthetic pathway of mevalonate and isoprenoids.

determines substrate specificity among the multiple prenylation enzymes. In a series of reactions to be described in more detail below, farnesyl pyrophosphate is enzymatically attached to Ras at the cysteine residue within the CAAX motif, preceding cleavage of the three terminal amino acids followed by methyl esterification of the newly created carboxy-terminal cysteine. Elucidation of the enzymatic events responsible for these chemical modifications of Ras have opened up a number of possibilities for therapeutic intervention.

4.2.2.1. Farnesylation. The cytoplasmic enzyme farnesyl protein transferase (FPT) catalyzes the reaction of farnesyl pyrophosphate, an isoprenoid derived from the mevalonate pathway (Fig. 2), with the CAAX cysteine residue on Ras to form a thioether linkage. FPT, which is ubiquitous among eukaryotic cells, is a heterodimer composed of 48- and 46-kDa subunits. The significant structural homology between mammalian and yeast FPT reveals a high degree of conservation. Three prenylation enzymes have been described. Whereas FPT catalyzes the addition of a 15-carbon farnesyl group, two other prenylation enzymes recognize a 20-carbon isoprenoid substrate and are responsible for protein geranylgeranylation. FPT shares a common subunit, its pyrophosphate binding (α) subunit, with geranylgeranyl protein transferase I (GGPT type 1). The β subunit provides CAAX box specificity; the occurrence of farnesylation as opposed to geranylgeranylation is dictated by the identity of the terminal amino acid in the CAAX motif. Whereas CAAX appears to be invariant for FPT, the geranylgeranylation enzymes recognize CAAL, XXCC, XCXC, and CCXX (*76*).

Table 2
Inhibitors of *ras* Farnesyl Protein Transferase

Inhibitor	*IC_{50}, μM*	*Reference*
CAAX analogs		
CVFM and related tetrapeptides	≥0.012	*79*
L-731,735	0.018	*80*
B581	0.021	*81*
BZA-2B	0.00085	*82*
Cys-AMBA-Met	0.15	*83*
L-739,750	0.018	*84*
Phenol tripeptides	63–157	*85*
Biphenyl nonpeptides	0.15	*86*
FPP analogs		
(α-Hydroxyfarnesyl) phosphonic acid	0.030	*87*
PD 083176	0.017	*88*
Farnesylamine	—	*89*
Natural products		
10′-Desmethoxy-stretonigrin	21	*90*
Manumycin	5	*91*
Gliotoxin	1.1	*92*
Chaetomellic acid A	0.055	*87*
Zaragozic acid	0.216	*87*
Pepticinnamin E	0.30	*93*
Fusidienol	0.30	*94*
Preussomerin	1.2	*95*

Interest in FPT as a pharmacological target was initially triggered by the finding that mutants lacking a CAAX motif do not associate with the plasma membrane and furthermore do not transform cells to malignancy (*77,78*). This has led to an intensive search in numerous laboratories for potent inhibitors of FPT. A comprehensive list of inhibitors reported to date have been compiled in Table 2 along with their relative potencies against Ras FPT. Reviews appear elsewhere regarding the chemistry of these compounds, as well as further discussion on their potential usefulness as anticancer agents (*96–101*). I will now focus on six key compounds (structures shown in Fig. 3) to illustrate the progress that has been made in the last five years in achieving cellular activity.

In their initial report describing the identification and purification of FPT, peptides as short as four residues were found by Reiss et al. to act as alternative substrates competing with full-length Ras for farnesylation (*79*). The tetrapeptide CVFM was found to be a potent inhibitor of FPT (IC_{50} = approx 40 n*M*) and acted as a true inhibitor since it was not a substrate for farnesylation (*102*). CVFM and other structurally related tetrapeptides were not serious drug candidates because of inefficient cellular uptake and rapid proteolytic degradation. However, they have served well as first-generation peptide inhibitors of FPT for the design of improved peptidomimetic derivatives of the CAAX motif.

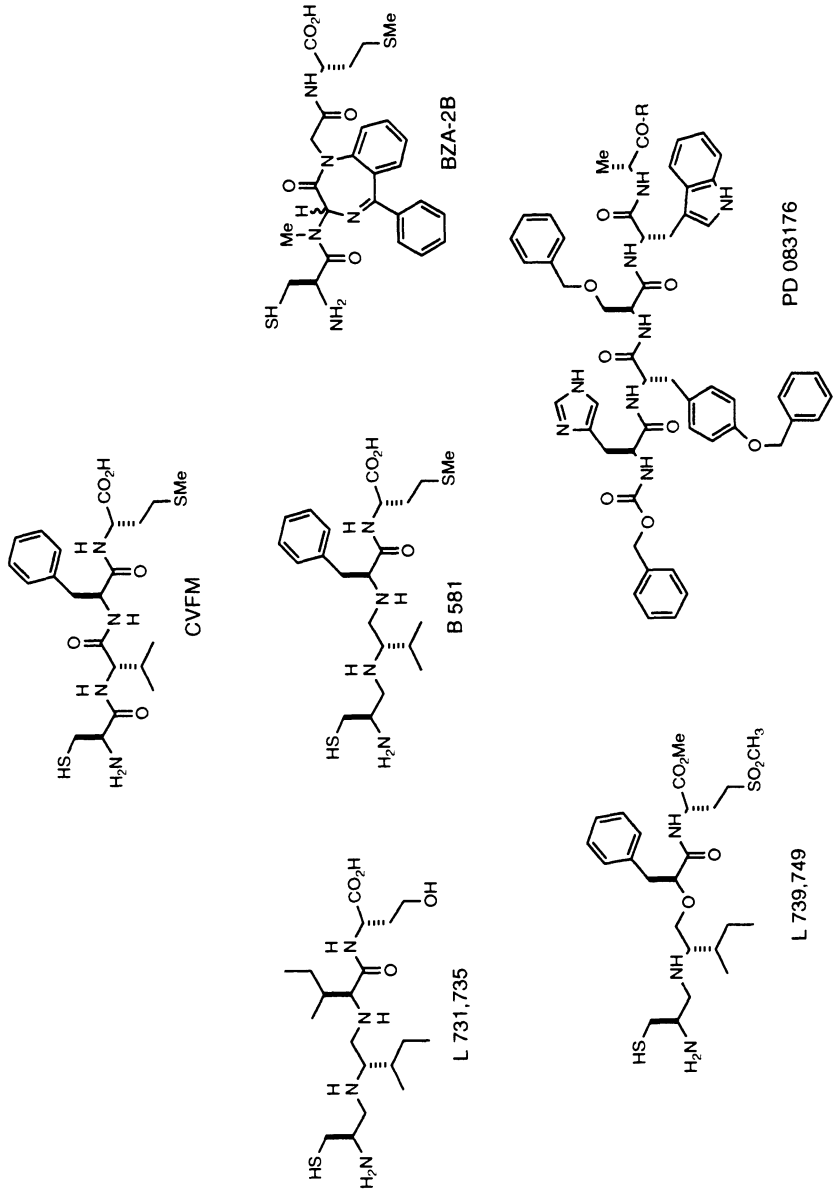

Fig. 3 Structures of key inhibitors of *ras* FPT.

The pseudo-tetrapeptide L 731, 735 (*see* Fig. 3) was designed as a CIIM analog in which the two N-terminal amide bonds were reduced and methionine replaced with homoserine in a successful effort to improve potency (*80*). When the corresponding lactone derivative was synthesized and evaluated, cellular activity could be detected, both with respect to modulation of Ras processing as well as selective inhibition of anchorage-independent growth of *ras*-transformed cells (*80*). Although these synthetic organic inhibitors still lacked sufficient potency to be viable drug candidates, they nevertheless provided evidence that inhibition of FPT can in fact modulate Ras function in whole cells.

B581 represents another CAAX analog that resulted from systematic modification of CVFM by replacement of the amino-terminal amide bonds (*81*). This compound proved to be significantly more stable than CVFM and resulted in cellular modulation of posttranslational processing of prenylated proteins, although at high concentrations (50 μM). Again, limited permeability may have had a detrimental effect on cellular potency. Particularly encouraging was the finding that B581 appeared to be selective for FPT in treated cells; this was reflected in part by a lack of inhibition in the processing of the geranylgeranylated Rap 1A protein (*81,103*).

Replacement of the two aliphatic residues in the CAAX motif with a benzodiazepine-derived mimic of a dipeptide turn led to a subnanomolar inhibitor of FPT (*82*). This compound was designed to allow both the N-terminal cysteine and the C-terminal methionine to coordinate the zinc ion present at the peptide binding site. Cellular activity was observed when the corresponding methyl ester was synthesized, although a 50-fold reduction in enzyme potency accompanied this chemical modification.

A novel compound to emerge from high-volume screening was PD 083176, a protected pentapeptide characterized by the sequence CBZ-his-(*O*-benzyl)tyrosine-(*O*-benzyl)serine-tryptophan-D-alanine-NH_2 (*88*). This nonthiol-containing peptide, although highly potent against FPT (IC_{50} = 20 nM), proved to be too impermeable for cellular studies. However, if PD 083176 was microinjected into *Xenopus* oocytes, a block in maturation induced by insulin, but not progesterone, was observed, indicating specific inhibition of Ras function. Truncation of PD 083176 has led to a series of modified peptide analogs, e.g. PD 152440, which exhibit a modest reduction in potency against purified FPT relative to PD 083176, but are cell-permeable (*104*). Inhibition of Ras farnasylation was observed when *ras*-transformed cells were treated with concentrations of PD 152440 as low as 1–5 μM.

The first Ras FPT inhibitor reported to have in vivo activity was L-739,750 (*84*). This compound was synthesized in an attempt to decrease the chemical instability of the prodrug of L-731,735 by replacing the nucleophilic nitrogen with an ether oxygen. The absence of systemic toxicity in this study is particularly important as discussed below.

Collectively, evaluation of these six prototype compounds have provided the foundation for an initial determination of the feasibility of developing a Ras FPT inhibitor as a pharmacological agent. First, the studies cited above clearly establish that FPT inhibitors can be designed to enter cells readily and modulate the putative target. Next comes the issue of enzyme specificity, where it is important to show that FPT can be specifically inhibited over other prenylation enzymes. Current dogma dictates that a

desirable FPT inhibitory candidate drug should be as selective as possible against FPT relative to GGPT. Since the vast majority of proteins of the Ras superfamily, critical to a wide variety of cellular functions, are prenylated by GGPT, this appears to be a reasonable premise. However, until a successful drug emerges against this target, it remains unclear exactly to what degree FPT prenylation needs to be selectively inhibited. Clearly most of the inhibitors discussed here proved to be highly specific for FPT when tested in vitro against purified enzymes. In some cases, data have begun to emerge at the cellular level supporting the concept that FPT inhibitors can be specific and not result in the concurrent inhibition of processing of geranylgeranylated proteins (*81*).

It is imperative that the toxicity profile of FPT inhibitors also includes an evaluation of their activity against non-Ras-related substrates for farnesylation. Most notably, the structural protein lamin B and proteins involved in visual transduction, i.e., transducin and rhodopsin kinase, are also farnesylated. Therefore, pharmacokinetic parameters, such as whether the inhibitor crosses the blood–brain barrier, may prove important. We still have very little information regarding the relative physiological levels of all of the pertinent substrates for prenylation, nor do we know how their relative substrate affinities compare, what their turnover rates are, and so forth. In the case of irreversible inhibitors, this issue may prove critical. Again, without a clinical precedent directed against this target, we must remain open-minded regarding criteria establishment for advancement of active compounds. Initial indications that administration of an FPT inhibitor to tumor-bearing mice results in efficacy without appreciable systemic toxicity are encouraging (*84*).

Finally, there is the issue of whether FPT inhibitors will selectively target mutant Ras as opposed to endogenous Ras function. It is therefore encouraging that normal fibroblast cells, for instance, continue to grow in monolayer culture at concentrations of BZA-5B that are significantly inhibitory to the growth of *ras*-transformed cells (*82*). The basis for this selectivity, which has been observed for several of the inhibitors reported here, largely remains an enigma.

In addition to specific inhibitors of FPT, some general inhibitors of isoprenylation are actively being investigated for potential clinical utility. Lovastatin, an HMG CoA reductase inhibitor used clinically to lower blood cholesterol levels, has also been reported to inhibit the growth of *ras*-transformed cells in animals (*105*). However, this agent is quite cytotoxic and is 100-fold more potent against cholesterol biosynthesis compared to isoprenylation (*106*). In other studies, phenylacetate, which is the deaminated metabolite of phenylalanine, also appears to be a general inhibitor of protein prenylation (*107*); Phase I testing of phenylactate against glioblastomas has shown early promising signs of activity (*108*). Finally, limonine is a monocyclic monoterpene that along with its metabolites selectivity inhibits the isoprenylation of p21Ras and other 21- to 26-kDa cell-growth-associated proteins (*109*). Preclinical studies have led to current exploration into the feasibility of pursuing limonene as a chemopreventative agent against breast cancer (*110*).

4.2.2.2. Proteolytic Cleavage. On farnesylation, the three terminal amino acids of the CAAX motif are cleaved by proteolytic processing (*111*). Two microsomal enzymes that perform this function have been described; the first is an endoprotease from bovine liver that cleaves a carboxy-terminal tripeptide (*112*), whereas the second

enzyme described is a rat brain carboxypeptidase that sequentially removes the terminal amino acids (*113*). Very few inhibitors of these enzymes have been reported. The notable exceptions are farnesyl-CAAX analogs, distinguished by isosteric replacements for the scissile peptide bond, which have significant activity against the endoprotease with K_is as low as 64 n*M* (*114*). From an anticancer drug standpoint, interest in this target is diminished by the fact that a CAAX mutant of *ras* that did not undergo proteolysis, while compromised in its ability to associate with the plasma membrane, showed a minimal loss in transforming activity (*78*).

4.2.2.3. Methylesterification. Subsequent to proteolytic removal of the three terminal amino acids of the CAAX motif, a protein methyltransferase catalyzes the methyl esterification at the newly terminal cysteine residue (*115,116*). S-farnesyl mercaptopropionic acids have been reported to inhibit weakly methyltransferase with $K_i > 25$ μM. As with the protease, this enzyme modification does not appear to be essential for transforming activity (*78*).

4.2.2.4. Palmitoylation. Farnesylation alone provides Ras proteins with a fairly weak affinity for membrane binding. However, the association of farnesylated Ras with the plasma membrane is enhanced by other structural features of the Ras protein. These may include, in the case of certain K-Ras proteins, a stretch of six lysine residues upstream from the carboxy-terminal CAAX motif; protonation of this polybasic region likely confers a positive charge that provides affinity to the negatively charged phosphate surface of the membrane bilayer (*117*). Cysteine palmitoylation sites may also exist upstream of the CAAX motif, at residues 181 and 184 in the case of H-Ras (*118*). Little is known about the palmitoyl-CoA transferase activity that is responsible for this modification, except that it is associated with an internal membrane compartment (*119*). A potential role for palmitoyl-CoA inhibitors as anticancer drugs does not seem likely based on the studies of Hancock and his colleagues showing that mutant proteins that lack cysteine palmitoylation sites nevertheless retain transforming activity (*117*). Furthermore, most K-Ras proteins, which are most prevalent among human tumors, do not contain sites for palmitoylation.

5. SUMMARY AND FUTURE DIRECTIONS

During the last decade, cytotoxic agents have been de-emphasized in our search for a new generation of anticancer drugs. Certainly it has not been for lack of effort that very few new agents have joined the ranks of the dozen or so drugs from which oncologists have to choose. Conventional chemotherapy has generally exploited the more aggressive growth kinetics exhibited by cancer cells relative to their normal counterparts; we have seen many examples of initially promising drug candidates, often within the DNA intercalator and antimetabolite class, that subsequently fail because tumors become resistant. The tide has therefore shifted toward focusing on the discovery of less toxic compounds designed to exploit fundamental differences between malignant and nonmalignant cells. It is in this context that the *ras* oncogene, expressed in approximately one-third of all human tumors, has become an attractive target for anticancer drug design. Until recently, attempts to discover Ras inhibitors were impeded by a lack of clear understanding of the precise role of Ras in fulfilling its critical obligation to a number of cellular processes, proliferation nonwithstanding. During the last decade,

basic research in the signal transduction field has progressed at an astounding rate with Ras emerging from its black box. As a result, the critical Ras interactions that are amenable to therapeutic intervention have been defined, and Ras inhibitors are now being discovered at a rapid rate. In some cases, in vitro studies demonstrating cellular modulation of the putative Ras-directed target have been followed by encouraging reports of in vivo activity, e.g., farnesyl protein transferase inhibitors. We are now faced with many pharmacological questions, previously not germane to the development of cytotoxic agents; the necessary requirements for a signaling antagonist to be worthy of advancement to the clinical testing stage remain to be defined. The next few years are likely to focus on showing that a sufficiently nontoxic Ras inhibitor can target oncogenic *ras* selectively and give a therapeutically meaningful response. If this proves to be the case, Ras inhibitors will likely represent a significant advance in the treatment of many solid tumors for which there is currently no effective treatment.

ACKNOWLEDGMENTS

I would like to thank W. R. Leopold, Gary Bolton, and Alan Saltiel for their assistance with this manuscript. I also wish to express my appreciation to Robert Jackson for introducing me to the challenges of anticancer drug development. I consider myself truly fortunate to have benefited from his mentorship.

REFERENCES

1. Zubrod CG. Chemical control of cancer. *Proc Natl Acad Sci USA* 1972; 69:1042-1047.
2. Stacey DW, DeGudicibus SR, Smith MR. Cellular Ras activity and tumor cell proliferation. *Exp Cell Res* 1987; 171:232.
3. Kung HF, Smith MR, Bekesi E, Stacey DW. Reversal of transformed phenotype by monoclonal antibodies against Ha-Ras p21 proteins. *Exp Cell Res* 1986; 162:363-371.
4. Bar Sagi D, Feramisco JR. Microinjection of the Ras oncogene protein into PC12 cells induces morphological differentiation. *Cell* 1985; 42:841-848.
5. Sadler SE, Maller JL, Gibbs JB. Transforming Ras proteins accelerate hormone-induced maturation and stimulate cyclic AMP phosphodiesterase in *Xenopus* oocytes. *Mol Cell Biol* 1990; 10:1689-1696.
6. Schlessinger J, Ullrich A. Growth factor signaling by receptor tyrosine kinases. *Neuron* 199; 2: 9:383-391.
7. Schlessinger J. SH2/SH3 signaling proteins. *Curr Opinion Genet Dev* 1994; 4:25-30.
8. Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar-Sagi D, Schlessinger J. The SH2 and SH3 domain-containing protein Grb2 links receptor tyrosine kinases to Ras signalling. *Cell* 1992; 70:431-442.
9. Buday L, Downward J. Epidermal growth factor regulates p21Ras through the formation of a complex of receptor Grb2 adaptor protein, and Sos nucleotide exchange facator. *Cell* 1973: 611-620.
10. Gale NW, Kaplan S, Lowenstein EJ, Schlessinger J, Bar-Sagi D. Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. *Nature* 1993; 363:88-92.
11. Osterop AP, Medema RH, v.d.-Zon GC, Bos JL, Moller W, Maassen JA. Epidermal growth factor receptors generate Ras. GTP more efficiently than insulin receptors. *Eur J Biochem* 1993; 212:477-482.
12. Trahey M, McCormick, F. A cytoplasmic protein stimulates normal N-Ras p21 GTPase, but does not affect oncogenic mutants. *Science* 1987; 238:542-554.
13. Cales C, Hancock JF, Marshall CJ, Hall A. The cytoplasmic protein GAP is implicated as the target for regulation by the *ras* gene product. *Nature* 1988; 332:548-551.

14. Wong G, Muller O, Clark R, Conroy L, Moran MF, Polakis P, McCormick F. Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. *Cell* 1992; 69:551-558.
15. Settleman J, Narasimhan V, Foster LC, Weinberg RA. Molecular cloning cDNAs encoding the GAP-associated protein p190: implications for a signalling pathway from Ras to the nucleus. *Cell* 1992; 69:539-549.
16. Hall A. Ras-related GTPases and the cytoskeleton. *Mol Biol Cell* 1992; 3:475-479.
17. Pendergast GC, Gibbs JB. Ras regulatory interactions: novel targets for anti-cancer intervention. *BioEssays* 1994; 16:187-191.
18. Moodie SA, Willumsen BM, Weber MJ, Wolfman A. Complexes of Ras-GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* 1993; 260:1658-1661.
19. Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF. Activation of Raf as a result of recruitment to the plasma membrane. *Science* 1994; 264:1463-1467.
20. Leevers SJ, Paterson JF, Marshall CJ. Requirement for Ras in Raf activation is overcome by targeting raf to the plasma membrane. *Nature* 1994; 369:411-441.
21. Gille H, Sharrocks AD, Shaw PE. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-*fos* promoter. *Nature* 1992; 358:414-417.
22. Sturgill TW, Ray LB, Erikson E, Maller JL. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature* 1988; 334:715-718.
23. Khosravi-Far R, Der CJ. The Ras signal transduction pathway. *Cancer and Metastasis Rev* 1994; 13:67-89.
24. Cook SJ, Rubinfeld B, Albert A, McCormick F. RapV12 antagonizes Ras-dependent activation of ERK-1 and ERK-2 by LPA and EGF in rat-1 fibroblasts. *EMBO J* 1993; 366:3475-3485.
25. Boguski MS, McCormick F. Proteins regulating Ras and its relatives. *Nature* 1993; 366:43-653.
26. Barbacid M. *Ras* genes. *Ann Rev Biochem* 1987; 56:779-827.
27. Shih C, Padhy LC, Murray M, Weinberg RA. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* 1981; 290:261-264.
28. Krontiris TG, Cooper GM. Transforming activity of human tumor DNA. *Proc Natl Acad Sci USA* 1981; 78:1181-1184.
29. Wilson DM, Yang D, Dillberger JE, Dietrich SE, Maher VM, McCormick JJ. Malignant transformation of human fibroblasts by a transfected N-*ras* oncogene. *Cancer Res* 1990; 50: 5587-5593.
30. Adams JM, Cory S. Transgenic models of tumor development. *Science* 1991; 254:1161-1167.
31. Bos JL. *Ras* oncogenes in human cancer: a review. *Cancer Res* 1989; 49:4682-4689.
32. Gumerlock P, Poonamallee U, Meyers F. Activated *ras* alleles in human carcinoma of the prostate are rare. *Cancer Res* 1991; 51:1632-1637.
33. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; 61:759-767.
34. Fearon ER. K-*ras* gene mutation as a pathogenic and diagnostic marker in human cancer. *J Natl Cancer Inst* 1993; 85:1978-1980.
35. Pretlow TP, Brasitus TA, Fulton NC, Cheyer C, Kaplan EL. K-*ras* mutations in putative preneoplastic lesions in human colon. *J Natl Cancer Inst* 1993; 85:2004-2007.
36. Yanagisawa A, Ohtake K, Ohashi K, et al. Frequent c-Ki-*ras* oncogene activation in mucous cell hyperplasias of pancreas suffering from chronic inflammation. *Cancer Res* 1993; 53:953-956.
37. Goretzki PE, Lyons J, Stacy-Phipps S, Rosenau W, Demeure M, Clark OH, McCormick F, Roher HD, Bourne HR. Mutational activation of *ras* and *gsp* oncogenes in differentiated thyroid cancer and their biological implications. *World J Surg* 1992; 16:576-581.
38. Anwar K, Nakakuki K, Naiki H, Inuzuka M. *Ras* gene mutations and HPV infection are common in human laryngeal carcinoma. *Int J Cancer* 1993; 53:22-28.
39. Su ZZ, Austin VN, Zimmer SG, Fisher PB. Defining the critical gene expression changes associated with expression and suppression of the tumorigenic and metastatic phenotype in Ha-*ras*-transformed cloned rat embryo fibroblast cells. *Oncogene* 1993; 8:1211-1219.
40. Zebrenetzky V, Harris CC, Steeg PS, Roberts DD. Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines. *Int J Cancer* 1994; 59:191-195.

41. Sanz L, Berra E, Municio MM, Dominguez I, Lozano J, Johansen T, Moscat J. Diaz-Meco MT. Zeta PKC plays a critical role during stromelysin promoter activation by platelet-derived growth factor through a novel palindromic element. *J Biol Chem* 1994; 269:10,044-10,049.
42. Sklar MD. The *ras* oncogenes increase the intrinsic resistance of NIH 3T3 cells to ionizing radiation. *Science* 1988; 239:645-647.
43. Miller AC, Kariko K, Myers CE, Clark EP, Samid D. Increased radioresistance of EJ *ras*-transformed human osteosarcoma cells and its modulation by lovastatin, an inhibitor of p21Ras isoprenylation. *Int J Cancer* 1993; 53:302-307.
44. Hermens AF, Bentvelzen PA. Influence of the H-*ras* oncogene on radiation responses of a rat rhabdomyosarcoma cell line. *Cancer Res* 1992; 52:3073-3082.
45. Grant ML, Bruton RK, Byrd PJ, Gallimore PH, Steele JC, Taylor AM, Grand RJ. Sensitivity to ionising radiation of transformed human cells to mutant *ras* genes. *Oncogene* 1990; 5: 1159-1164.
46. Garden AS, Meyn RE, Weil MM, Lebovitz RM, Lieberman MW. The influence of *ras* oncogene expression on radiation response in the cell. *Int J Radiat Biol* 1992; 307-311.
47. Su LN, Little JB. Transformation and radiosensitivity of human diploid skin fibroblasts transfected with activated *ras* oncogene and SV40 T-antigen. *Int J Radiat Biol* 1992; 62:201-210.
48. Mendonca MS, Boukamp P, Stanbridge EJ, Redpath JL. The radiosensitivity of human keratinocytes: influence of activated c-H- *ras* oncogene expression and tumorigenicity. *Int J Radiat Biol* 1991; 59:1195-1206.
49. Alapetite C, Baroche C, Remvikos Y, Goubin X, Moustacchi E. Studies on the influence of the presence of an activated *ras* oncogene on the in vitro radiosensitivity of human mammary epithelial cells. *Int J Radiat Biol* 1991; 59:385-396.
50. Pirollo KF, Tong YA, Villegas Z, Chen Y, Chang EH. Oncogene-transformed NIH 3T3 cells display radiation resistance indicative of a signal transduction pathway leading to the radiation-resistant phenotype. *Radiat Res* 1993; 135:234-243.
51. McKenna WG, Weiss MC, Endlich B, Ling CC, Bakanauskas VJ, Kelsten ML, Muschel RJ. Synergistic effect of the v-*myc* oncogene with H- *ras* on radioresistance. *Cancer Res* 1990; 50: 97-102.
52. Warenius HM, Browning PG, Britten RA, Peacock JA, Rapp UR. C-*raf*-1 proto-oncogene expression relates to radiosensitivity rather than radioresistance. *Eur J Cancer* 1994; 30A:369-375.
53. Cheong N, Wang Y, Iliakis G. Radioresistance induced in rat embryo cells by transfection with the oncogenes H-*ras* v-*myc* is cell cycle dependent and maximal during S and G2. *Int J Radiat Biol* 1993; 63:623-629.
54. Haeuptle MT, Frank R, Dobberstein B. Translation arrest by oligodeoxynucleotides complementary to mRNA coding sequences yields polypeptides of predetermined length. *Nucleic Acids Res* 1986; 14:1427-1428.
55. Walder RY, Walder JA. Role of RNase H in hybrid-arrested translation by antisense oligonucleotides. *Proc Natl Acad Sci USA* 1988; 85:5011-5015.
56. Chang EH, Miller PS, Cushman C, Devadas K, Pirollo KF, Ts'o PO, Yu ZP. Antisense inhibition of *ras* p21 expression that is sensitive to a point mutation. *Biochemistry* 1991; 30:8283-8286.
57. Chang EH, Miller PS. Ras, an inner membrane transducer of growth stimuli. In: Wilkstrom E, ed. *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS.* New York: Wiley-Liss. 1991:115-124.
58. Saison-Behmoaras T, Tocque B, Rey I, Chassignol M, Thuong NT, Helene C. Short modified antisense oligonucleotides directed against Ha-*ras* mutation induce selective cleavage of the mRNA and inhibit T24 cells proliferation. *EMBO J* 1991; 10:1111-1118.
59. Gray GD, Hernandez OM, Hebel D, Root M, Pow-Sang JM, Wickstrom E. Antisense DNA inhibition of tumor growth induced by c-Ha-*ras* oncogene in nude mice. *Cancer Res* 1993; 53: 577-580.
60. Zhang Y, Mukhopadhyay T, Donehower LA, Georges RN, Roth JA. Retroviral vector-mediated transduction of K-*ras* antisense RNA into human lung cancer cells inhibits expression of the malignant phenotype. *Human Gene Therapy* 1993; 4:451-460.
61. Georges RN, Mukhopadhyay T, Zhang Y, Yen N, Roth JA. Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K-*ras* construct. *Cancer Res* 1993; 53:1743-1746.

62. Drug & Market Development 1994; 4:235.
63. Bayever B, Iversen PL, Bishop MR, Sharp JG, Tewary HK, Arneson MA, Pirrucello SJ, Ruddon RW, Kessinger A, Zon G, Armitage JO. Systemic administration of a phosphorothioate oligonucleotide with a sequence complementary to p53 for acute myelogenous leukemia and myelodysplastic syndrome: initial results of a phase I trial. *Antisense Res Dev* 1993; 3:383–390.
64. Kashani-Sabel M, Funato T, Florenes VA, Fodstad O, Scanlon KJ. Suppression of the neoplastic phenotype in vivo by an anti-*ras* ribozyme. *Cancer Res* 1994; 54:900–920.
65. Koizumi M, Kamiya H, Ohtsuka E. Inhibition of c-Ha-*ras* gene expression by hammerhead ribozymes containing stable C(UUCG)G hairpin loop. *Biol Pharm Bull* 1993; 16:879–883.
66. Tone T, Kashani-Sabet M, Funato T, Shitara T, Yoshida E, Kashfian BI, Horng M, Fodstadt O, Scanlon KJ. Suppression of EJ cells tumorigenicity. *In Vivo* 1993; 7:471–476.
67. Kashini-Sabet M, Funato T, Tone T, Jiao L, Wang W, Yoshida E, Kashfinn BI, Shitara T, Wu AM, Moreno JG, Scanlon KJ, et al. Reversal of the malignant phenotype by an anti-*ras* ribozyme. *Antisense Res Dev* 1992; 2:3–15.
68. Efrat S, Leiser M, Wu YJ, Fusco-DeMane D, Emran OA, Surana M, Jetton TL, Magnuson MA, Weir G, Fleischer N. Ribozyme-mediated attenuation of pancreatic beta-cell glucokinase expression in transgenic mice results in impaired glucose-induced insulin secretion. *Proc Natl Acad Sci USA* 1994; 91:2051–2055.
69. Huber HE, Koblan KS, Heimbrook DC. Protein-protein interactions as therapeutic targets for cancer. *Curr Med Chem* 1994; 1:13–34.
70. Gibbs JB. Pharmacological probes of Ras function. *Sem Cancer Biol* 1992; 3:383–390.
71. Gilmer TG, Rodriguez M, Jordan S, Crosby R, Alligood K, Green M, Kimery M, Wagner C, Kinder D, Charifson P, Hassell AM, Willard D, Luther M, Rusnak D, Sternbach DD, Mehrotra M, Peel M, Shampine L, Davis R, Robbins J, Patel IR, Kassel D, Burkhart W, Moyer M, Bradshaw T, Berman J. Peptide inhibitors of src SH3-SH2-phosphoprotein interactions. *J Biol Chem* 1994; 269:31,711–31,719.
72. Vojtek AB, Hollenberg SM, Cooper JA. Mammalian ras interacts directly with the serine/threonine kinase Raf. *Cell* 1993; 74:205–214.
73. Duchesne M, Schweighoffer F, Parker F, Clerc F, Frobert Y, Thang MN, Tocque B. Identification of the SH3 domain of GAP as an essential sequence for Ras-GAP-mediated signaling. *Science* 1993; 259:525–528.
74. Schafer WR, Kim R, Sterne R, Thorner J, Kim S-H, Rine J. Genetic and pharmacological suppression of oncogenic mutations in RAS genes of yeast and humans. *Science* 1989; 245:379–385.
75. Casey PJ, Solski PA, Der CJ, Buss JE. p21Ras is modified by a farnesyl isoprenoid. *Proc Natl Acad Sci USA* 1989; 86:8323–8327.
76. Gibbs JB. Lipid modifications of proteins in the Ras superfamily. In: Dickey BF, Birnbaumer L, eds. *GTPases in Biology I.* New York: Springer-Verlag. 1993:335–344.
77. Jackson JH, Cochrane CG, Bourne JR, Solski PA, Buss JE, Der CJ. Farnesol modification of Kirsten-*ras* exon 4B protein is essential for transformation. *Proc Natl Acad Sci USA* 1990; 87:3042–3046.
78. Kato K, Cox AD, Hisaka MM, Graham SM, Buss JE, Der CJ. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. *Proc Natl Acad Sci USA* 1992; 89:6403–6407.
79. Reiss Y, Goldstein JL, Seabra MC, Casey PJ, Brown MS. Inhibition of purified p21Ras farnesyl: protein transferase by cys-AAX tetrapeptides. *Cell* 1990; 62:81–88.
80. Kohl NE, Mosser SD, deSolms SJ, Giuliani EA, Pompliano DL, Graham SL, Smith RL, Scolnick EM, Oliff A, Gibbs JB. Selective inhibition of Ras-dependent transformation by a farnesyltransferase inhibitor. *Science* 1993; 260:1934–1937.
81. Garcia AM, Rowell C, Ackermann K, Kowalczyk JJ, Lewis MD. Peptidomimetic inhibitors of Ras farnesylation and function in whole cells. *J Biol Chem* 1993; 268:18,415–18,418.
82. James GL, Goldstein JL, Brown MS, Rawson TE, Somers TC, McDowell RS, Crowley CW, Lucas BK, Levinson AD, Marsters JC. Benzodiazepine peptidomimetics: potent inhibitors of Ras farnesylation in animal cells. *Science* 1993; 260:1937–1942.
83. Nigam M, Seong C-M, Qian Y, Hamilton AD, Sebti SM. Potent inhibition of human tumor p21Ras farnesyltransferase by A_1A_2-lacking p21*ras* CA_1A_2X peptidomimetics. *J Biol Chem* 1993; 268:20,695–20,698.

84. Kohl NI, Wilson FR, Mosser SD, Giuliani E, DeSolms SJ, Conner MW, Anthony NJ, Holtz WJ, Gomez RP, Lee T, Smith RL, Graham SL, Hartman GD, Gibbs JB, Oliff A. Protein farnesyltransferase inhibitors block the growth of Ras-dependent tumors in nude mice. *Proc Natl Acad Sci USA* 1994; 91:9141-9145.
85. Patel DV, Patel MM, Robinson SS, Gordon EM. Phenol based tripeptide inhibitors of Ras farnesyl protein transferase. *Bioorganic Med Chem Lett* 1994; 4:1883-1888.
86. Vogt A, Qian Y, Blaskovich MA, Fossum RD, Hamilton AD, Sebti SM. A non-peptide mimetic of Ras-CAAX:selective inhibition of farnesyltransferase and Ras processing. *J Biol Chem* 1994; 270:660-664.
87. Gibbs JB, Pompliano DL, Mosser SD, Rands E, Lingham RB, Singh SB, Scolnick EM, Kohl NE, Oliff A. Selective inhibition of farnesyl-protein transferase blocks Ras processing in vivo. *J Biol Chem* 1993; 268:7617-7620.
88. Sebolt-Leopold JS, Gowan R, Su T-Z, Leonard D, Sawyer T, Bolton G, Hodges J, Hupe D. Inhibition of Ras farnesyltransferase by a novel class of peptides containing no cysteine or thiol moieties. *Proc Am Assoc Cancer Res* 1994; 35:3535.
89. Kothapalli R, Guthrie N, Chambers AF, Carroll KK. Farnesylamine: an inhibitor of farnesylation and growth of Ras-transformed cells. *Lipids* 1993; 28:969.
90. Liu WC, Barbacid M, Bulgar M, Clark JM, Crosswell AR, Dean L, Doyle TW, Fernandes PB, Huang S, Manne V, Pirnik DM, Wells JS, Meyers E. 10'-Desmethoxystreptonigrin, a novel analog of streptonigrin. *J Antibiotics* 1992; 45:454-457.
91. Hara M, Akasaka K, Akinaga S, Okabe M, Nakano H, Gomez R, Wood D, Uh M, Tamanoi F. Identification of Ras farnesyltransferase inhibitors by microbial screening. *Proc Natl Acad Sci USA* 1993; 90:2281-2285.
92. Van der Pyl D, Inokoshi J, Shiomi K, Yang H, Takeshima H, Omura S. Inhibition of farnesyl-protein transferase by gliotoxin and acetylgliotoxin. *J Antibiotics* 1992; 45:1802-1805.
93. Omura S, Van Der Pyl D, Inokoshi J, Takahashi Y, Takeshima H. Pepticinnamins, new farnesyl-protein transferase inhibitors produced by an actinomycete. *J Antiobiotics* 1993; 46:222-234.
94. Singh SB, Jones ET, Goetz MA, Bills GF, Nallin-Omstead M, Jenkins RG, Lingham RB, Silverman KC, Gibbs JB. Fusidienol: a novel inhibitor of Ras farnesyl-protein transferase from *Fusidium griseum*. *Tetrahedron Lett* 1994; 35:4693-4696.
95. Singh SB, Zink DL, Liesch JM, Ball RG, Goetz MA, Bolessa EA, Giacobbe RA, Silverman KC, Bills GF, Pelaez F, Cascales C, Gibbs JB, Lingham RB. Preussomerins and deoxypreussomerins: novel inhibitors of Ras farnesyl-protein transferase. *J Org Chem* 1994; 59:6296-5302.
96. Bolton GL, Sebolt-Leopold JS, Hodges JC. *Ras* oncogene directed approaches in cancer chemotherapy. In: Plattner J, ed. *Annual Reports in Medicinal Chemistry*. New York: Academic. 1994:165-174.
97. Prendergast GC, Gibbs JB. Ras regulatory interactions: novel targets for anticancer intervention? *BioEssays* 1994; 16:187-191.
98. Khosravi-Far R, Cox AD, Kato K, Der CJ. Protein prenylation: key to Ras function and cancer intervention? *Cell Growth & Differ* 1992; 3:461-469.
99. Gibbs JB. Ras C-terminal processing enzymes-new drug targets? *Cell* 1991; 65:1-4.
100. Gibbs JB, Oliff A, Kohl NE. Farnesyltransferase inhibitors: Ras research yields a potential cancer therapeutic. *Cell* 1994; 77:175-178.
101. Omura S, Takeshima H. Farnesyl-protein transferase inhibitors. *Drugs of the Future* 1994; 19:751-755.
102. Goldstein JL, Brown MS, Stradley SJ, Reiss Y. Gierasch LM. Nonfarnesylated tetrapeptide inhibitors of protein farnesyltransferase. *J Biol Chem* 1991; 266:15,575-15,578.
103. Cox AD, Garcia AM, Westwick JK, Kowalczyk JJ, Lewis MD, Brenner DA, Der CJ. The CAAX peptidomimetic compound B581 specifically blocks farnesylated, but not geranylgeranylated or myrisylated, oncogenic Ras signaling and transformation. *J Biol Chem* 1994; 269:19,203-19,206.
104. Sebolt-Leopold JS. The therapeutic potential of Ras-targeted protein farnesyltransferase inhibitors. In: *Emerging Drugs: The Prospect for Improved Medicines*. London: Ashley. 1996: 219-239.
105. Sebti SM, Tkalcevic GT, Jani JP. Lovastatin, a cholesterol biosynthesis inhibitor, inhibits the growth of human H-*ras* oncogene transformed cells in nude mice. *Cancer Commun* 1991; 3: 141-147.

106. Sinensky M, Beck LA, Leonard S, Evans R. Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis.*J Biol Chem* 1990; 265:19,937–19,941.
107. Samid D, Ram Z, Hudgins WR, Shack S, Liu L, Walbridge S, Oldfield EH, Myers CE. Selective activity of phenylacetate against malignant gliomas:resemblance to fetal brain damage in phenylketonuria. *Cancer Res* 1994; 54:891–895.
108. Thibault AM, Cooper MR, Figg WD, Venzon DJ, Oliver Sartor VA, Tompkins AC, Weinberger MS, Headlee DJ, McCall NA, Samid D, Myers CE. A phase I and pharmacokinetic study of intravenous phenylacetate in patients with cancer. *Cancer Res* 1994; 54:1690–1694.
109. Crowell PL, Lin S, Vedejs E, Gould MN. Identification of metabolites of the antitumor agent *d*-limonene capable of inhibiting protein isoprenylation and cell growth. *Cancer Chem Pharmacol* 1992; 31:205–212.
110. Gould MN, Moore CJ, Zhang R, Wang B, Kennen WS, Haag JD. Limonene chemoprevention of mammary carcinoma induction following direct in situ transfer to v-Ha-*ras*. *Cancer Res* 1994; 54:3540–3543.
111. Ashby MN, King DS, Rine J. Endoproteolytic processing of a farnesylated peptide in vitro. *Proc Natl Aci USA* 1992; 89:4613–4617.
112. Ma Y-T, Rando RR. A microsomal endoprotease that specifically cleaves isoprenylated peptides. *Proc Natl Acad Sci USA* 1992; 89:6275–6279.
113. Akopyan TN, Couedel Y, Beaumont A, Foumie-Saluski MC, Roques BP. Cleavage of farnesylated COOH-terminal heptapeptide of mouse N-ras by brain microsomal membranes: evidence for a carboxypeptidase which specifically removes the COOH-terminal methionine. *Biochem Biophys Res Commun* 1991; 187:1336–1339.
114. Ma Y-T, Gilbert, Rando RR. Inhibitors of the isoprenylated protein endopeptidase. *Biochemistry* 1993; 32:2386.
115. Clarke S, Vogel JP, Deschenes RJ, Stock J. Post-translational modification of the H-*ras* oncogene protein: evidence for a third class of protein carboxyl methyltransferase. *Proc Natl Acad Sci USA* 1988; 85:4643–4647.
116. Perez-Sala D, Tan EW, Canada FJ, Rando RR. Methylation and demethylation reactions of guanine nucleotide-binding proteins of retinal rod outer segments. *Proc Natl Acad Sci USA* 1991; 88:3043–3046.
117. Hancock JF, Paterson H, Marshall CJ. A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21Ras to the plasma membrane. *Cell* 1990; 63:133–139.
118. Hancock JF, Magee AI, Childs JE, Marshall CJ. All Ras proteins are polyisoprenylated but only some are palmitoylated. *Cell* 1989; 57:1167–1177.
119. Gutierrez L, Magee AI. Characterization of an acyltransferase acting on p21N-Ras protein in a cell-free system. *Biochim Biophys Acta* 1991; 1078:147–154.

19 Gene Therapy

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1. INTRODUCTION

Cancer is one of the acquired diseases finding its origin in multiple genetic alterations. From studies on several forms of hereditary cancer, we know that part of the alterations are present constitutively in the germ line, leading to a predisposition for getting cancer (*1*). These alterations are heterozygous and affect an allele of one or more tumor suppressor genes, which play a role in controlling cell proliferation and differentiation. Another part of the alterations occurs during life-time as a result of spontaneous mutations in the remaining intact allele, ultimately leading to the formation of the transformed cell. In this development also alterations in proto-oncogenes contribute to the formation of the tumor cell (*2,3*). Sporadic forms of cancer involve similar genetic alterations in tumor suppressor genes and proto-oncogenes, but they usually take much longer to develop. The notion of cancer being a disease anchored in the genes of the cancer cell makes it an attractive target for strategies involving gene therapy.

Gene therapy is commonly defined as "the transfer of new genetic material to cells of an individual with resulting therapeutic benefit to the individual" (*4*). In our view, this definition should be more broadly interpreted, i.e., do not restrict gene therapy only to transfer of new genetic material, but also include manipulation of existing genetic material. This holds true especially for cancer cells in the situation that dominantly activated oncogenes have to be eliminated. In principle, germ line cells as well as somatic cells are targets for genetic alterations. Germ line therapy in humans, however, is for the moment being considered as unethical and not safe, at least as long the experience with the various forms of somatic therapy is limited.

In cancer patients, gene therapy has to be directed to the tumor or, in the case of known hereditary defects, to the (organ) sites were tumor formation is likely to occur.

From: *Cancer Therapeutics: Experimental and Clinical Agents*
Edited by: B. Teicher Humana Press Inc., Totowa, NJ

Strikingly, in the case of cancer, the gene therapy is not confined to the proto-oncogenes or tumor suppressor genes affected. Various other approaches for genetic manipulation allow new forms of cancer therapy, e.g., the transfer of new antigenic determinants into cancer cells, aimed at an enhanced immunogenicity and subsequent destruction by the patient's immune system. These approaches will be first discussed in Section 2. The focus of this chapter, however, will be the implications of somatic gene therapy with regard to interactions of the immune system with neoplastic cells.

2. APPROACHES FOR GENE THERAPY OF CANCER

2.1. Overview

2.1.1. Why Gene Therapy?

Since sophisticated tools developed by molecular biologists to modify single genes in individual cells became easily accessible to the field of medical practice, a new area of research opened, aimed at curing diseases by altering human genetic material. This approach has several advantages when compared to conventional drug therapy. First, DNA of the vector carrying the gene can be stably integrated in the host genome, and the subsequent constitutive expression makes the therapy more effective and longer lasting. This will be particularly true for hereditary diseases, where a gene correction is made. Second, vectors can be targeted to certain cells or tissues by making use of tissue-specific promoters regulating the transcription of the transferred genes. Here, one can, for instance, think of altering normal bone marrow stem cells by introducing a multidrug resistance gene to limit myelosuppression of cytostatic drugs in cancer therapy. However, also tumor cells themselves can be modified to produce, for example, enzymes converting prodrugs, or proteins that make the cells more immunogenic. Third, whole cells, modified in vitro to serve as factories of proteins involved in a therapeutic action, can be delivered to patients by adoptive transfer or by surgery. These cells can even be targeted to certain organs or tumors, as in the case of tumor-infiltrating lymphocytes (TIL) homing specifically to the tumor. A number of examples relevant to the treatment of various forms of cancer will be discussed later (cf Section 2.1.3.).

2.1.2. Methods of Gene Transfer

Various delivery systems suitable for gene therapy have been developed over recent years (Table 1). The most simple one is physical transfer of DNA (or RNA) to cells. This can be achieved in vivo by injection of naked DNA in tumors (*5*) or by making use of liposome-encapsulated DNA in order to make access of the DNA into tumor cells easier (*6*). Recently, new techniques using a bombardment of DNA-coated particles into tissues were developed, sometimes referred to as "gene gun" experiments (*7*). Ex vivo, other techniques are available, including the classical calcium phosphate precipitation method (*8*), electroporation (*9*), and lipofection (*10*). The latter method makes use of liposomes, as in the case of the in vivo situation. The efficiencies of these methods are highly variable and depend on the cells to be transferred. For instance, the calcium phosphate precipitation method works well, with reasonable transfection efficiencies, provided the cells are well dividing in an adherent monolayer. Lipofection can be more efficient, but is more laborious and expensive (*10,11*). However, in

Table 1
Delivery Systems Used in Gene Therapy

Method	*Application in gene therapy*		*Transient, T, or stable, S, expression*
	ex vivo	*in vivo*	
Physical			
Direct injection of DNA	−	+	T
$CaPO_4$ precipitation	+	−	S
Lipofection	±	+	T
Transferrinfection	±	+	T
Vector-based			
Retroviruses	+	+	T
Adenovirus	±	+	T
Adeno-associated virus	−	+	S
Herpes virus	±	±	?
Vaccinia virus	±	±	T

+ : major application; ± ; some application; − : minor or no application; S: Stable expression; T: Transient expression; ?: not (yet) known. Data are adapted from ref. (*14*).

the case that tumors are treated, in fact a 100% efficiency is required, unless a bystander effect is taking place (*see* Section 2.3.1.). The relatively low transfection efficiency makes the physical methods less attractive, because, for instance, selected clones may not express all tumor antigens owing to heterogeneity of the tumor.

Other methods to deliver DNA specifically in dividing cells can be more efficient. One example is the transferrinfection method, which is based on receptor-mediated transfer (*12*). In this method, DNA is coupled to a transferrin–polylysin conjugate and, thus, results in targeting dividing cells expressing the transferrin receptor. The uptake of the complex can be largely enhanced by including an adenovirus–polylysin conjugate in the complex (*13*).

Techniques using viral vectors have been widely explored. In the first instance, murine retroviral vectors became the most popular, because of their high efficiency of transduction as a result of high-titered virus stocks (*14*). These viruses are defective in packaging and can only replicate in a helper cell line harboring the packaging sequences. After infection and reverse transcription to cDNA, the viral genome is integrated in the host genome, and constitutive expression can be obtained for more than a year (*14*). The gene to be transduced is cloned behind a stong (eukaryotic) promoter, and usually also a selectable marker gene is present allowing selection of transduced cells. The ability of the vector DNA to integrate randomly in the genome, however, can be considered as a potential danger: oncogenes may be activated or tumor suppressor genes inactivated and a new cancer cell may develop. The chance this will happen, however, seems small, since the development of cancer is a multistep process involving multiple alterations in proto-oncogenes as well as in tumor suppressor genes.

One of the major disadvantages of retroviruses is that their integration in the host genome and the subsequent expression of the recombinant gene are limited to dividing cells. This has prompted many investigators to develop viral vectors expressed

in nondividing cells. Replicating vectors are preferable, because they usually promote high expression of the recombinant gene (*15*). Examples being explored now are human adenoviruses. They are hardly toxic, and on infection, they accomplish a high gene expression (*16,17*). However, this expression is transient, and more seriously, an immune response against the virus may develop. Adenovirus-associated viruses may be a better choice, because they stably integrate in the genome of the host. The development of these vectors, however, has only recently started (*18*).

2.1.3. Specific Approaches for Cancer

Although gene therapy initially had not been designed specifically for cancer, many of its applications have first been tried in cancer patients. Several factors may have contributed to this. First, in the cancer field, there is a large experience with clinical trials, because in many cases, no regular therapy that has been proven successful exists. Second, because of the lack of an alternative, many cancer patients are willing to cooperate in these clinical trials and give their informed consent. Finally, in cancer, the gene therapy is directed to destroying malignant cells, rather than making corrections to improve survival, as is the case for nonneoplastic diseases. This makes the therapy less intrusive for the patient, since, with the exception of corrections made for defects in tumor suppressor genes, no long-term genetic alterations are implemented in the individual.

Several very different concepts, making use of gene therapy in cancer, have been worked out during recent years (Table 2). A distinction can be made between "gene marking" and "gene modification." The former was the first being applied to humans to test the feasibility of gene modification of human cells. TIL from melanomas were transduced with a retroviral vector comprising the neomycin phosphotransferase gene and reinfused into the autologous patients. G418-resistant cells could be recovered from tumor biopsies up to 2 mo after the adoptive transfer (*19*). Later, this technique has been used to assay whether malignant cells were relapsing from purged bone marrow after autologous bone marrow transplantation (BMT). Studies with acute myeloid leukemia (AML) and neuroblastoma patients showed that, in all cases where relapses occurred, the recurrent tumors came from the transplanted bone marrow (*20–22*). This gene marking has also been applied for the labeling of adoptively transferred HIV-specific cytotoxic T-cells (CTL) to patients with non-Hodgkin's lymphoma to prevent HIV infection of healthy donor bone marrow. The transferred CTL were labeled with a resistance gene as well as with the HSV *tk* gene to allow killing off the CTL with ganciclovir in case of unexpected growth (*4*). A similar experiment has been performed with Epstein-Barr virus (EBV)-specific CTL in a patient with post-BMT EBV-related lymphoproliferative disease to allow killing of the EBV-specific CTL (*23*). More examples of the introduction of such a "suicide gene" in tumor cells will be given below (Section 2.3.).

The second group of gene transfer techniques used in cancer consists of those involving gene modification (Table 2). They all aim at the in vivo or in vitro genetic alteration of either normal cells or tumor cells in such a way that the modification will be beneficial for the patient. There are many, conceptually very different, variations possible on this theme. First, one can try to direct the therapy directly to the genes responsible for the oncogenic transformation, and either add deleted genes or replace defect genes. Second, one can try to deliver genes encoding potential therapeutical

Table 2
Approaches of Gene Therapy of Cancer

Form	*Example*	*Goal*	*Status*
Gene marking			
Cell labeling	Labeling TIL cells with a selectable marker	Determine homing	C
	Labeling bone marrow after purging	Determine origin relapse after autologous BMT	C
Cell tracking	Transfer of HSV tk gene in CTL	Killing after adoptive transfer	C
Gene modification			
Addition/replacement	Correct oncogene defect	Kill tumor cell	E
Delivery			
Direct			
Suicide	Transfer of HSV tk gene in tumor cell	Kill tumor cell	C
Intensivation	Transfer of enzymes that activate drugs	Enhance chemotherapy	E
Indirect			
Protection	Transfer of a drug resistance gene	Allow high dose of chemotherapy	E
Immunomodulation	Transfer cytokine gene in tumor cell	Enhance immunogenicity of tumor cell	C

C: Clinically applied in humans.
E: Experimental.

proteins either to tumor cells or normal cells. In the first case, these can be proteins altering the sensitivity of tumor cells to cytostatic drugs (cf Table 2, direct gene therapy). In the second case, these can be proteins protecting normal cells against harmful effects of therapeutical drugs (indirect gene therapy). These forms of gene therapy will be discussed briefly below (Sections 2.2. and 2.3.). Finally, a large number of therapeutical trials making use of gene transfer techniques are focused on the alteration of the immune reactivity of either the tumor cells or the effector cells of the patient. This field of research will be the subject of a separate section (Section 3.).

2.2. Gene Correction

2.2.1. Genes Involved in Cancer

Genetic defects in proto-oncogenes as well as tumor suppressor genes, in principle, form a good target for gene correction therapy. In recent years, two classes of these tumor-promoting genes have been discerned, the so-called oncogenes and tumor suppressor genes (*1*). Genetic alterations in the former lead per definition to a "gain of function," and these are genotypically and phenotypically dominant. Such alterations are supposed to be lethal when present in the germ line and are therefore never

found as a hereditary property. Alterations in the second type of genes, the tumor suppressor genes, lead to a "loss of function" and may segregate in families as a recessive trait, the lost function being exerted by the nonaffected allele. However, once the intact allele is also affected by sporadic mutation, which probability is considerable during a life-time, cancer will develop. Therefore, in this case, the recessive defect behaves as a dominantly inherited trait.

In the early 1970s, molecular mechanisms of cell transformation were beginning to be unraveled by studies using viruses as transforming agents (*24,25*). Transforming genes of murine retroviruses, capable of transforming murine cells, were isolated and characterized. Instead of using the viable virus for the induction of transformation, it became feasible to transform cultured cells with purified genes (*8*). Prominent transforming genes are the oncogenes of acute transforming oncogenic retroviruses, named v-*onc* genes. Well-known examples of retroviral oncogenes are v-*ras* and v-*myc*, encoding proteins involved in signal transduction and transcription, respectively. More than a hundred of these types of genes have been characterized, and all these genes were found to possess cellular counterparts, termed proto-oncogenes or c-*onc* genes. They invariably turned out to play a role in the regulation of cell proliferation and differentiation of normal, noncancerous cells. They mostly mediate the transfer of growth-stimulating signals to the cell nucleus, or are directly involved in the transcription of genes involved in cell proliferation or differentiation.

Several mutated proto-oncogenes were picked up as the transforming genes present in DNA isolates from human tumors, when assayed in an in vitro transformation assay (*26*). Since only dominantly acting transforming genes could be detected by this assay, our knowledge of tumor suppressor genes emerged largely from cytogenetic and restriction fragment length polymorphism (RFLP) analysis of hereditary forms of cancer. Well-known examples of such tumor suppressor genes are *Rb* and *p53*, the former deleted retinoblastoma, and the latter inactivated by deletion or mutation in numerous forms of human cancer.

2.2.2. Correction of Oncogenes

Correcting activated oncogenes by gene therapy is difficult, one of the major problems being that all cells in the tumor have to be hit. Replacing genes by homologous recombination, as performed for germ line cells, seems not to be very effective for this purpose. Also, forcing high expression of the normal products (*27*) does not seem to be very attractive, since the capacity of the vectors is often limited and, moreover, the alteration in the oncogene is often highly dominant and cannot be simply overruled. Therefore, alternative strategies have been initiated. One of these is making use of antisense constructs, encoding transcripts that counteract expression of the mutated products. Also antisense oligonucleotides have been tried. The feasibility of these methods has been demonstrated for the H-*ras*, c-*myc*, N-*myc*, *bcr-abl*, c-*fos*, and c-*sis* oncogenes (*28–33*), but for most cases, successful applications in vivo have not been reported. Only for *ras* have the in vitro studies been extended to an in vivo model using human lung cancer cells in nude mice (*34*). Interestingly, it was recently shown that rat glioma cells could be rendered nontumorigenic in rats by expression of antisense constructs inhibiting insulin-like growth factor (IGF)-1 (*35*). Strikingly, parental tumor cells at distant sites were also affected, suggesting that inhibition of the growth factor leads to increased expression of immunogenic proteins, raising an

immune response also effective against the parental cells (reviewed in *27*). This situation is reminiscent of the effect of transfected cytokine genes in tumor cells on their immune reactivity as will be discussed below.

To counteract oncogene-encoded mRNA effectively in another way, so-called ribozymes have been explored. These RNA molecules offer antisense as well as catalytic properties and allow cleavage of specific RNA sequences. Constructs comprising sequences encoding these ribozymes can be transferred to tumor cells. Effective inhibition of H-*ras* with an activating point mutation at amino acid codon 12 has been shown in melanoma cells, resulting in morphological changes suggestive of a lower degree of malignancy (*27,36*). Similar findings have been reported for the mRNA encoding the tumor-specific *bcr-abl* epitope (*37*).

2.2.3. Correction of Tumor Suppressor Genes

As compared to strategies to counteract activated oncogenes, the introduction of lost or affected tumor suppressor genes into tumor cells seems more attractive and easier to accomplish. An attractive candidate is p53 because:

1. It is affected in numerous forms of human cancer (*38,39*);
2. It is involved in cell-cycle arrest as well as in the induction of apoptosis (*40*); and
3. A number of in vitro studies have shown that (re)introduction of the p53 gene in cancer cells leads to immediate growth arrest (*see 41,42* for review).

Moreover, expression of *p53* in tumor cells has been shown to act synergistically with ionizing radiation or cytostatics in the induction of apoptosis, which makes the combination of gene therapy for *p53* and conventional therapy attractive (*39, 43–46*). Recently, adenoviral vectors comprising the *p53* gene were constructed and shown to be capable of abolishing tumor formation of human tumor cells in nude mice (*45,47*). Another tumor suppressor gene candidate target for therapy is the *Rb* gene, originally discovered in retinoblastoma, but affected in many other forms of human cancer (*48,49*). Furthermore, the product of this gene is involved in cell-cycle regulation, and in fact, its state of phosphorylation controls G1 to S phase transition through its interaction with the nuclear transcription factor E2F (*50,51*). Also, here it has been shown that introduction of an active *Rb* gene may inhibit proliferation of tumor cells (*52*) and vascular proliferative disorders (*53*). Altogether, these experiments show that attractive gene therapy approaches to correct altered oncogene and tumor suppressor gene expression are available and have been proven successful already in experimental tumor models. Their way to the clinic, however, is proceeding slowly, and eventual success in curing cancer patients has to be awaited.

2.3 Drug Delivery

2.3.1. Suicide Genes

A large application field in gene therapy does not intend to alter directly genes involved in the origin of cancer as described above, but conducts alterations of either cancer cells or normal cells in such a way that therapy can be improved. The various approaches are summarized in Table 2. A rather spectacular form that is being tested in patients with brain tumors is transfer of the herpes simplex virus thymidine kinase (*tk*) gene into tumor allowing suicide of the cells. The *tk* gene confers sensitivity of human cells to ganciclovir by phosphorylating the drug to a form that is ultimately

incorporated in the DNA, leading to inhibition of DNA synthesis and cell death (reviewed in *23*). The ganciclovir can be given to the patient systemically. The *tk* gene can be transferred to the tumor cells in the brain by stereotactic injection either of retrovirus-containing supernatants with a high virus titer or of a murine fibroblast cell line producing a defective retrovirus comprising the *tk* gene (*54,55*). In rat studies, this approach was shown to be extremely effective in destroying the tumor cells macroscopically, as well as microscopically (*56,57*). An important phenomenon observed was that infection of all tumor cells with the virus was not necessary to eradicate the tumor. Apparently, neighboring nontransduced cells were also destroyed, referred to as a "bystander" or "field" effect. This could well be owing to diffusion of toxic products or to disrupture of the tumor vasculature, but another interesting observation was a high invasion of T-lymphocytes and macrophages in *tk* gene-transduced tumors, suggesting that the cells become targets for an enhanced immune reactivity (*58*). The precise mechanism of this is not known, but the effect clearly depended on an intact immune system, because the effect was not seen in nude or irradiated mice (*23,58–60*).

2.3.2. Improvement of Drug Action

Other forms of gene therapy directly targeted to the tumor cells make use of intensification of the action of conventional cytostatic drugs. For example, 5F-uracil (5FU), which has a high systemic toxicity, can be administered in the form of the nontoxic 5F-cytosine when the tumor cells are capable of converting this compound to 5FU. This is possible through transfer of the bacterial enzyme cytosine deaminase (*61*). This transfer can be done by making use of retroviral systems and this type of therapy is therefore named virus-directed enzyme/prodrug therapy (VDEPT) (*62*). Strikingly, also in this case of suicide therapy, an immune component has been shown to be involved in the response, and protective immunity against subsequent challenges with the parental cell line could be gained (*63*). The VDEPT therapy can be made more specific by targeting expression of the enzyme to certain types of tumors by making use of tissue-specific promoters, such as the *CEA* promoter, highly expressed in colon carcinoma, or the *erbB2* or *α-fetoprotein* promoter driving high expression of a transduced gene in hepatocellular and in breast carcinoma, respectively (*62,64*). This approach of enhancing differential transcription is very attractive, since it allows many variations on the theme: various tissues do often express specific genes, and this is reflected in tumors derived from these genes. A prominent example here is the *tyrosinase* gene only expressed in cells of the melanocytic linage. Making use of the *tyrosinase* promotor, genes of choice can be expressed in melanoma (*5,65*).

2.3.3. Directing T-cells

Another way to deliver anticancer agents to the tumor site is by making use of specifically homing cells, such as TIL. These can be isolated from surgically removed tumors and transduced with genes encoding, for instance, TNF-α or IFN-γ (*66,67*). After reinfusion in the patient, they home to the tumor and produce locally large amounts of TNF, leading to tumor destruction, in combination with the cytolytic activity of the TIL. One of the problems encountered is the limited production of TNF by the TIL, which can possibly be enhanced by making use of better vectors. Another possibility is to engineer the TNF gene in such a way that a more stable or better secreted protein is produced (*67*). Another problem is that the homing of TIL

is not exclusively tumor-specific, causing considerable toxicity of the TIL therapy. To circumvent this problem, one can think of engineering the T-cells in such a way that they express receptors recognizing the tumor cells. Such receptors may be a hybrid of the T-cell receptor with its variable (peptide-recognizing) part replaced by the variable region of a tumor-specific monoclonal antibody (Mab). This approach using the chimeric receptors has been shown to be effective in redirecting cytolytic T-cells to cancer cells (*68–70*).

2.3.4. Protection of Bone Marrow

In addition to these direct ways to influence gene expression in tumor cells or cells targeted to the tumor, gene modification can be used in a more indirect way, such as to protect bone marrow from being destroyed by conventional chemotherapy. So far, bone marrow stem cell growth factors have been successfully used in combination with high-dose conventional chemotherapy. An alternative strategy may be to protect the bone marrow of the patient by transducing it with genes encoding proteins involved in drug resistance, such as the *mdr-1* gene encoding the P-glycoprotein. This protein functions as a membrane pump to remove toxic compounds out of the cell and is easily induced by a number of different cytostatics rendering tumor cells resistant to further therapy. Animal and human models to explore the feasibility of this system have been reported (*71,72*). Mice reconstituted with *mdr-1*-engineered bone marrow showed less reduction in white blood cell counts than control mice after treatment with taxol. Using this approach, one should keep in mind that *mdr-1* is often also expressed in cancer cells and, therefore, protecting the bone with *mdr-1* makes sense only when the cancer does not express *mdr-1* on treatment. For these reasons, one has sought to modify the P-glycoprotein in such a way that transduced protein can be discerned from endogenous mdr-1 with respect to sensitivity to agents that may reverse its action (*71*). For example, when a Gly185Val mutant is used for transduction bone marrow, verapamil might be used to reverse the resistance of the tumor cells (containing normal mdr-1), because the mutated protein is insensitive to the action of verapamil. In this way, the design of specific mutations altering the properties of mdr-1 may allow very selective action. Another application of *mdr-1* gene transduction is to use it as a selectable marker. For instance, in strategies explained below, where engineered tumor cell vaccines are used, it might be desirable to increase the resistance of the engineered cells as opposed to the parental tumor cells, as to increase exposition of the immune system to these cells, while simultaneously the parental cells are killed off by the used drug. Outgrowth of the engineered tumor cells might be prevented by cotransduction of a suicide gene. In this way, an effective concert action of drug therapy and immune therapy might be obtained. In the next section, we will further elaborate on the use of engineered tumor cell vaccines.

3. THE IMMUNE SYSTEM AS VEHICLE

3.1. Immune Defense Against Cancer Cells

3.1.1. T-cells

Over the past few years, exciting new developments in the involvement of the immune system in the defense against neoplastic cells have drawn much attention. Although the results of classical vaccinations with irradiated autologous tumor cells

were only of limited success (*73–76*), a new era started when recombinant cytokines, in particular interleukin-2 (IL-2), were introduced in the clinic (*77,78*). These were applied in particular in patients with melanoma and renal cell cancer, which types of cancer had previously been shown to be rather immunogenic, judged on the basis of spontaneous regressions observed (*79–81*). Although the response rates were relatively high, these still did not exceed 40% (*see 82,83* for review). In the earliest studies, lymphokine-activated killer cells (LAK cells) emerging after treatment of peripheral blood lymphocytes (PBL) with IL-2 in vitro were used (*77*). LAK activity is mediated primarily by natural killer (NK) cells and to a lesser extent by MHC-unrestricted T-cells (*84*). LAK cell therapy has been improved by using TIL isolated from fresh tumor specimens rather than the patient's PBL for activation with IL-2 (*78*). In TIL cells, the active effector cells are predominantly T-cells with specific antitumor activity (*85*). In humans, these cells consist of HLA Class II-restricted $CD4^+$ helper T-cells as well as $CD8^+$ MHC Class I-restricted cytotoxic T-cells.

3.1.2. Tumor Antigens

Since MHC antigens are involved in the presentation of altered peptides to reactive T-cells, tumor-reactive T-cells in the TIL cell population interact with tumor-specific peptides bound to the groove of HLA Class I proteins (*86–89*). HLA Class I-restricted T-cell cultures or T-cell clones reacting with the tumor could be established from patients with various forms of human cancer, including melanoma (*90–94*), renal cell carcinoma (*95–98*), ovarian carcinoma (*95–98*), gastric carcinoma (*99*), and pancreatic carcinoma (*100*). Most importantly, specificity of some of the T-cell cultures for allogeneic tumors was found, suggesting that common tumor antigens are involved (*101,102*).

Recently, several tumor antigens recognized by melanoma-specific T-cell clones or T-cell lines have been characterized. These include MAGE-1 and MAGE-3 (*103,104*), Mart-1, also called Melan-A (*105,106*), tyrosinase (*107*), and gp100 (*108,109*). In all these cases, the genes encoding the antigens were not mutated as compared to the genes in normal tissues, indicating that nonmutated self-peptides may serve as CTL target. Tyrosinase is a key enzyme in the melanin synthesis pathway in pigmented cells. The function of the other antigens is not known. With the exception of the testis, MAGE antigens are expressed exclusively in the tumor. This may explain why CTL can be raised against it: inappropriate expression at high density in the tumor cells may elicit an autoimmune reaction. On the contrary, tyrosinase and gp100 are also expressed in normal melanocytes, and here, tumor-specific CTL induction is apparently triggered by bulk expression on tumor cells. The MAGE and Mart antigens are not specific for melanoma, but are also expressed in other tumor types, such as breast carcinoma, neuroblastoma, and lung carcinoma (*110–112*).

3.1.3. Induction of Tumor-Specific CTL

The restriction elements for T-cell recognition of these antigens identified so far were HLA-A*0101, HLA-A*0201, HLA-A*2401, and HLA-Cw*1601 (*105,113–116*). The identification of these targets and the discovery of unique allele-specific peptide binding motifs in the groove of the HLA Class I molecules (*117,118*) made it feasible to determine which peptides in a tumor antigen potentially bind to an HLA Class I allele, and these peptides subsequently could be tested for induction of a T-cell

response (*119*). This was done for a number of tumor antigens, such as MAGE-3 and tyrosinase (*120,121*), but also for mutated oncogene products, such as p53 and p21ras (*122,123*). CTL can also be raised against native p53 (*122*), which largely extends the grip of the immune system on tumor cells, because apparently nonmutated proteins present in elevated expression in tumor cells can also serve as a target. Finally, viral proteins in virus-associated human tumors, such as cervical carcinoma, are good targets for T-cells because the viral epitopes are unique and do not resemble cellular proteins (*124,125*). In this latter case, peptide vaccination in an animal model has been shown to be effective in inducing long-term protection of animals against a subsequent challenge with tumor cells (*126*). The power of peptide vaccination for elimination of established tumor, however, has still has to be shown.

An effective method of peptide vaccination may be loading the peptides on professional antigen-presenting cells. In this case, gene technology can play a role because high expression of immunogenic peptides may be reached by transfer of peptide-encoding sequences linked to a signal peptide-encoding sequence, making transport to the endoplasmatic reticulum and assembly into HLA Class I molecules very effective (*127*). Even the HLA Class I expression can be engineered by transfer of individual HLA Class I alleles in cells with an endogenously low HLA Class I expression. Ultimately, this approach may lead to allogeneic vaccination of patients with engineered cells expressing a high density of tumor peptide on the cell surface. Allogenic determinants on these cells are not necessarily a disadvantage, because it has been shown that transfection of syngeneic tumor cells with foreign MHC Class I molecules raises their immunogenicity and may lead to an immune reaction against parental tumor cells owing to the bystander effect (*128–130*). Methods to improve the immunogenicity of tumor cells by genetic engineering as a tool to fight the parental cancer cells will be discussed below.

3.2. Natural Immune Response Against Human Tumor Is Often Ineffective

Despite the apparent potential of the human immune system to develop a cytotoxic reaction against tumors, an obvious immune reaction seems usually to be absent in patients with cancer. Several reasons may account for this (*see also 131,132* for comprehensive reviews). First, expansion of tumors may be so fast that effector cells are not capable of killing the tumor properly. Second, tumor cells might have escaped an immune response by downmodulation of tumor-specific antigens or MHC Class I antigens in experimental tumors, as well as in humans (*89,133*). For instance, in melanoma, one of the most immune reactive human tumors, downmodulation of particular HLA Class I alleles is a common phenomenon (*134*). Third, suppressor cells consisting mostly of $CD4^+$ T-cells may be activated by tumor cells. This has been clearly shown in animal tumor models (*135*) as well as for human melanomas (*93,136*). Fourth, alterations in the T-cell repertoire leading to aberrant T-cell receptor expression has been found in renal cell carcinomas (*137*). The mechanism leading to this phenomenon is far from clear, but the result is an anergic T-cell population in the tumor. Fifth, the presentation of tumor antigens at the cell surface may be disturbed. This may happen through a defect in genes involved in peptide processing in

the cell, required for the generation of peptides and the subsequent assembly of peptide-HLA Class I complexes (*138*). Sixth, local immunosuppressive factors secreted by the tumor, such as transforming growth factor-β (TGF-β) might be involved in the suppression of cytotoxic T-cells (*139,140*). This view is supported by the observation that highly immunogenic tumor cells expressing a transfected TGF-β gene did indeed escape immune surveillance (*141*). Finally, the immunogenicity of spontaneously arising tumors, as human tumors usually are, may be in general too low to elicit a proper immune response (*142*). Altogether, these factors, alone or in combination, may impede recruitment of a proper T-cell helper circuit necessary for proliferation of tumor-specific cytotoxic T-cells. Below, we will discuss several strategies of gene therapy of cancer patients to improve their immune response to the tumor.

3.3. Improving Immunogenicity of Tumor Cells

As mentioned before, naturally occurring tumors, except virally induced ones, have a low immunogenicity. If these tumor cells could be manipulated in such a way that a better response could be evoked by the immune system of the host, vaccination with the manipulated cells could be tested as treatment modality. Recently, attempts to enhance the immunogenicity of murine tumor cells by gene transfer have been remarkably successful. Transfection of allogeneic MHC Class I genes in tumor cells did elicit immune responses directed against the nontransfected tumor cells and could prevent tumor take after immunization (*128,129,143,144*). This situation is reminiscent of immunization of mice with mutated highly immunogenic variants of an originally nonimmunogenic tumor (*145*). The immunized mice became resistant to the mutated tumor cells, as well as to the nonimmunogenic parental tumor. This is probably owing to an adjuvant effect of the mutated antigens on the generation of an immune response against poorly immunogenic (cryptic) antigens present in the mutated tumor cells as well as in the parental tumor. A similar approach was explored by infection of tumor cells with an immunogenic virus, e.g., the Newcastle Disease Virus, or by transfection of a gene encoding an immunogenic viral protein, e.g., hemagglutinin (*146,147*). In all cases, protection was acquired against challenge with the original nonmanipulated tumor cells.

A promising new approach came about with the discovery of so-called accessory molecules, such as B7.1, which plays a role in the physical interaction between antigen-presenting cells and immune effector cells (*148*). This protein represents an essential part of the helper arm of the immune response. Tumor cells are usually devoid of this helper machinery and, therefore, are crippled in their capacity to elicit an onset of an effective immune response. Transfection of the *B7.1* gene in B7.1-negative cells largely stimulates the interaction of effector and target (*149*). This prompted several investigators to see whether transfer of the *B7* gene would make tumor cells more immunogenic. It turned out that, indeed, the introduction of the *B7* gene in nonimmunogenic tumor cells made them less tumorigenic (*150–152*). Moreover, the immune response mounted did protect the animals from a subsequent challenge with the parental cells. Also, long-term memory against the *B7*-engineered as well as the parental cells could be obtained. This approach works particularly well with MHC Class II-negative tumor cells, suggesting that indeed the helper arm of the immune response is restored (*153*). Also in the induction of human tumor-specific CTL in vitro, transfer of the B7 molecule into several human tumor cell lines largely enhances

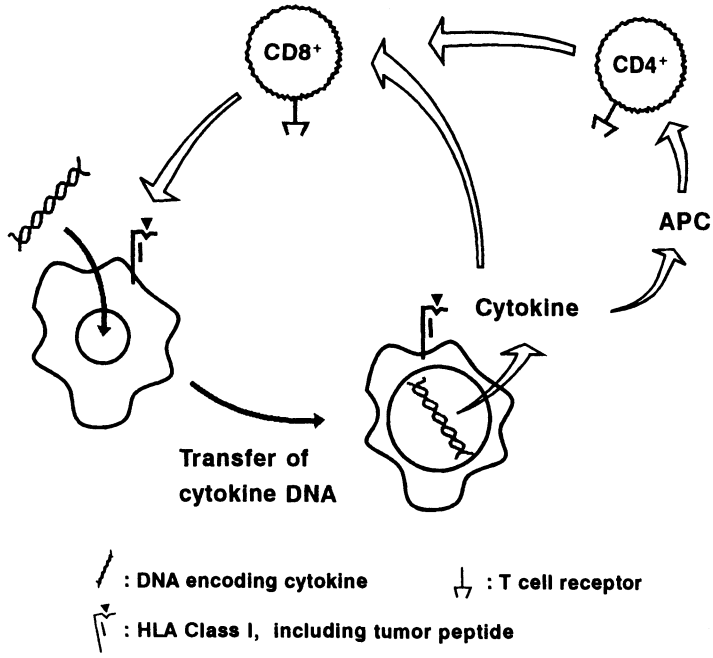

Fig. 1. Schematic representation of the effect of vaccination with cytokine-modified tumor cells. Two alternative routes are depicted: 1. The cytokine in combination with HLA Class I-bound tumor peptide enhances stimulation of $CD8^+$ T-cells. 2. The cytokine enhances the stimulation of antigen-presenting cells (APC) that present tumor peptide derived from degraded tumor protein to $CD4^+$ T-cells.

proliferation of tumor-specific CTL in MLTC (*154*). The resulting CTL were not only reactive with the B7-transfected cells, but also with the parental cell line, indicating that B7 is involved in the inductive phase of the immune response.

These experiments clearly show that structural alterations of tumor cells that in some way change their interaction with the immune system may largely improve their immune recognition and enhance the induction of tumor-specific CTL. The most exciting observation is that, in many cases, these CTL recognize not only the modified cells, but also the parental, unaltered, tumor cell. This is also the case when the tumor cells are transduced in order to let them produce cytokines. Since this has been pioneered by many groups over the past few years and has already made its entrance in the cancer clinic, we will discuss this approach in more detail in the next section.

3.4. Enhancing Immunogenicity of Tumor Cells by Cytokine Gene Transfer

3.4.1. Cytokines

Very effective protection may be obtained when tumor cells are transfected or transduced with genes encoding growth factors or cytokines. The principle of this approach is schematically represented in Fig. 1. The manifest advantage of this system is the local production and high concentration of immune-modulating factors at the site of the administered tumor cells. Moreover, the side effects of the produced cytokines can be expected to be much lower than on systemic administration. To date, no

data have been reported for humans. In animal models, however, promising results have been obtained with the cytokines IL-1 (*155*), IL-2 (*156–161*), IL-4 (*160, 162–164*), IL-6 (*165,166*), IL-7 (*160,167,168*), IL-10 (*155*), IL-12 (*169*), IFN-γ (*160, 170–174*), TNF-α (*66,160, 175–178*), G-CSF (*179*), and GM-CSF (*180,181*). In these cases, at least a significant reduction of tumorigenicity of the cells in syngeneic animals was observed, but in most cases tumor formation was completely abolished. Usually, the reduced tumorigenicity was highly dependent on the amount of cytokine produced: high amounts of cytokine are required for abolishment of tumorigenicity, although bell-shaped dose responses have been reported (*157,182*).

3.4.2. Mechanism

The mechanism of blocking of tumor growth has not been made unequivocally clear, but definitely depends on the modification made to the tumor cell. The general idea is that the production of the cytokine at the vaccination site attracts immune effector cells, mainly macrophages, neutrophils, NK cells, and $CD4^+$ and $CD8^+$ T-cells. The $CD8^+$ T-cells are ultimately the most important contributors in eliminating the tumor cells, suggesting that one of the main reasons for the effectiveness of the secreted cytokines is the bypassing of the helper arm of the immune system, comprising the antigen presentation through HLA Class II to $CD4^+$ T-cells (Fig. 1). Other effector cells, such as NK cells, may be important too, in particular, in the initial phase of the rejection (*160,183*). It should be stressed, however, that the actual mechanism cannot be generalized, but is largely dependent on the cytokine and on the tumor model used (reviewed in *184,185*). Whatever the actual effectors are, in the above presented view, the cytokine-producing tumor cell interacts directly with the immune system of the host. In an alternative view, however, tumor cells are broken down at the vaccination site, and owing to the local cytokine production of yet intact cells, an effective presentation of tumor antigens is initiated by antigen-presenting cells of the host. This view is substantiated by recent experiments showing that allogeneic cytokine-producing tumor cells are also effective in mounting protective immunity against a subsequent challenge with a syngeneic tumor (*186*). This would actually decimate the necessity of matching for tumor antigen-presenting MHC Class I alleles in vaccination protocols, since the tumor antigens from allogeneic tumor cells would be presented by the autologous antigen-presenting cells and subsequently induce an immune response against the autologous tumor (also *see* Section 4.1.).

Most importantly, in all models studied, animals treated with cytokine gene-engineered tumor cells developed long-term memory against the engineered cells as well as against the parental tumor cells. This effect could not be reached by administration of tumor cells mixed with cytokine or by tumor cells only. In some systems, however, the effect of the cytokine-producing tumor cells could be mimicked by mixing the tumor cells with autologous fibroblasts engineered to produce the cytokine (*186*). In most systems, treatment with live tumor cell vaccines was superior to irradiated cells (*166,187*), stressing that the success of vaccination with engineered tumor cells in human cancer patients may turn out to be limited when the cells are irradiated, as presently required by medical ethical committees.

3.4.3. Comparison of Efficacy

There is still much discussion about what cytokine is the most effective on transfer in tumor cells in raising an immune response against parental cells. In a series of

experiments by various groups, a number of cytokines were compared, and variable results were obtained (*160,166,180,188*). Good choices seem to be IL-2 and GM-CSF, though results are undoubtedly dependent on the tumor model used. Surprisingly, in one study (*187*), the cytokine transfection seemed not to be superior above a mixture of nonmodified tumor cells and *Corynebacterium Parvum* (*C. Parvum*), an adjuvant frequently used in the past as immunostimulator (*189*). The general validity of this observation, however, is hard to evaluate. In particular, for the human situation, vaccination with BCG/tumor cell mixtures was only shown to be effective in rare instances, making a comparison with the animal studies difficult.

Finally, a distantly related, but interesting approach making use of the immunostimulating properties of cytokines is the engineering of immunogenic epitopes that are target for a B-cell response. Constructs harboring sequences encoding tumor-specific idiotypes have been fused to sequences encoding GM-CSF, IL-4, or IL-2, and fusion proteins were synthesized. Such fusion proteins are capable of eliciting significant levels of specific antibodies against the idiotype and can elicit significant antitumor immunity (*190,191*). This strategy is used for the preparation of protein vaccines, but one can speculate that cellular vaccines producing these types of recombinant proteins may be useful too.

In conclusion, vaccination using cytokine transfer techniques seems very promising, although a lot of more research has to be done to fine-tune the experimental conditions. The results so far have encouraged clinicians to initiate clinical studies in patients with melanoma and renal cell carcinoma. In the next section, we will discuss the rationale and outline of these studies.

4. CLINICAL STUDIES

4.1. Rationale

The molecular biology techniques for the modification of tumor cells, discussed in detail before, enable us to explore the new strategies to enhance the immunogenicity of human tumor cells and use these cells for vaccination. Most of the tumor-specific antigens, candidates for such an enhancement in the individual patients, have not yet been identified. The most optimal situation, therefore, is to transfer autologous tumor cells of the patient, either in vivo or *ex vivo*, and let them work as a vaccine. This circumvents the determination of matching for tumor-specific antigens and for the HLA Class I alleles required to present the tumor-specific peptides.

The feasibility and safety of gene therapy with genetically modified cells have been demonstrated by the pioneering work of Rosenberg and coworkers (*19,67,192*). In applying the strategies of cytokine transfection as discussed above, two approaches can be envisioned: (1) transfer of the cytokine genes in vivo locally at the site of the tumor(s), and (2) perform the transfer *ex vivo* on cultured cells and re-administer them to the patient. Both methods have, however, certain disadvantages. Transfer to the tumor *in situ* may be technically difficult, if there is any tumor there. Also, however, the *ex vivo* approach may cause complications. Culturing autologous tumor cells is time-consuming and not always successful. The identification of common tumor antigens presented by either HLA-A*0101 or HLA-A*0201 allows the use of HLA-A*0101 or HLA-A*0201-positive allogeneic cells as a vaccine in melanoma patients harboring these alleles. Approximately 60–70% of white patients will be

HLA-A*0101 and/or HLA-A*0201. Therefore, such a vaccine is expected to be applicable in a considerable number of melanoma patients. Furthermore, the presence of allogeneic HLA Class I or II molecules may be even more advantageous, because it has been shown in animal experiments that foreign MHC genes may induce a similar raise in immunogenicity as obtained with cytokine gene therapy (*see* Section 3.3.). Moreover, as a practical advantage, the allogeneic cells will most likely be rejected by the immune system of the patient, avoiding any risk of tumor recurrences by cells surviving the irradiation. It even may allow vaccination with live tumor cells in patients with a well-functioning immune system, which may be more effective on the basis of the experience with animal vaccination studies (*see* Section 3.4.2.).

Following immunization of patients with genetically modified allogeneic tumor cells that share an HLA Class I allele, the cells may in vivo directly present the shared tumor peptides to HLA Class I-restricted CTL of the patient, provided that the shared HLA Class I allele presents a shared immunodominant peptide. Alternatively, the cells will be degraded and serve as a source of tumor antigens presented by autologous antigen-presenting cells (Fig. 2). These cells will then process and select the appropriate epitopes, which will enter the HLA Class II processing route to stimulate $CD4^+$ T-cells, or even enter the HLA Class I route to induce directly proliferation of $CD8^+$ cytotoxic T-cells (*186*). In this line, one can even think of using a fully allogeneic cell line for vaccination. In this case, a shared tumor antigen is required, and the corresponding tumor peptides can only be presented by the autologous antigen-presenting cells (Fig. 2).

4.2. Trials

A number of clinical trials using gene modification for immune modulatory purposes have been initiated (*193–198*). The studies focused on HLA Class I genes and cytokine genes. The various modes of administration of the "vaccines" are:

1. Genes packed in liposomes directly transferred into the tumor *in situ* (*196*);
2. Cytokine-producing fibroblasts admixed with tumor cells (*198*);
3. Autologous tumor cells cultured and transduced with cytokine; and
4. Allogeneic cytokine-transduced or transfected tumor cells (*194,195,197*).

In February 1992, we initiated the first clinical study in which HLA-A*0101 or HLA-A*0201-positive melanoma patients were immunized with an HLA-A*0101, A*0201,B*0801-positive melanoma cell line, transfected with the IL-2 gene. The cell line expresses high levels of HLA Class I. Moreover, the cell line expresses MAGE-1, 2, and 3, tyrosinase, Mart-1, and gp100. The choice of IL-2 was based on the fact that clinical remissions were obtained in patients with metastatic melanoma following IL-2 based treatment regimens. Furthermore, ample evidence exists for the presence of CTL precursors in melanoma patients, and the animal studies testing different cytokines for gene transfer indicated that IL-2 worked well. The IL-2 gene under control of an immediate early cytomegalovirus (CMV) promoter was transfected using the calcium phosphate precipitation technique. This promoter is well expressed in melanoma cells, and the cells produce high amounts of IL-2 up to at least 100 ng IL-2/10^6 cells/24 h. After 100 gy of irradiation, a dose that completely inhibited the proliferation of the cells in vitro, the secretion of biologically active IL-2 on a per-cell basis increased in the first days, decreased thereafter, but after 10 d IL-2 production was

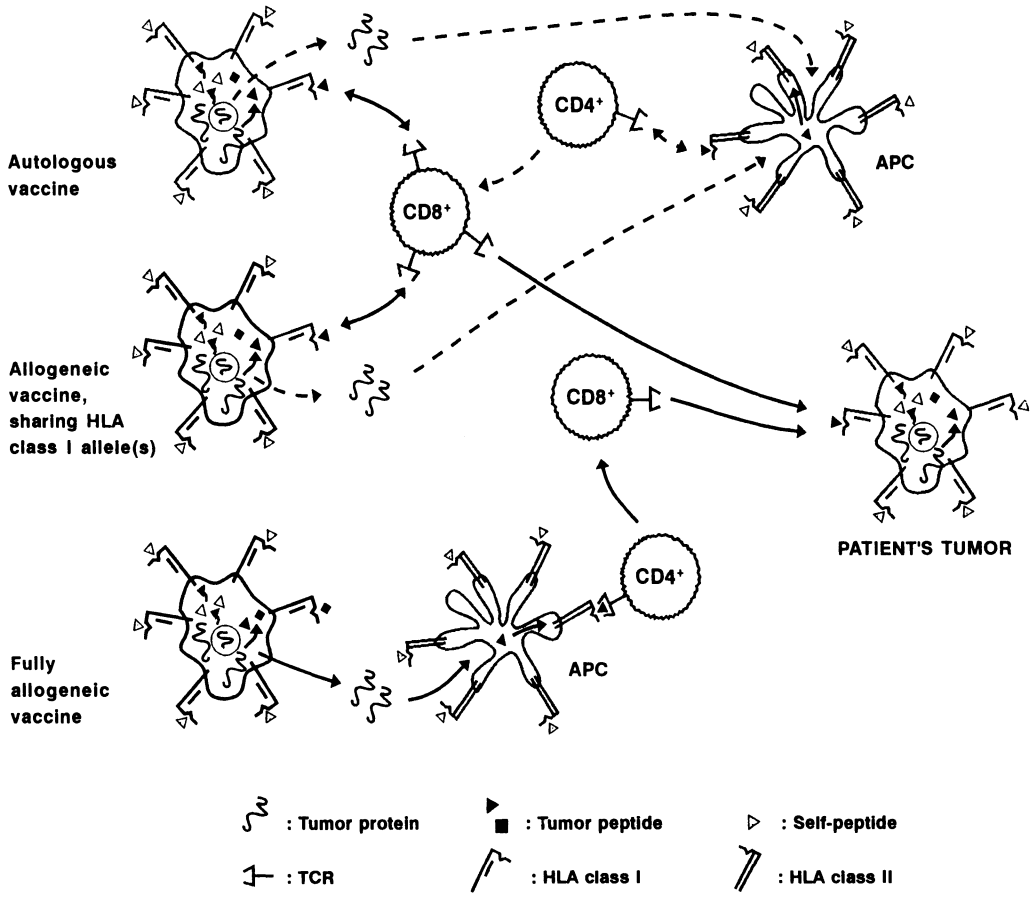

Fig. 2. Induction of a $CD8^+$ T-cell-mediated antitumor response by vaccination with gene-modified tumor cells. Three situations are shown as indicated: 1. autologous tumor cells, 2. allogeneic tumor cells, sharing one or more HLA Class I alleles, or 3. fully allogeneic tumor cells. All vaccination strategies lead to a $CD8^+$ -mediated immune response against the patient's own tumor (shown on the right side). Autologous tumor cells or autologous HLA molecules are depicted in gray, and allogeneic tumor cells or allogeneic HLA molecules are depicted in black.

still measurable. The purpose of our study was to evaluate toxicity and antitumor efficacy of weekly subcutaneous injections of the IL-2-secreting irradiated cells. So far, no toxicities have been observed, though we did observe inflammatory reactions at the site of vaccination as well as at the site of distant metastases. Regressions of metastases were observed, but these always represented mixed responses.

Nabel and coworkers (*6*) reported the enhancement of antitumor immune response by in vivo injection directly in tumors of allogeneic HLA-B7 using DNA–liposome complexes. HLA-B7 protein expression was demonstrated in the tumor cells near the site of injection. No systemic toxicity was observed, whereas a fivefold increase in the frequency of HLA-B7-reactive CTL precursors was demonstrated in one patient following immunization. Furthermore, a distant lung metastasis regressed in one of the five reported patients, suggesting that the allogeneic effects may indeed have enhanced the antitumor immune response.

Altogether these experiments look promising and merit further investigation of gene therapy in cancer patients.

5. EPILOGUE

The present insight into the genetics of cancer development allows an active intervention by means of genetic manipulation. This may be either replacement of affected genes or addition of inactivated or lacking genes. It has been shown that random integration of, for instance, tumor suppressor genes in cancer cells is effectively inhibiting cell-cycle progression. This shows that, in principle, this type of addition therapy can work. Many barriers have to be removed before the delivery of such genes has the power to reach every malignant cell in the body, an absolute requirement for the cure of metastatic cancer. For the moment, however, these techniques can be explored as debulking tools, allowing more effective methods of cancer therapy to be successful. This may, for instance, be triggering the immune system to attack the cancer cell.

The new developments in genetic engineering, in particular the design of new eukaryotic expression vectors, have disclosed an array of possibilities to deliver genes specifically to cancer cells, leaving the surrounding normal cells intact. This has, for instance, led to the clinical use of suicide genes. Another step forward toward precise delivery of genes has been made by invoking the help of the highly selective immune system: on one hand, cytotoxic T-cells have been genetically altered in such a way that they can more effectively interact with the tumor cell; on the other hand, tumor cells have been made immunogenic to enhance the immune system of the host. An intriguing observation has recently been made, i.e., cells harboring suicide genes evoke an immune reaction, leading to a situation that killing by the drug in combination with an immune response may be particularly effective in eradicating the tumor. The gene transfer techniques can be applied to normal cells, too: purged bone marrow of the cancer patient can be engineered is such a way that it becomes resistant to cytostatic drugs, allowing high-dose chemotherapy.

In the clinical setting, the conditions for vaccination will have to be very carefully investigated and evaluated. Based on the animal experiments, the optimal situations for cellular vaccines to be effective were at very low tumor load. Since the clinical trials now performed are all on patients with advanced metastatic disease and probably considerable tumor burden, the outcome may give an underestimated view on the power of the therapy. Therefore, arguments have to be gathered for treating patients with a more favorable prognosis, for instance, in an adjuvant setting. Also, a combination with an adoptive transfer of in vitro expanded tumor-specific CTLs induced by the vaccine might be fruitful. For the moment, however, important data can be gained from the treatment of metastatic patients. First, a careful histological evaluation of biopsies of macroscopic metastases before and after vaccination may give insight into the immune reaction elicited. Second, a minute analysis of tumor-specific CTL in the peripheral blood mononuclease cells (PBMC) of the patients before and after vaccination may reveal whether an increased T-cell reactivity can be induced and toward what antigens these T-cells are directed. On the basis of the outcoming data, further studies can be undertaken to optimize the conditions for this form of gene therapy.

By and large, the search for gene therapy approaches for the treatment of cancer has yielded a number of exciting new developments, with highlights in the "suicide" therapy and immunotherapy. Very powerful new techniques are finding their way to the clinic now. The preliminary clinical data, however, should be evaluated with care and used to allude to new directions for improvement of the present protocols. It is our sincere expectation that the almost indefinite possibilities of the new genetic techniques ultimately will survive in the struggle against cancer.

REFERENCES

1. Bishop JM. Molecular themes in oncogenesis. *Cell* 1991; 64:235-248.
2. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; 61:759-767.
3. Hunter T. Cooperation between oncogenes. *Cell* 1991; 64:249-270.
4. Morgan RA, Anderson WF. Human gene therapy. *Annu Rev Biochem* 1993; 62:191-217.
5. Hart IR, Vile RG. Targeted therapy for malignant melanoma. *Curr Opinion Oncol* 1994; 6: 221-225.
6. Nabel GJ, Nabel EG, Yang ZY, Fox BA, Plautz GE, Gao X, Huang L, Shu S, Gordon D, Chang AE. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. *Proc Natl Acad Sci USA* 1993; 90:11,307-11,311.
7. Williams RS, Johnston SA, Riedy M, DeVit MJ, McElligott SG, Sanford JC. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. *Proc Natl Acad Sci USA* 1991; 88:2726-2730.
8. Graham FL, Van der Eb AJ. A new technique for the assay of infectivity of human Adenovirus 5 DNA. *Virology* 1973; 52:456-467.
9. Keating A, Toneguzzo F. Gene transfer by electroporation: a model for gene therapy. *Prog Clin Biol Res* 1990; 333:491-498.
10. Ray J, Gage FH. Gene transfer into established and primary fibroblast cell lines: comparison of transfection methods and promoters. *Biotechniques* 1992; 13:598-603.
11. Jiang CK, Connolly D, Blumenberg M. Comparison of methods for transfection of human epidermal keratinocytes. *J Invest Dermatol* 1991; 97:967-973.
12. Cotten M, Wagner E, Birnstiel ML. Receptor-mediated transport of DNA into eukaryotic cells. *Methods Enzymol* 1993; 217:618-644.
13. Wagner E, Zatloukal K, Cotten M, Kirlappos H, Mechtler K, Curiel DT, Birnstiel ML. Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. *Proc Natl Acad Sci USA* 1992; 89:6099-6103.
14. Mulligan RC. The basic science of gene therapy. *Science* 1993; 260:926-932.
15. Russell SJ. Replicating vectors for cancer therapy: A question of strategy. *Semin Cancer Biol* 1994; 5:437-443.
16. Haddada H, Ragot T, Cordier L, Duffour MT, Perricaudet M. Adenoviral interleukin-2 gene transfer into P815 tumor cells abrogates tumorigenicity and induces antitumoral immunity in mice. *Hum Gene Ther* 1993; 4:703-711.
17. Kozarsky KF, Wilson JM. Gene therapy: Adenovirus vectors. *Curr Opinion Genet Dev* 1993; 3:499-503.
18. Flotte TR, Afione SA, Conrad C, McGrath SA, Solow R, Oka H, Zeitlin PL, Guggino WB, Carter BJ. Stable in vivo expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. *Proc Natl Acad Sci USA* 1993; 90:10,613-10,617.
19. Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC, Topalian SL, Merino MJ, Culver K, Miller AD, Blaese RM, Anderson WF. Gene transfer into humans - Immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med* 1990; 323:570-578.
20. Rill DR, Buschle M, Foreman NK, Bartholomew C, Moen RC, Santana VM, Ihle JN, Brenner MK. Retrovirus-mediated gene transfer as an approach to analyze neuroblastoma relapse after autologous bone marrow transplantation. *Hum Gene Ther* 1992; 3:129-136.

21. Brenner MK, Rill DR, Moen RC, Krance RA, Mirro J, Jr., Anderson WF, Ihle JN. Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* 1993; 341:85,86.
22. Brenner MK, Rill DR, Heslop HE, Rooney CM, Roberts WM, Li C, Nilson T, Krance RA. Gene marking after bone marrow transplantation. *Eur J Cancer* 1994; 30A:1171-1176.
23. Tiberghien P. Use of suicide genes in gene therapy. *J Leukocyte Biol* 1994; 56:203-209.
24. Bishop JM. Viral oncogenes. *Cell* 1985; 42:23-38.
25. Weinberg RA. The action of oncogenes in the cytoplasm and nucleus. *Science* 1985; 230: 770-776.
26. Land H, Parada LF, Weinberg RA. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 1983; 304:596-602.
27. Vile RG. Tumor-specific gene expression. *Semin Cancer Biol* 1994; 5:429-436.
28. Wickstrom EL, Bacon TA, Gonzalez A, Freeman DL, Lyman GH, Wickstrom E. Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-*myc* mRNA. *Proc Natl Acad Sci USA* 1988; 85:1028-1032.
29. Rosolen A, Whitesell L, Ikegaki N, Kennett RH, Neckers LM. Antisense inhibition of single copy N-*myc* expression results in decreased cell growth without reduction of c-myc protein in a neuroepithelioma cell line. *Cancer Res* 1990; 50:6316-6322.
30. Saison-Behmoaras T, Tocque B, Rey I, Chassignol M, Thuong NT, Helene C. Short modified antisense oligonucleotides directed against Ha-*ras* point mutation induce selective cleavage of the mRNA and inhibit T24 cells proliferation. *EMBO J* 1991; 10:1111-1118.
31. Szczylik C, Skorski T, Nicolaides NC, Manzella L, Malaguarnera L, Venturelli D, Gewirtz AM, Calabretta B. Selective inhibition of leukemia cell proliferation by *bcr-abl* antisense oligodeoxynucleotides. *Science* 1991; 253:562-565.
32. Nitta T, Sato K. Inhibition of c-sis protein synthesis and cell growth with antisense oligonucleotides in human glioma cells. *No Shinkei Geka* 1992; 20:857-863.
33. Robinson-Benion C, Li YX, Holt JT. Gene transplantation: combined antisense inhibition and gene replacement strategies. *Leukemia* 1994; 8:S152-S155.
34. Mukhopadhyay T, Tainsky M, Cavender AC, Roth JA. Specific inhibition of K-*ras* expression and tumorigenicity of lung cancer cells by antisense RNA. *Cancer Res* 1991; 51:1744-1748.
35. Trojan J, Johnson TR, Rudin SD, Ilan J, Tykocinski ML. Treatment and prevention of rat glioblastoma by immunogenic C6 cells expressing antisense insulin-like growth factor I RNA. *Science* 1993; 259:94-97.
36. Ohta Y, Tone T, Shitara T, Funato T, Jiao L, Kashfian BI, Yoshida E, Horng M, Tsai P, Lauterbach K, Kashani-Sabet M, Florenes VA, Fodstad O, Scanlon KJ. *H-ras* ribozyme-mediated alteration of the human melanoma phenotype. *Ann NY Acad Sci* 1994; 716:242-253.
37. Lange W, Cantin EM, Finke J, Dolken G. In vitro and in vivo effects of synthetic ribozymes targeted against *bcr/abl mRNA*. *Leukemia* 1993; 7:1786-1794.
38. Weinberg RA. Tumor suppressor genes. *Science* 1991; 254:1138-1146.
39. Hall PA, Lane DP. Genetics of growth arrest and cell death: Key determinants of tissue homeostasis. *Eur J Cancer* 1994; 30A:2001-2012.
40. Yonish-Rouach E, Grunwald D, Wilder S, Kimchi A, May E, Lawrence JJ, May P, Oren M. p53-mediated cell death—relationship to cell cycle control. *Mol Cell Biol* 1993; 13:1415-1423.
41. Roemer K, Friedman T. Mechanisms of action of the p53 tumor suppressor and prospects for cancer gene therapy by reconstitution of p53 function. *Ann NY Acad Sci* 1994; 716:265-280.
42. Lane DP. The regulation of p53 function. *Int J Cancer* 1994; 57:623-627.
43. Lotem J, Sachs L. Regulation by *bcl*-2, c-*myc*, and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and differentiation-defective myeloid leukemic cells. *Cell Growth Differ* 1993; 4:41-47.
44. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 1993; 362:847-849.
45. Clayman GL, Elnaggar AK, Roth JA, Zhang WW, Goepfert H, Taylor DL, Liu TJ. In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. *Cancer Res* 1995; 55:1-6.

46. Oren M. Relationship of p53 to the control of apoptotic cell death. *Semin Cancer Biol* 1994; 5: 221-227.
47. Wills KN, Maneval DC, Menzel P, Harris MP, Sutjipto S, Vaillancourt MT, Huang WM, Johnson DE, Anderson SC, Wen SF, Bookstein R, Shepard HM, Gregory RJ. Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer. *Hum Gene Ther* 1994; 5:1079-1088.
48. Cobrinik D, Dowdy SF, Hinds PW, Mittnacht S, Weinberg RA. The retinoblastoma protein and the regulation of cell cycling. *Trends Biochem Sci* 1992; 17:312-315.
49. Hollingsworth RE Jr, Hensey CE, Lee WH. Retinoblastoma protein and the cell cycle. *Curr Opinion Genet Dev* 1993; 3:55-62.
50. Cao L, Faha B, Dembski M, Tsai LH, Harlow E, Dyson N. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. *Nature* 1992; 355:176-179.
51. Shirodkar S, Ewen M, Decaprio JA, Morgan J, Livingston DM, Chittenden T. The transcription factor E2F interacts with the retinoblastoma product and a p107-Cyclin-A complex in a cell cycle-regulated manner. *Cell* 1992; 68:157-166.
52. Xu HJ, Xu K, Zhou YL, Li J, Benedict WF, Hu SX. Enhanced tumor cell growth suppression by an N-terminal truncated retinoblastoma protein. *Proc Natl Acad Sci USA* 1994; 91: 9837-9841.
53. Chang MW, Barr E, Seltzer J, Jiang YQ, Nabel GJ, Nabel EG, Parmacek MS, Leiden JM. Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. *Science* 1995; 267:518-522.
54. Oldfield EH, Ram Z, Culver KW, Blaese RM, Devroom HL. Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir. *Hum Gene Ther* 1993; 4:39-69.
55. Culver KW, Van Gilder J, Link CJ, Carlstrom T, Buroker T, Yuh W, Koch K, Schabold K, Doornbas S, Wetjen B, et al. Gene therapy for the treatment of malignant brain tumors with in vivo tumor transduction with the herpes simplex thymidine kinase gene/ganciclovir system. *Hum Gene Ther* 1994; 5:343-379.
56. Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, Blaese RM. In vivo gene transfer with retroviral vector producer cells for treatment of experimental brain tumors. *Science* 1992; 256:1550-1552.
57. Ram Z, Walbridge S, Shawker T, Culver KW, Blaese RM, Oldfield EH, The effect of thymidine kinase transduction and ganciclovir therapy on tumor vasculature and growth of 9L gliomas in rats. *J Neurosurg* 1994; 81:256-260.
58. Caruso M, Panis Y, Gagandeep S, Houssin D, Salzmann JL, Klatzmann D. Regression of established macroscopic liver metastases after in situ transduction of a suicide gene. *Proc Natl Acad Sci USA* 1993; 90:7024-7028.
59. Barba D, Hardin J, Sadelain M, Gage FH. Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. *Proc Natl Acad Sci USA* 1994; 91:4348-4352.
60. Vile RG, Nelson JA, Castleden S, Chong H, Hart IR. Systemic gene therapy of murine melanoma using tissue specific expression of the HSVtk gene involves an immune component. *Cancer Res* 1994; 54:6228-6234.
61. Mullen CA, Kilstrup M, Blaese RM. 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 USA* 1992; 89:33-37.
62. Huber BE, Richards CA, Austin EA, Virus-directed enzyme/prodrug therapy (VDEPT). Selectively engineering drug sensitivity into tumors. *Ann NY Acad Sci* 1994; 716:104-114.
63. Mullen CA, Coale MM, Lowe R, Blaese RM. 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* 1994; 54:1503-1506.
64. Huber BE, Richards CA, Krenitsky TA. Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. *Proc Natl Acad Sci USA* 1991; 88:8039-8043.
65. Yasumoto K, Yokoyama K, Shibata K, Tomita Y, Shibahara S. Microphthalmia-associated

transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. *Mol Cell Biol* 1994; 14:8058–8070.

66. Hwu P, Yannelli JR, Kriegler M, Anderson WF, Perez C, Chiang YWL, Schwarz S, Cowherd R, Delgado C, Mule JJ, et al. Functional and molecular characterization of tumor-infiltrating lymphocytes transduced with tumor necrosis factor-alpha cDNA for the gene therapy of cancer in humans. *J Immunol* 1993; 150:4104–4115.
67. Hwu P, Rosenberg SA. The use of gene-modified tumor-infiltrating lymphocytes for cancer therapy. *Ann NY Acad Sci* 1994; 716:188–197.
68. Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci USA* 1993; 90:720–724.
69. Hwu P, Shafer GE, Treisman J, Schindler DG, Gross G, Cowherd R, Rosenberg SA, Eshhar Z. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the F_C-receptor gamma-chain. *J Exp Med* 1993; 178:361–366.
70. Stancovski I, Schindler DG, Waks T, Yarden Y, Sela M, Eshhar Z. Targeting of T-lymphocytes to *neu/her2*-expressing cells using chimeric single chain F_V receptors. *J Immunol* 1993; 151:6577–6582.
71. Cottesman MM, Germann UA, Aksentijevich I, Sugimoto Y, Cardarelli CO, Pastan I. Gene transfer of drug resistance genes. Implications for cancer therapy. *Ann NY Acad Sci* 1994; 716:26–38.
72. Boesen JJ, Nooter K, Valerio D. Circumvention of chemotherapy-induced myelosuppression by transfer of the *mdr1* gene. *Biotherapy* 1993; 6:291–302.
73. Morton DL. Active immunotherapy against cancer. Present status. *Semin Oncol* 1986; 13: 180–185.
74. McCune CS, O'Donnell RW, Marquis DM, Sahasrabudhe DM. Renal cell carcinoma treated by vaccines for active specific immunotherapy: Correlation of survival with skin testing by autologous tumor cells. *Cancer Immunol Immunother* 1990; 32:62–66.
75. Mitchell MS, Harel W, Kempf RA, Hu E, Kan-Mitchell J, Boswell WD, Dean G, Stevenson L. Active-specific immunotherapy for melanoma. *J Clin Oncol* 1990; 8:856–869.
76. Morton DL, Foshag LJ, Hoon DSB. Prolongation of survival in metastatic melanoma after active specific immunotherapy with a new polyvalent melanoma vaccine. *Ann Surg* 1993; 217:309.
77. Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, Linehan WM, Robertson CN, Lee RE, Rubin JT, Seipp CA, Simpson CG, White DE. A progress report on the treatment of 157 patients with advanced cancer with lymphokine-activated killer cells and interleukin-2 or high dose interleukin-2 alone. *N Engl J Med* 1987; 316:889.
78. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Lotze MT, Yang JC, Seipp CA, Simpson C, Carter C, Bock S, Schwarzentruber D, Wei JP, White DE. Use of tumor-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. *N Engl J Med* 1988; 319:1676–1680.
79. Cole WH. Spontaneous regression of cancer and the importance of finding its cause. *Natl Cancer Inst Monogr* 1976; 44:5–9.
80. Nathanson A. Spontaneous regression of malignant melanoma: a review of the literature on incidence, clinical features, and possible mechanisms. *Natl Cancer Inst Monogr* 1976; 44: 67–76.
81. Nishimura K, Okada Y, Okada K, Yoshida O, Amitani R, Kubo Y, Ushida S. Spontaneous regression of pulmonary metastasis from renal pelvic cancer. *Urol Int* 1987; 42:461–463.
82. Rosenberg SA. Immunotherapy and gene therapy of cancer. *Cancer Res* 1991; 51:S5074–S5079.
83. Linehan WM, Walther MM, Alexander RB, Rosenberg SA. Adoptive immunotherapy of renal cell carcinoma: studies from the Surgery Branch, National Cancer Institute. *Semin Urol* 1993; 11:41–43.
84. Ortaldo JR, Longo DL. Human natural lymphocyte effector cells: Definition, analysis of activity, and clinical effectiveness. *J Natl Cancer Inst* 1988; 80:999–1010.
85. Topalian SL, Solomon D, Rosenberg SA. Tumor-specific cytolysis by lymphocytes infiltrating human melanomas. *J Immunol* 1989; 142:3714–3725.

86. Bjorkman PJ, Parham P. Structure, function, and diversity of Class I major histocompatibility complex molecules. *Annu Rev Biochem* 1990; 59:253-288.
87. Hoglund P, Ljunggren HG, Kärre K, Jay G. Role of major histocompatibility complex Class I molecules in tumor rejection—new insights from studies with synthetic peptides and transgenic mice. *Immunol Res* 1990; 9:298-313.
88. Browning MJ, Bodmer WF. MHC antigens and cancer-implications for T-cell surveillance. *Curr Opinion Immunol* 1992; 4:613-618.
89. Mellief CJM, Kast WM. Lessons from T-cell responses to virus-induced tumors for cancer eradication in general. *Cancer Surveys* 1992; 13:81-91.
90. Degiovanni G, Lahaye T, Herin M, Hainaut P, Boon T. Antigenic heterogeneity of a human melanoma detected by autologous CTL clones. *Eur J Immunol* 1988; 18:671-676.
91. Anichini A, Mazzocchi A, Fossati G, Parmiani G. Cytotoxic T-lymphocyte clones from peripheral blood and from tumor site detect intratumor heterogeneity of melanoma cells. Analysis of specificity and mechanisms of interaction. *J Immunol* 1989; 142:3692-3701.
92. Wölfel T, Klehmann E, Müller CA, Schütt KH, Meyer zum Büschenfelde KH, Knuth A. Lysis of human melanoma cells by autologous cytolytic T-cell clones. Identification of human histocompatibility leukocyte antigen A2 as a restriction element for three different antigens. *J Exp Med* 1989; 170:797-810.
93. Mukherji B, Chakraborty NG, Sivanandham M. T-cell clones that react against autologous human tumors. *Immunol Rev* 1990; 116:33-62.
94. Knuth A, Wölfel T, Meyer zum Büschenfelde KH. T-cell responses to human malignant tumours *Cancer Surveys* 1992; 13:39-52.
95. Koo AS, Tso CL, Shimabukuro T, Peyret C, Dekernion JB, Belldegrun A. Autologous tumor-specific cytotoxicity of tumor-infiltrating lymphocytes derived from human renal cell carcinoma. *J Immunother* 1991; 10:347-354.
96. Finke JH, Rayman P, Edinger M, Tubbs RR, Stanley J, Klein E, Bukowski R. Characterization of a human renal cell carcinoma specific cytotoxic $CD8^+$ T-cell line. *J Immunother* 1992; 11:1-11.
97. Schendel DJ, Gansbacher B, Oberneder R, Kriegmair M, Hofstetter A, Riethmüller G, Segurado OG. Tumor-specific lysis of human renal cell carcinomas by tumor-infiltrating lymphocytes. I. HLA-A2-restricted recognition of autologous and allogeneic tumor lines. *J Immunol* 1993; 151:4209-4220.
98. Bernhard H, Maeurer MJ, Wölfel T, Karbach J, Schneider J, Storkus W, Huber C, Meyer zum Büschenfelde KH, Knuth A. HLA-A2 restricted cytotoxic T-lymphocytes clones recognize a shared peptide epitope on human renal cancer cells. *Proc Am Assoc Cancer Res* 1994; 35:494.
99. Shimizu Y, Weidmann E, Iwatsuki S, Heberman RB, Whiteside TL. Characterization of human autotumor-reactive T-cell clones obtained from tumor-infiltrating lymphocytes in liver metastasis of gastric carcinoma. *Cancer Res* 1991; 51:6153-6162.
100. Wölfel T, Herr W, Coulie P, Schmitt U, Meyer zum Büschenfilde KH, Knuth A. Lysis of human pancreatic adenocarcinoma cells by autologous HLA- class-I-restricted cytolytic T-lymphocyte (CTL) clones. *Int J Cancer* 1993; 54:636-644.
101. Crowley NJ, Darrow TL, Quinn-Allen MA, Seigler HF. MHC-restricted recognition of autologous melanoma by tumor-specific cytotoxic T-cells—Evidence for restriction by a dominant HLA-A allele. *J Immunol* 1991; 146:1692-1696.
102. Kawakami Y, Zakut R, Topalian SL, Stotter H, Rosenberg SA. Shared human melanoma antigens—recognition by tumor-infiltrating lymphocytes in HLA-A2.1-transfected melanomas. *J Immunol* 1992; 148:638-643.
103. Van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T-lymphocytes on a human melanoma. *Science* 1991; 254:1643-1647.
104. Gaugler B, Van den Eynde B, Van der Bruggen P, Romero P, Gaforio JJ, De Plaen E, Lethe B, Brasseur F, Boon T. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytotolytic T-lymphocytes. *J Exp Med* 1994; 179:921-930.
105. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Rivoltini L, Topalian SL, Miki T, Rosenberg SA. Cloning of the gene for a shared human melanoma antigen recognized by autologous T-cells infiltrating into tumor. *Proc Natl Acad Sci USA* 1994; 91:3515-3519.

106. Coulie PG, Brichard V, Van Pel A, Wolfel T, Schneider J, Traversari C, Mattei S, De Plaen E, Lurquin C, Szikora JP, Boon T. A new gene coding for a differentiation antigen recognized by autologous cytolytic lymphocytes on HLA-A2 melanomas. *J Exp Med* 1994; 180:35–42.
107. Brichard V, Van Pel A, Wölfel C, De Plaen E, Lethe B, Coulie P, Boon T. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T-lymphocytes on HLA-A2 melanomas. *J Exp Med* 1993; 178:489–495.
108. Bakker ABH, Schreurs MJW, De Boer AJ, Kawakami Y, Rosenberg SA, Adema GJ, Figdor CG. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor infiltrating lymphocytes. *J Exp Med* 1994; 179:1005–1009.
109. Kawakami Y, Eliyahu S, Delgado CH, Robbins PF, Sakaguchi K, Appella E, Yannelli JR, Adema GJ, Miki T, Rosenberg SA. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc Natl Acad Sci USA* 1994; 91:6458–6462.
110. Brasseur F, Marchand M, Van Wijck R, Herin M, Lethe B, Chomez P, Boon T. Human gene MAGE-1, which codes for a tumor rejection antigen, is expressed by some breast tumors. *Int J Cancer* 1992; 52:839–841.
111. Rimoldi D, Romero P, Carrel S. The human melanoma antigen-encoding gene, MAGE-1, is expressed by other tumour cells of neuroectodermal origin such as glioblastomas and neuroblastomas. *Int J Cancer* 1993; 54:527–528.
112. Weynants P, Lethe B, Brasseur F, Marchand M, Boon T, Expression of Mage genes by non-small-cell lung carcinomas. *Int J Cancer* 1994; 56:826–829.
113. Traversari C, Van der Bruggen P, Leuscher IF, Lurquin C, Chomez P, Van Pel A, De Plaen E, Amarcostesec A, Boon T. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T-lymphocytes directed against tumor antigen-MZ2-E. *J Exp Med* 1992; 176:1453–1457.
114. Wölfel T, Van Pel A, Brichard V, Schneider J, Seliger B, Meyer zum Büschenfelde KH, Boon T. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T-lymphocytes. *Eur J Immunol* 1994; 24:759–764.
115. Robbins PF, El-Gamil M, Kawakami Y, Stevens E, Yannelli JR, Rosenberg SA. Recognition of tyrosinase by tumor-infiltrating lymphocytes from a patient responding to immunotherapy. *Cancer Res* 1994; 54:3124–3126.
116. Van der Bruggen P, Szikora JP, Boel P, Wildmann C, Somville M, Sensi M, Boon T. Autologous cytolytic T-lymphocytes recognize a Mage-1 nonapeptide on melanomas expressing HLA-Cw*1601. *Eur J Immunol* 1994; 24:2134–2140.
117. Falk K, Rötzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 1991; 351:290–296.
118. Hunt DF, Henderson RA, Shabanowitz J, Sakaguchi K, Michel H, Sevilir N, Cox AL, Appella E, Engelhard VH. Characterization of peptides bound to the Class-I MHC molecule HLA-A21.1 by mass spectrometry. *Science* 1992; 255:1261–1263.
119. Nijman HW, Houbiers JGA, Vierboom MPM, Van der Burg SH, Drijfhout JW, Damaro J, Kenemans P, Melief CJM, Kast WM. Identification of peptide sequences that potentially trigger HLA-A2.1-restricted cytotoxic T-lymphocytes. *Eur J Immunol* 1993; 23:1215–1219.
120. Visseren MJW, Van Elsas A, Van der Voort EIH, Ressing ME, Kast WM, Schrier PI, Melief CJM. Cytotoxic T-lymphocytes specific for the tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells. *J Immunol* 1995; 154:3991–3998.
121. Celis E, Tsai V, Crimi C, DeMars R, Wentworth PA, Chesnut RW, Grey HM, Sette A, Serra HM. Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. *Proc Natl Acad Sci USA* 1994; 91:2105–2109.
122. Houbiers JGA, Nijman HW, Van der Burg SH, Drijfhout JW, Kenemans P, Van de Velde CJH, Brand A, Momburg F, Kast WM, Melief CJM. In vitro induction of human cytotoxic T-lymphocyte responses against peptides of mutant and wild-type p53. *Eur J Immunol* 1993; 23:2072–2077.
123. Van Elsas A, Nijman HW, Van der Minne CE, Mourer SJ, Kast WM, Melief CJM, Schrier PI. Induction and characterization of cytotoxic T-lymphocytes recognizing mutated p21*ras* peptide presented by HLA-A*0201. *Int J Cancer* 1995; 6:389–396.

124. Stauss HJ, Davies H, Sadovnikova E, Chain B, Horowitz N, Sinclair C. Induction of cytotoxic T-lymphocytes with peptides in vitro—identification of candidate T-cell epitopes in human papilloma virus. *Proc Natl Acad Sci USA* 1992; 89:7871-7875.
125. Kast WM, Brandt RMP, Drijfhout JW, Melief CJM. Human leukocyte antigen-A2.1 restricted candidate cytotoxic T-lymphocyte epitopes of human papillomavirus type-16 E6-protein and E7-protein identified by using the processing-defective human cell line-T2. *J Immunother* 1993; 14:115-120.
126. Feltkamp MCW, Smits HL, Vierboom MPM, Minnaar RP, De Jongh BM, Drijfhout JW, Ter Schegget J, Melief CJM, Kast WM. Vaccination with cytotoxic T-lymphocyte epitope-containing peptide protects against a tumor induced by Human Papillomavirus type-16-transformed cells. *Eur J Immunol* 1993; 23:2242-2249.
127. Minev BR, McFarland BJ, Spiess PJ, Rosenberg SA, Restifo NP. Insertion signal sequence fused to minimal peptides elicits specific $CD8^+$ T-cell responses and prolongs survival of thymoma-bearing mice. *Cancer Res* 1994; 54:4155-4161.
128. Gelber C, Plaksin D, Vadai E, Feldman M, Eisenbach L. Abolishment of metastasis formation by murine tumor cells transfected with "foreign" H-2K genes. *Cancer Res* 1989; 49:2366-2373.
129. Hui KM, Sim TF, Foo TT, Oei AA. Tumor rejection mediated by transfection with allogeneic Class-I histocompatibility gene. *J Immunol* 1989; 143:3835-3843.
130. Ostrand-Rosenberg S, Roby C, Clements VK, Cole GA. Tumor-specific immunity can be enhanced by transfection of tumor cells with syngeneic MHC-Class-II genes or allogeneic MHC-Class-I genes. *Int J Cancer* 1991; 61-68.
131. Greenberg PD. Adoptive T-cell therapy of tumors—mechanisms operative in the recognition and elimination of tumor cells. *Adv Immunol* 1991; 49:281-355.
132. Melief CJM. Tumor eradication by adoptive transfer of cytotoxic T-lymphocytes. *Adv Cancer Res* 58:143-175.
133. Elliott BE, Carlow DA, Rodricks AM, Wade A. Perspectives on the role of MHC antigens in normal and malignant cell development. *Adv Cancer Res* 1989; 53:181-245.
134. Versteeg R, Noordermeer IA, Krüse-Wolters KM, Ruiter DJ, Schrier PI. c-*Myc* downregulates class I HLA expression in human melanomas. *EMBO J* 1988; 7:1023-1029.
135. North RJ, Awwad M, Dunn PL. The immune response to tumors. *Transplant. Proc.* 1989; 21:575-577.
136. Radrizzani M, Benedetti B, Castelli C, Longo A, Ferrara GB, Herlyn M, Parmiani G, Fossati G. Human allogeneic melanoma-reactive T-helper lymphocyte clones—functional analysis of lymphocyte-melanoma interactions. *Int J Cancer* 1991; 49:823-830.
137. Finke JH, Zea AH, Stanley J, Longo DL, Mizoguchi H, Tubbs RR, Wiltrout RH, Oshea JJ, Kudoh S, Klein E, Bukowski RM, Ochoa AC. Loss of T-cell receptor zeta-chain and p56(*lck*) in T-cells infiltrating human renal cell carcinoma. *Cancer Res* 1993; 53:5613-5616.
138. Restifo NP, Esquivel F, Kawakami Y, Yewdell JW, Mule JJ, Rosenberg SA, Bennink JR. Identification of human cancers deficient in antigen processing. *J Exp Med* 1993; 177:265-272.
139. Ranges GE, Figari IS, Espevik T, Palladino MA, Jr. Inhibition of cytotoxic T-cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. *J Exp Med* 1987; 166:991-998.
140. Inge TH, Hoover SK, Susskind BM, Barrett SK, Bear HD. Inhibition of tumor-specific cytotoxic T-lymphocyte responses by transforming growth factor-beta 1. *Cancer Res* 1992;·52: 1386-1392.
141. Torre-Amione G, Beauchamp RD, Koeppen H, Park BH, Schreiber H, Moses HL, Rowley DA. A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance. *Proc Natl Acad Sci USA* 1990; 87:1486-1490.
142. Hewitt HB, Blake ER, Walder AS. *Br J Cancer* 1976; 33:241-259.
143. Isobe K, Hasegawa Y, Iwamoto T, Hasegawa T, Kawashima K, Ding L, Nakashima I. Induction of antitumor immunity in mice by allo-Major Histocompatibility Complex Class-I gene transfectant with strong antigen expression. *J Nat Cancer Inst* 1989; 81:1823-1828.
144. Maio M, Altomonte M, Tatake R, Zeff RA, Ferrone S. Reduction in susceptibility to natural killer cell mediated lysis of human FO-1 melanoma cells after induction of HLA Class-I antigen expression by transfection with β_2m gene. *J Clin Invest* 1991; 88:282-289.

145. Boon T, Van Pel A, De Plaen E. Tum$^-$ transplantation antigens, point mutations and antigenic peptides: a model for tumor-specific transplantation antigens. *Cancer Cells* 1989; 1: 25–28.
146. Fearon ER, Itaya T, Hunt B, Vogelstein B, Frost P. Induction in a murine tumor of immunogenic tumor variants by transfection with a foreign gene. *Cancer Res* 1988; 38:2975–2980.
147. Schirrmacher V, Schlag P, Liebrich W, Patel BT, Stoeck M. Specific immunotherapy of colorectal carcinoma with newcastle-disease virus-modified autologous tumor cells prepared from resected liver metastasis. *Ann NY Acad Sci* 1993; 690:364–366.
148. Azuma M, Cayabyab M, Buck D, Phillips JH, Lanier LL. Involvement of CD28 in MHC-unrestricted cytotoxicity mediated by a human natural killer leukemia cell line. *J Immunol* 1992; 149:1115–1123.
149. Hayashi H, Matsubara H, Yokota T, Kuwabara I, Kanno M, Koseki H, Isono K, Asano T, Taniguchi M. Molecular cloning and characterization of the gene encoding mouse melanoma antigen by cDNA library transfection. *J Immunol* 1992; 149:1223–1229.
150. Chen LP, Ashe S, Brady WA, Hellstrom I, Hellstrom KE, Ledbetter JA, Mcgowan P, Linsley PS. Costimulation of antitumor immunity by the B7 counterreceptor for the T-lymphocyte molecules CD28 and CTLA-4. *Cell* 1992; 71:1093–1102.
151. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8+ T-cells by B7-transfected melanoma cells. *Science* 1993; 259:368–370.
152. Chen LP, Mcgowan P, Ashe S, Johnston J, Li YW, Hellstrom I, Hellstrom KE. Tumor immunogenicity determines the effect of B7 costimulation on T-cell-mediated tumor immunity. *J Exp Med* 1994; 179:523–532.
153. Baskar S, Ostrand-Rosenberg S, Nabavi N, Nadler LM, Freeman GJ, Glimcher LH. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class-II molecules. *Proc Natl Acad Sci USA* 1993; 90:5687–690.
154. Dohring C, Angman L, Spagnoli G, Lanzavecchia A. T-helper- and accessory-cell-independent cytotoxic responses to human tumor cells transfected with a B7 retroviral vector. *Int J Cancer* 1994; 57:754–759.
155. Douvdevani A, Huleihel M, Zoller M, Segal S, Apte RN. Reduced tumorigenicity of fibrosarcomas which constitutively generate IL-1-alpha either spontaneously or following IL-1-alpha gene transfer. *Int J Cancer* 1992; 51:822–830.
156. Fearon ER, Pardoll DM, Itaya T, Golumbek P, Levitsky HI, Simons JW, Karasuyama H, Vogelstein B, Frost P. Interleukin-2 production by tumor cells bypasses T-helper function in the generation of an antitumor response. *Cell* 1990; 60:397–403.
157. Gansbacher B, Zier K, Daniels B, Cronin K, Bannerji R, Gilboa E. Interleukin-2 gene transfer into tumor cells abrogates tumorigenicity and induces protected immunity. *J Exp Med* 1990; 172:1217–1224.
158. Ley V, Langlade-Demoyen P, Kourilsky P, Larsson-Sciard EL. Interleukin-2-dependent activation of tumor-specific cytotoxic T-lymphocytes in vivo. *Eur J Immunol* 1991; 21:851–854.
159. Connor J, Bannerji R, Saito S, Heston W, Fair W, Gilboa E. Regression of bladder tumors in mice treated with interleukin-2 gene-modified tumor cells. *J Exp Med* 1993; 177:1127–1134.
160. Hock H, Dorsch M, Kunzendorf U, Qin ZH, Diamantstein T, Blankenstein T. Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin-2, interleukin-4, interleukin-7, tumor necrosis factor, or interferon-gamma. *Proc Natl Acad Sci USA* 1993; 90: 2774–2778.
161. Porgador A, Gansbacher B, Bannerji R, Tzehoval E, Gilboa E, Feldman M, Eisenbach L. Anti-metastatic vaccination of tumor-bearing mice with IL-2-gene-inserted tumor cells. *Int J Cancer* 1993; 53:471–474.
162. Blankenstein T, Li W, Müller W, Diamantsein T. Retroviral Interleukin-4 gene transfer into a Interleukin-4-dependent cell line results in autocrine growth but not in tumorigenicity. *Eur J Immunol* 1990; 20:935–938.
163. Golumbek PT, Lazenby AJ, Levitsky HI, Jaffee LM, Karasuyama H, Baker M, Pardoll DM. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. *Science* 1991; 254:713–716.
164. Tepper RI, Pattengale PK, Leder P. Murine interleukin-4 displays potent anti-tumor activity in vivo. *Cell* 1989; 57:503–512.

165. Mullen CA, Coale MM, Levy AT, Stetler-Stevenson WG, Liotta LA, Brandt S, Blaese RM. Fibrosarcoma cells transduced with the IL-6 gene exhibited reduced tumorigenicity, increased immunogenicity, and decreased metastatic potential. *Cancer Res* 1992; 52:6020–6024.
166. Porgador A, Tzehoval E, Vadai E, Feldman M, Eisenbach L. Immunotherapy via gene therapy - comparison of the effects of tumor cells transduced with the interleukin-2, interleukin-6, or interferon-gamma genes. *J Immunother* 1993; 14:191–201.
167. Hock H, Dorsch M, Diamantstein T, Blankenstein T. Interleukin-7 induces $CD4^+$ T-cell-dependent tumor rejection. *J Exp Med* 1991; 174:1291–1298.
168. Miller AR, McBride WH, Dubinett SM, Dougherty GJ, Thacker JD, Shau HG, Kohn DB, Moen RC, Walker MJ, Chiu R, Schuck BL, Rosenblatt JA, Huang M, Dhanani S, Rhoades K, Economou JS. Transduction of human melanoma cell lines with the human interleukin-7 gene using retroviral-mediated gene transfer - comparison of immunologic properties with interleukin-2. *Blood* 1993; 82:3686–3694.
169. Tahara H, Zeh HJ, Storkus WJ, Pappo I, Watkins SC, Gubler U, Wolf SF, Robbins PD, Lotze MT. Fibroblasts genetically engineered to secrete interleukin-12 can suppress tumor growth and induce antitumor immunity to a murine melanoma in vivo. *Cancer Res* 1994; 54: 182–189.
170. Watanabe Y, Kuribayashi K, Miyatake S, Nishihara K, Nakayama E, Taniyama T, Sakata T. Exogenous expression of mouse interferon-γ cDNA in mouse C1300 neuroblastoma cells results in reduced tumorigenicity by augmented anti-tumor immunity. *Proc Natl Acad Sci USA* 1989; 86:456–9460.
171. Gansbacher B, Bannerji R, Daniels B, Zier K, Cronin K, Gilboa E. Retroviral vector-mediated γ-interferon gene transfer into tumor cells generates potent and long lasting anti-tumor immunity. *Cancer Res* 1990; 50:7820–7825.
172. Restifo NP, Spiess PJ, Karp SE, Mulé JJ, Rosenberg SA. A nonimmunogenic sarcoma transduced with the cDNA for interferon-gamma elicits $CD8^+$ T-cells against the wild-type tumor — correlation with antigen presentation capability. *J Exp Med* 1992; 175:1423–1431.
173. Porgador A, Bannerji R, Watanabe Y, Feldman M, Gilboa E, Eisenbach L. Antimetastatic vaccination of tumor-bearing mice with 2 types of IFN-gamma gene-inserted tumor cells. *J Immunol* 1993; 150:1458–1470.
174. Shiloni E, Karp SE, Custer MC, Shilyansky J, Restifo NP, Rosenberg SA, Mule JJ. Retroviral transduction of interferon-gamma cDNA into a nonimmunogenic murine fibrosarcoma: generation of T-cells in draining lymph nodes capable of treating established parental metastatic tumor. *Cancer Immunol Immunother* 1993; 37:286–292.
175. Blankenstein T, Qin Z, Überla K, Müller W, Rosen H, Volk H-D, Diamantstein T. Tumor suppression after tumor cell-targeted tumor necrosis factor-α gene transfer. *J Exp Med* 1991; 173:1047–1057.
176. Itoh Y, Kohgo Y, Watanabe N, Kanisawa Y, Sakamaki S, Takahashi M, Hirayama Y, Ono H, Himeno T, Niitsu Y. Human tumor-infiltrating lymphocytes transfected with tumor necrosis factor gene could augment cytotoxicity to autologous tumor cells. *Jpn J Cancer Res* 1991; 82: 1203–1206.
177. Hoon DSB, Hayashi Y, Morisaki T, Foshag LJ, Morton DL. Interleukin-4 plus tumor necrosis factor alpha augments the antigenicity of melanoma cells. *Cancer Immunol Immunother* 1993; 37:378–384.
178. Marincola FM, Ettinghausen S, Cohen PA, Cheshire LB, Restifo NP, Mule JJ, Rosenberg SA. Treatment of established lung metastases with tumor-infiltrating lymphocytes derived from a poorly immunogenic tumor engineered to secrete human TNF-alpha. *J Immunol* 1994; 152: 3500–3513.
179. Colombo MP, Ferrara G, Stoppacciaro A, Parenza M, Rodolfo M, Mavilio F, Parmiani G. Granulocyte colony-stimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma in vivo. *J Exp Med* 1991; 173:889–897.
180. Dranoff G, Jaffee EM, Lazenby AJ, Golumbek PT, Levitsky HI, Brose K, Jackson V, Hamada H, Pardoll DM, Mulligan RC. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci USA* 1993; 90:3539–3543.
181. Levitsky HI, Lazenby A, Hayashi RJ, Pardoll DM. In vivo priming of two distinct antitumor

effector populations: the role of MHC class I expression. *J Exp Med* 1994; 179:1215–1224.
182. Cavallo F, Giovarelli M, Gulino A, Vacca A, Stoppacciaro A, Modesti A, Forni G. Role of neutrophils and CD4$^+$ T-lymphocytes in the primary and memory response to nonimmunogenic murine mammary adenocarcinoma made immunogenic by IL-2 gene. *J Immunol* 1992; 149:3627–3635.
183. Visseren MJW, Koot M, Van der Voort EIH, Gravestein LA, Schoenmakers HJ, Kast WM, Zijlstra M, Melief CJM. Production of Interleukin-2 by EL4 tumor cells induces natural killer cell- and T-cell-mediated immunity. *J Immunother* 1994; 15:119–128.
184. Gilboa E, Lyerly HK, Vieweg J, Saito S. Immunotherapy of cancer using cytokine gene-modified tumor vaccines. *Semin Cancer Biol* 1994; 5:409–417.
185. Blankenstein T. Increasing tumor immunogenicity by genetic modification. *Eur J Cancer* 1994; 30A:1182–1187.
186. Huang A, Golumbek P, Ahmann DL, Jaffee EM, Pardoll DM, Levitsky HI. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* 1994; 264: 961–965.
187. Hock H, Dorsch M, Kunzendorf U, Uberla K, Qin Z, Diamantstein T, Blankenstein T. Vaccinations with tumor cells genetically engineered to produce different cytokines: effectivity not superior to a classical adjuvant. *Cancer Res* 1993; 53:714–716.
188. Vieweg J, Rosenthal FM, Bannerji R, Heston WD, Fair WR, Gansbacher B, Gilboa E. Immunotherapy of prostate cancer in the Dunning rat model: use of cytokine gene modified tumor vaccines. *Cancer Res* 1994; 54:1760–1765.
189. Oettgen HF, Old LJ. The history of cancer immunotherapy. In: De Vita VT, Helman S, Rosenberg SA, eds. *Biologic Therapy of Cancer, Principles and Practice*. Philadelphia: JB Lippincott, 1991:87–119.
190. Tao MH, Levy R. Idiotype/granulocyte-macrophage colony-stimulating factor fusion protein as a vaccine for B-cell lymphoma. *Nature* 1993; 362:755–758.
191. Chen TT, Tao MH, Levy R. Idiotype-cytokine fusion proteins as cancer vaccines. Relative efficacy of IL-2, IL-4, and granulocyte-macrophage colony-stimulating factor. *J Immunol* 1994; 153:4775–4787.
192. Lotze MT, Custer MC, Bolton ES, Wiebke EA, Kawakami Y, Rosenberg SA. Mechanisms of immunologic antitumor therapy: lessons from the laboratory and clinical applications. *Hum Immunol* 1990; 28:198–207.
193. Anonymous. TNF/TIL human gene therapy clinical protocol. *Hum Gene Ther* 1990; 1:441–480.
194. Gansbacher B, Houghton A, Livingston P, Minasian L, Rosenthal F, Gilboa E, Golde D, Oettgen H, Steffens T, Yang SY, Wong G. A pilot study of immunization with HLA-A2 matched allogeneic melanoma cells that secrete interleukin-2 in patients with metastatic melanoma—Memorial Sloan-Kettering Cancer Center. *Hum Gene Ther* 1992; 3:677–690.
195. Gansbacher B, Motzer R, Houghton A, Bander N, Minasian L, Gastl G, Rosenthal F, Gilboa E, Scheinfeld J, Yang SY, Wong G, Golde D, Reuter V, Livingston P, Bosl G, Nanus D, Fair WR. A pilot study of immunization with interleukin-2 secreting allogeneic HLA-A2 matched renal cell carcinoma cells in patients with advanced renal cell carcinoma—Memorial Sloan-Kettering Cancer Center. *Hum Gene Ther* 1992; 3:691–703.
198. Nabel GJ, Chang A, Nabel EG, Plautz G, Fox BA, Huang L, Shu S. Immunotherapy of malignancy by in vivo gene transfer into tumors. *Hum Gene Ther* 1992; 3:339–410.
197. Osanto S, Brouwenstijn N, Vaessen N, Figdor CG, Melief CJM, Schrier PI. Immunization with interleukin-2 transfected melanoma cells—a phase-I-II study in patients with metastatic melanoma. *Hum Gene Ther* 1993; 4:323–330.
198. Lotze MT, Rubin JT, Carty S, Edington H, Ferson P, Landreneau R, Pippin B, Posner M, Rosenfelder D, Watson C, Carlos T, Kirkwood J, Lembersky B, Logan T, Rosenstein M, Rybak ME, Whiteside TL, Elder E, Moen RC, Jacob W, Chen YW, Pinkus RL, Bryant J. Gene therapy of cancer—a pilot study of IL-4-gene-modified fibroblasts admixed with autologous tumor to elicit an immune response. *Hum Gene Ther* 1994; 5:41–55.

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