Concepts, Clinical Developments, and Therapeutic Advances in Cancer Chemotherapy

Cancer Treatment and Research

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Cancer Treatment and Research

Foreword

Where do you begin to look for a recent, authoritative article on the diagnosis or management of a particular malignancy? The few general oncology textbooks are generally out of date. Single papers in specialized journals are informative but seldom comprehensive; these are more often preliminary reports on a very limited number of patients. Certain general journals frequently publish good indepth reviews of cancer topics, and published symposium lectures are often the best overviews available. Unfortunately, these reviews and supplements appear sporadically, and the reader can never be sure when a topic of special interest will be covered.

Cancer Treatment and Research is a series of authoritative volumes which aim to meet this need. It is an attempt to establish a critical mass of oncology literature covering virtually all oncology topics, revised frequently to keep the coverage up to date, easily available on a single library shelf or by a single personal subscription.

We have approached the problem in the following fashion. First, by dividing the oncology literature into specific subdivisions such as lung cancer, genitourinary cancer, pediatric oncology, etc. Second, by asking eminent authorities in each of these areas to edit a volume on the specific topic on an annual or biannual basis. Each topic and tumor type is covered in a volume appearing frequently and predictably, discussing current diagnosis, staging, markers, all forms of treatment modalities, basic biology, and more.

In Cancer Treatment and Research, we have an outstanding group of editors, each having made a major commitment to bring to this new series the very best literature in his or her field. Martinus Nijhoff Publishers has made an equally major commitment to the rapid publication of high quality books, and world-wide distribution.

Where can you go to find quickly a recent authoritative article on any major oncology problem? We hope that Cancer Treatment and Research provides an answer.

WILLIAM L. MCGUIRE
Series Editor

Preface

As in the preceding two volumes on cancer chemotherapy published in this series, this book represents but a glimpse at the vast and expanding area of cancer chemotherapy. When considered in its broadest sense, biologic response modifiers introduced to treat cancer in man are the latest class of agents being studied for their antiproliferative activities. It is fitting that the first of these drugs widely employed as a result of recombinant technology be reviewed in depth just as five years of investigational clinical experience have been completed. Although disappointing to those whose expected a universally successful translation of its heralded in vitro versatility, alpha interferon has established itself as an effective treatment of hairy cell leukemia and a promising adjunct in several other clinical areas. Coincidentally we review deoxycoformycin, which is also proving to be effective in hairy cell leukemia. Deoxycoformycin, while failing to live up to expectations of unprecedented selectivity against lymphoid tumors, also holds promise if we learn to respect its toxicities; and it holds great theoretical interest. Another drug covered in depth is tiazofurin. Although this compound has not proven to have clinical utility, the experience in phase I trials is instructive, and its mechanism of action has been investigated in detail. Its antiviral properties should also prove of interest in this era of burgeoning hopes of controlling retroviral and other viral infections. Since viral etiology of malignancy is coming into prominence with the acquired immunodeficiency syndrome-related neoplasms, future relationships between antitumor activity and antiviral properties might emerge.

The evolving concepts and technical applications of locoregional therapy are covered by the San Diego group of Drs. Markman and Howell, who provide the perspective of several years and, by now, hundreds of patients' experiences. The sound pharmacologic basis of this approach was developed less then a decade ago by workers at the National Cancer Institute. The advantage achieved by intraperitoneal administration of drugs has been carefully studied utilizing a range of agents, and its theoretical foundations have been amply confirmed. This review therefore constitutes an excellent example of the optimal interface between the laboratory and the clinic in establishing new therapeutic modalities.

Another aspect of experimental therapeutics deals with the interface of animal and human pharmacology in the planning and execution of phase I studies. More closely linked to the laboratory are the principles of biochemical modulation, many of which have not yet been successfully applied clinically. Finally, clinicians and experimentalists alike might be interested in the overview of the DNA alkaline elution technique in drug development by its initiator, Dr. Kurt Kohn and his colleauges.

The review of clinical applications of chemotherapy focuses on two distinct areas, each of which merits in-depth attention at this time. Brain tumors, which first yielded partially to chemotherapy more than 18 years ago, must be reevaluated with an eye towards capitalizing on recent advances in tumor biology. Mediastinal germ cell tumors represent a new entity which is now successfully treated with chemotherapy and is worthy of a thorough review. We hope that in-depth readers and surveyors of the chemotherapy field will find this blend of concepts, new clinical developments, and therapeutic landmarks useful to clinical and laboratory scientists on a recurrent basis.

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I

Concepts

1. DNA filter elution methods in anticancer drug development

Kurt W. Kohn

DNA filter elution methods are widely used to assess DNA lesions such as single- and double-strand breaks, alkali-labile sites, interstrand crosslinks, and DNA-protein crosslinks in mammalian cells [1]. Such DNA lesions are likely to be involved in the production of cytotoxic and mutagenic effects of drugs and chemicals, and the assays are often sensitive enough to detect actions at doses pertinent to the mechanisms of antitumor and carcinogenic effects. Recent work has utilized the technique to investigate the factors that determine the sensitivities of different cell types to anti-cancer drugs. This chapter reviews some recent results, mainly from my laboratory, that illustrate how the technique can be applied to studies of drug action mechanisms. Before going on to these findings, however, current knowledge of how the technique works will be summarized.

Experimental and theoretical bases of DNA filter elution assays

The methods of DNA filter elution assays are simple in principle, but must be carried out with care to avoid mechanically breaking very long DNA strands which are very fragile [2]. Appropriate controls and calibrations are needed to make the results quantitatively meaningful.

The basic procedure common to all of the DNA filter elution assays [1] is, briefly, as follows: Cells are deposited on a membrane filter and then lysed by means of a detergent solution. This lysis solution is allowed to run out through the filter and removes most of the cell protein and RNA, leaving the nuclear DNA intact on the filter. At this point in the procedure, proteinase K can be applied to digest protein that may be covalently linked to the DNA. An alkaline solution is then added and slowly pumped through the filter. Fractions are collected to determine the rate at which the DNA elutes through the filter. The DNA is either prelabeled and measured by scintillation counting or it is assayed fluorometrically.

The physical basis for the filter elution technique may be that the flow of solvent carries individual DNA strands into several filter pore channels, as depicted in figure 1 [2]. The time required for a DNA strand to elute from

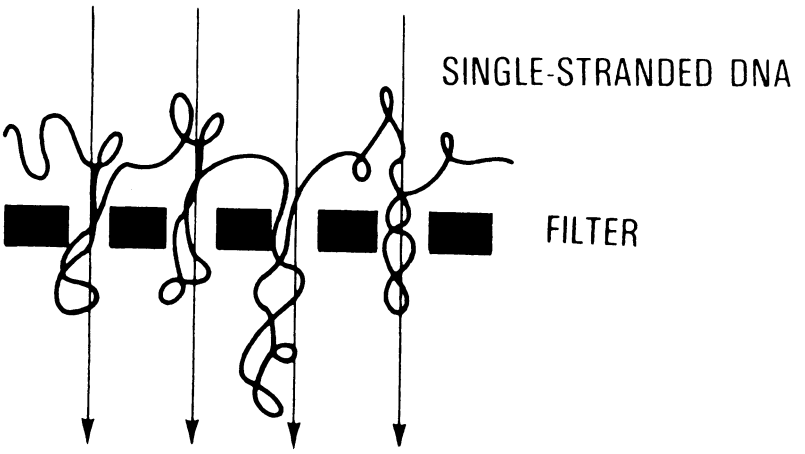


Figure 1. Possible mechanism of size-dependent elution of DNA strands from filters. A long DNA strand is carried by the solvent flow (arrows) simultaneously into several pore channels. The DNA strand will not elute until a large enough portion of the strand chances to be in one pore channel so that solvent flow will pull the entire strand through that channel [2].

the filter would depend on the length of the strand and on its initial configuration among the filter pores. The same principle may hold for the elution of DNA double strands and for the elution of alkali-separated single strands.

The basic procedure is modified in various ways to assay particular DNA lesion classes. For the assay of single-strand breaks, as well as for all of the other commonly assayed lesions except double-strand breaks, the alkaline eluting solution is above pH 12.0, so as to disrupt the hydrogen bonding between base pairs. The DNA single-strands can then disentangle and slide away from each other. This is shown by the fact that newly replicated DNA strand segments, which are relatively short, elute rapidly, leaving the long parental strands on the filter [3].

The assays above pH 12.0 are usually referred to as DNA alkaline elution, whereas the assays for double-strand breaks, usually carried out at about pH 10, are sometimes termed *neutral elution.*

The measurements of single-strand breaks, as well as of all other DNA lesions except DNA-protein crosslinks, are most consistent and reliable if care is taken to avoid any effects due to possible stable DNA-protein complexes. The best lysis detergent to dissociate any such complexes and to inhibit nucleases is sodium dodecyl sulfate. Proteinase K is active in this detergent and can be used in all assays except those of DNA-protein cross-links. Proteinase K, however, does not digest DNA-linked proteins to completion. Therefore it is also desirable to use filters that have a low capacity to adsorb proteins. In addition, sodium dodecyl sulfate is added to the alkaline eluting solution in order to further reduce the adsorption of pro-

teins to the filter. For the assay of DNA-protein crosslinks, of course, the converse conditions apply.

Single-strand breaks

When random single-strand breaks are produced by ionizing radiation, the shorter single-strand segments elute faster, on average, than do longer segments. Populations of single-strand segments produced by ionizing radiation elute with kinetics that are very close to exponential. The DNA retention, r, at time, t, closely obeys the relation $\log(r/r_0) = -kt$, and the slope k is proportional to the radiation dose, and thus to the strand break frequency [4]. From these facts plus minimal additional assumptions, the elution behavior of a homogenous strand population (strands all of the same length) can be mathematically derived (K.W. Kohn, unpublished). It can be shown that the elution kinetics of a homogenous strand population would not be exponential, but rather would be a step function: a constant number of strands would elute in each time interval from time zero until all of the strands have eluted. The magnitude of this constant elution rate is inversely proportional to the strand length and continues for a time period that is directly proportional to strand length.

These conclusions are important for two reasons. First, they show that alkaline elution cannot be used to sharply fractionate DNA strands according to size as in a gel filtration column. Secondly, it gives the basis for a theoretical account of the alkaline elution phenomenon. (A recently published theory [5] erroneously assumed exponential elution for uniform strand length populations.)

Double-strand breaks

For the assay of DNA double-strand breaks, the pH of the eluting solution is lowered to approximately 10, so as to retain the hydrogen-bonded double strand structure. It is necessary to digest the cell lysate with proteinase K in order to obtain optimum elution of double stranded DNA [6]. There seems to be a protein-containing structure that otherwise prevents the elution of DNA at pH 10. Although single strands elute independently of each other at pH 12, this not entirely true for double-strands at pH 10 (M.O. Bradley, personal communication). Thus when irradiated and unirradiated cells are mixed, each affects the pH 10 elution of the other (although no such interaction exists in pH 12 elution). This must be taken into account in the design of double-strand break experiments, especially in regard to the use of internal standards.

Alkali-labile sites

Some compounds, notably monofunctional alkylating agents and 2′-deoxyazacytidine, produce DNA alterations that are converted to single-strand breaks during the exposure to alkaline solutions in the course of filter elution assays. In the case of alkylating agents, the alkali-labile sites probably represent apurinic sites formed by the spontaneous or enzymatic release of alkylated purines from DNA. In the case of 2′-deoxyazacytidine, the alkali-lability is attributed to the alkaline decomposition of 5-azacytidine residues incorporated into DNA [7, 8]. The presence of alkali-labile sites is often signaled in pH 12 elution assays by elution curves (plotted as log [retention] versus time) that bend downward due to the progressive formation of strand breaks during the alkaline elution period [1]. The presence of alkali-labile sites is confirmed by an increase in elution rate when the pH of alkaline elution is raised, for example, from 12.1 to 12.6.

A recent observation by Nicolini et al. [9] suggests that an even more effective method would be to incubate (at room temperature) the cell lysate at pH 10 for 24–48 hours before elution at pH 12. The pH 10 incubation increased the sensitivity of detection of DNA damage in cells treated with methylemthane-sulfonate or dimethylnitrosamine. Nicolini et al. proposed to explain this effect in terms of DNA unfolding at pH 10. A.J. Fornace, (personal communication) recently confirmed this effect and obtained evidence that it is not due to DNA unfolding, but rather to the conversion of alkali-labile sites to strand breaks at pH 10.

Interstrand crosslinks

The assay for DNA interstrand crosslinks is based on the idea that crosslinked single-strand fragments would elute more slowly than the separate DNA fragments. The procedure is to fragment the cell DNA in a controlled manner by subjecting the cells to an appropriate dose of ionizing radiation, typically 300 rad, which introduces about one single-strand break per 3×10^6 nucleotides. The cells are kept on ice during and after irradiation so that the breaks are not repaired. Interstrand crosslinking is gauged by the degree to which elution at pH 12 is slowed relative to control cells subjected to the same radiation dose.

Interstrand crosslink frequency [ISC] is calculated using the formula

$$[\mathrm{ISC}] = \left(\sqrt{(1 - r_0)/(1 - r)} - 1\right) R$$

where r and r_0 are the fractional retentions of DNA from treated and control cells, respectively, and R is the radiation dose. This formula is based on a simplified model and has limitations [10]. The formula is useful empirically because it generally gives values that are proportional to drug dose and that are not very sensitive to the elution time endpoint used. The values

obtained are proportional estimates of interstrand crosslink frequency, but are subject to an unknown proportionality constant.

When a low frequency of strand breaks is present in addition to crosslinks, a partial correction can be made [10]. However when large numbers of both types of lesions coexist, a unique determination of each type is difficult.

DNA-protein crosslinks

The assay for DNA-protein crosslinks is based on a different principle from the other assays. Rather than being based on effects of strand length on elution kinetics, it is based on the adsorption of the DNA-linked proteins to the filters. The assay conditions are designed to maximize the efficiency of protein adsorption without allowing retention of free DNA fragments by the filters. In order to allow free DNA fragments to elute rapidly from the filters, cells are irradiated with a relatively high dose, typically 3,000 rad. The DNA fragments held up on the filters are assumed to be covalently linked to one or more protein molecules. This model leads to a simple accurate formula for the determination of DNA-protein crosslink frequency [DPC]:

$$[\mathrm{DPC}] = [(1 - r)^{-1/2} - 1]\,R$$

where r is the fraction of the DNA held up on the filter and R is the radiation dose. (For details see [1] or [11]).

DNA chlorethylating agents

The chloroethylnitrosoureas were among the first anticancer drugs to be studied by DNA filter elution methods [10, 12]. These drugs are extraordinarily active against a wide variety of experimental tumor systems in mice. The chloroethylnitrosoureas, BCNU, CCNU, and MeCCNU (see figure 2 for structures) are used clinically, usually combined with other drugs, in standard anticancer regimens and in many therapeutic trials. DNA filter elution experiments during the past five years have disclosed action mechanisms that bear on the question of how this class of drugs should be used and further developed.

These drugs can be viewed as generally reactive chemicals that undergo diverse reactions with biomolecules; among all of these chemical reactions, those responsible for antitumor activity may well constitute a minor part of the whole. One aspect of the drug development problem is to modify the drug structure so as to eliminate unnecessary chemical reactions while preserving the desired reactions. A second aspect, to which DNA filter elution studies have contributed, is the identification of those tumor cell populations

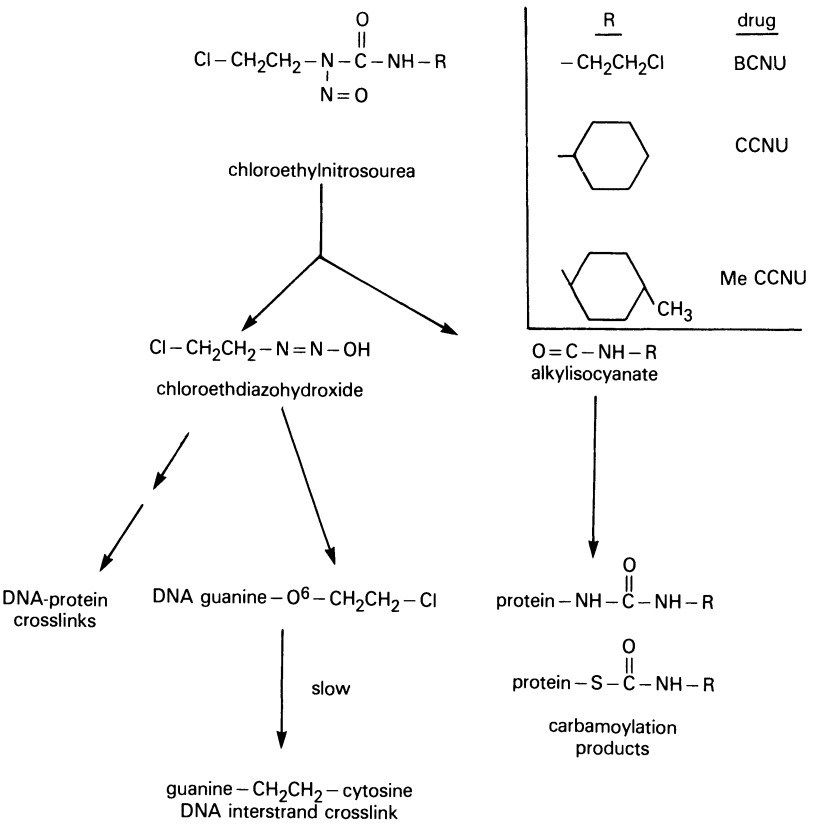


Figure 2. Reaction paths and products of chloroethylnitrosoureas. (Reactions leading to cyclic intermediates and hydroxyethylation products have been omitted.)

that may be potentially vulnerable to treatment with DNA chloroethylating agents.

Overview of reactions, products and antitumor activity

Chloroethylnitrosoureas such as BCNU, CCNU, and MeCCNU decompose spontaneously to produce two types of highly reactive compounds that engage in two different types of addition reactions: alkylations and carbamoylations (figure 2).

Alkylation reactions derive from (1) chloroethyldiazohydroxide, which adds a chloroethyl group to various sites on nucleic acids and proteins, and from (2) two possible cyclic intermediates (not shown in figure 2), which can add a hydroxyethyl group to these sites [13]. One of the reactions with DNA, the formation of chloroethyl adducts at guanine-06 positions, can lead to the production of possibly lethal interstrand crosslinks. The conver-

sion of 06-chloroethylguanine moieties to interstrand crosslinks, the chemistry of which has recently been elucidated [14] is a slow reaction; 6–12 hours are required for the reaction to go to completion at 37° [15, 16]. This gives enough time for a DNA repair enzyme to remove the monoadduct before it converts to a crosslink. Most human cells contain a guanine-06-alkyltransferase activity that rapidly removes small alkyl groups from guanine-06 positions of DNA [17, 18]. DNA filter elution experiments provided the first evidence that this activity can remove chloroethyl groups from guanine-06 positions and thereby prevent the formation of potentially cytolethal interstrand crosslinks [19]. The ability of guanine-06-alkyltransferase to prevent chloroethylnitrosourea-induced DNA interstrand crosslinking has now been confirmed using an enzyme extract from human leukemic cells [20] as well as using a purified enzyme preparation [21].

If this inference about the mechanism of cytotoxicity is correct, then it is the chloroethylation reaction that is responsible for the antitumor activity, whereas the hydroxyethylation reaction may only add to the carcinogenicity, mutagenicity, or other side effects of the drugs. Since chloroethylnitrosoureas produce more hydroxyethylation than chloroethylation [14, 22, 23], it would be desirable to develop a drug that would produce chloroethylations but no hydroxyethylations.

Carbamoylation reactions derive from alkylisocyanates, the second type of product formed by the spontaneous splitting of the nitrosourea moiety (figure 2). The isocyanates can add a carbamyol group to amino and sulfhydryl groups on proteins, but do not react with nucleic acids. Carbamoylation reactions may inhibit many enzyme reactions, including RNA splicing and DNA ligase in mammalian cells [24, 25, 26, 27, 28, 29]. The inhibition of DNA ligase activity, an essential step in most DNA repair processes, was demonstrated by DNA filter elution experiments.

The carbamoylation reactions do not contribute to antitumor activity. Chloroethylnitrosourea derivatives that lack carbamoylating activity are available and retain full activity in a wide range of animal tumor systems. Contrariwise, alkylnitrosoureas have been prepared that retain carbamoylating activity but lack alkylating activity, and these have no antitumor activity. It seems illogical, therefore, to continue to use carbamoylating nitrosoureas such as BCNU, CCNU, and MeCCNU in clinical therapy, especially in drug combinations where carbamoylation of critical enzymes (e.g., DNA repair enzymes) might impair the potential effectiveness of other drugs in the combination.

A logical direction for the further development of this class of drugs would be to develop compounds that produce the desired chloroethylation reactions but which lack the hydroxyethylation and carbamoylation activities. As will be summarized below, a new class of highly active antitumor drugs having these characteristics is now available for development.

DNA lesions and cell lethality

From the variety of the chemical reactions produced by chloroethylnitrosoureas, it is not surprising that multiple effects on DNA are observed. DNA filter elution experiments have indicated the production of DNA interstrand crosslinks and DNA-protein crosslinks, as well as DNA strand breaks and alkali-labile lesions [10,12,30]. In addition, the rejoining of DNA strand breaks produced by x-ray or ultraviolet light was observed to be inhibited by compounds that possess carbamoylating activity [24,29]. Studies (described below) have been conducted to determine which class of lesions is responsible for the antitumor activity and what biochemical characteristics make particular cell types vulnerable to these drugs.

Selectivity based on guanine-06-alkyltransferase deficiency

DNA filter elution experiments gave particularly striking results when interstrand crosslinks and DNA-protein crosslinks were assayed in human cell types that were either normal or deficient in guanine-06-alkyltransferase [19]. DNA-protein crosslinking was not significantly different in cells having normal or deficient transferase activity (figure 3). Interstrand crosslinking, however, was markedly reduced and often undetectable in the deficient cells (figure 4). DNA-protein crosslinking is fully developed within 1–2 hours of addition of the drug, whereas interstrand crosslinking continues to rise for 6–12 hours after a 1–2 hour drug exposure period. Cell killing was consistently greater in cells showing high interstrand crosslinking and did not correlate with DNA-protein crosslinking.

Current evidence indicates that, among human tumor cell strains in culture, approximately 20% are transferase deficient (Mer^-) [17,18,31,32]. Transferase-deficient tumor cell types show an enhanced killing by chloroethylnitrosoureas compared with normal cells (all of which so far have been Mer^+). Using human rhabdomyosarcoma xenograft tumors in mice, Brent et al. [33] found large differences among the tumor lines in guanine-06-methyltransferase activity, which were well correlated with the in vivo sensitivities of the tumors to MeCCNU.

The cell content of guanine-06-alkyltransferase is limited. It is possible to deplete normal or Mer^+ tumor cells of enzyme by sublethal treatment with a DNA methylating agent such as methylnitrosourea. For each methyl group removed from a guanine-06 position, an enzyme molecule becomes inactivated (i.e., the reaction is stoichiometric rather than catalytic). By pretreatment with a DNA methylating agent, it was possible to enhance the sensitivity of human Mer^+ cells to a subsequent treatment with a chloroethylnitrosourea [34,35,36]. Concomitantly, cells that did not form detectable interstrand crosslinks when treated with a chloroethylnitrosourea, did show such crosslinking when pretreated with a DNA methylating agent. The pretreatment did not affect DNA-protein crosslinking, nor did it affect

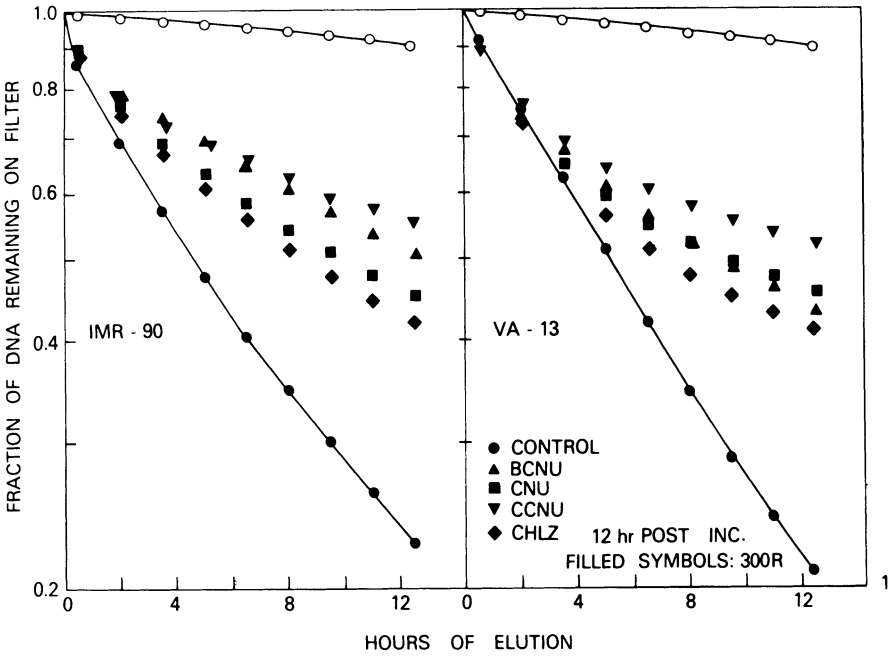


Figure 3. An example of DNA-protein crosslinking assays in human cells treated with chloroethylnitrosoureas. Polyvinylchloride filters that efficiently adsorb proteins were used. The elution was carried out at pH 12.1. IMR-90 (left) are normal human embryo cells having normal levels of guanine-06-alkyltransferase (Mer^+ phenotype). VA-13 (right) are transformed human embryo cells that are transferase deficient (Mer^- phenotype). Open circles show that DNA from untreated control cells elutes very little from the filters. Filled circles show the increase in elution caused by exposing control cells to 300 R of x-rays at 0° C. The other symbols show the reduced elution caused by treatment with chloroethylnitrosoureas in assays which included the 300 R x-ray exposure. In assays without x-ray, the drug treatment caused small increases in elution rate compared to the open symbol controls (data not shown). The cells were exposed to 50 μM of drug for one hour and then incubated without drug for 12 hours. The drugs used were BCNU, CCNU, chlorozotocin (CHLZ), and 1-(2-chloroethyl)-1-nitrosourea (CNU) [19].

interstrand crosslinking by nitrogen mustards or cisplatin. This interesting drug interaction between chloroethylnitrosoureas and DNA methylating agents, which might at an earlier time have seemed unexpected and puzzling, was predictable on the basis of the guanine-06-alkyltransferase mechanism.

One might ask whether this drug interaction provides a potential means of treating Mer^+ tumors by inactivating their guanine-06-alkyltransferase. Unfortunately this is unlikely to succeed, because the source of the differential between normal cells and sensitive tumor cells would be eliminated.

It seems likely that only tumors consisting predominantly of transferase-deficient cells would be potentially responsive to chloroethylnitrosoureas. Tumor tissues could be assayed for guanine-06-alkyltransferase, and treat-

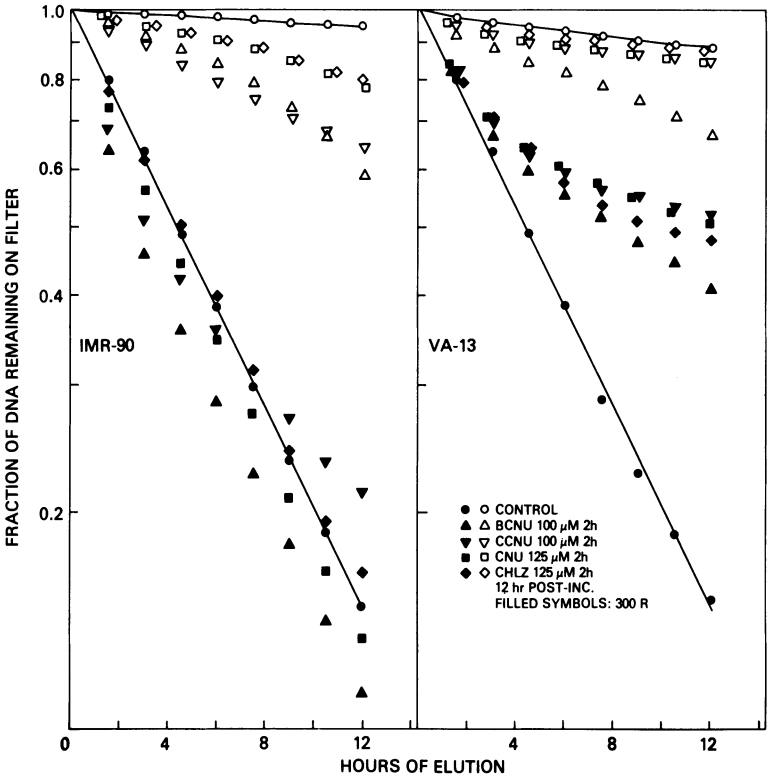


Figure 4. An example of DNA interstrand crosslinking assays in the same experiment as in figure 3. In order to eliminate the filter retention of DNA due to protein linking, polycarbonate filters are used, the cell lysates on the filter are digested with proteinase K, and 0.1% sodium dodecyl sulfate is added to the pH 12.1 eluting solution. Reduced elution in the assay that included a 300 R x-ray exposure at 0°C then is attributed to interstrand crosslinking. The transferase-deficient (Mer^-) VA-13 cells (right) show drug-induced interstrand crosslinking, whereas the IMR-90 Mer^+ cells (left) do not. Symbols are the same as the figure 3. In addition, the small drug-induced increases in elution rates in assays without x-ray are shown by the open symbols in the upper part of the figure; these are due to DNA strand breaks or alkali-labile lesions [19].

ment with these drugs could be confined to patients bearing transferase-deficient tumors.

Inhibition of DNA repair: An undesirable effect of carbamoylation

The effects of carbamoylating activity have been investigated by comparing some chloroethylnitrosoureas that possess this activity with others that do not. Both types of compounds are extraordinarily active against murine neoplasms (NCI antitumor screening data). In addition, compounds have been studied that have carbamoylating activity exclusively; these compounds are alkylisocyanates and derivatives of 1,3-bis(cyclohexyl)-1-nitrosourea and

are devoid of alkylating activity. Both of these types of pure carbamoylating agents, as well as the carbamoylating chloroethylnitrosoureas, have been found to slow the rate of rejoining of x-ray-induced strand breaks in mammalian cells [24, 28, 37], and to synergise x-ray-induced cell killing. Carbamoylation also has been shown to block DNA strand rejoining in the nucleotide excision repair of ultraviolet light damage [29]. Noncarbamoylating nitrosoureas do not have these effects.

An apparent exception to this rule is 1-(2-chloroethyl)-1-nitrosourea which liberates cyanate, a carbamoylating species, but does not inhibit DNA repair in cells. Cyanate, however, is ionic, contrary to the nonionic alkylisocyanates liberated by 3-substituted nitrosoureas and, on this basis, would be expected to be excluded from cells.

Since the chloroethylnitrosoureas produce an excess of various DNA lesions other than guanine-06 chloroethylations, the repair of these other lesions would be crucial if selective toxicity based on the guanine-06 reaction is to be expressed. Alkylations of DNA bases other than at guanine-06 positions would be expected to be repaired by a nucleotide excision mechanism in which the rejoining of DNA strand breaks by a DNA ligase is an essential step. By blocking nucleotide excision repair in normal as well as tumor cells, carbamoylation may obscure any potential selectivity based on differences in guanine-06-alkyltransferase.

One would then expect that selective cell killing of transferase-deficient human tumor cells would be reduced in the case of carbamoylating compared with noncarbamoylating chloroethylnitrosoureas. We have verified this effect in a pair of transferase-competent and deficient human embryo cells (IMR-90 and VA-13) [19], and more recent work in our laboratory has confirmed this in human tumor cells. Furthermore, the main cause of the reduction in selectivity of cell killing by the carbamoylating drugs is an increased killing of the normal cells [37]. As a possible explanation, Sariban et al. [37] observed the accumulation of strand breaks in IMR-90 cells treated with carbamoylating chloroethylnitrosoureas, but not with the noncarbamoylating drugs. The strand breaks may represent nucleotide excision repair intermediates whose rejoining was inhibited by the carbamoylating reaction. The evidence indicates that carbamoylation may nonspecifically interfere with the repair of cytotoxic DNA lesions unrelated to the guanine-06 lesions responsible for the potential selectivity against transferase-deficient tumor cells.

2-chloroethyl (methanesulfonyl)methanesulfonate (clomesone): A pure chloroethylating agent

In order to construct active new antitumor compounds that would produce exclusively chloroethylations, Shealy et al. [38, 39] prepared 2-chloroethyl (methanesulfonyl)methanesulfonate (NSC 338947) as a promising new drug candidate. The compound was found to be as effective as chloro-

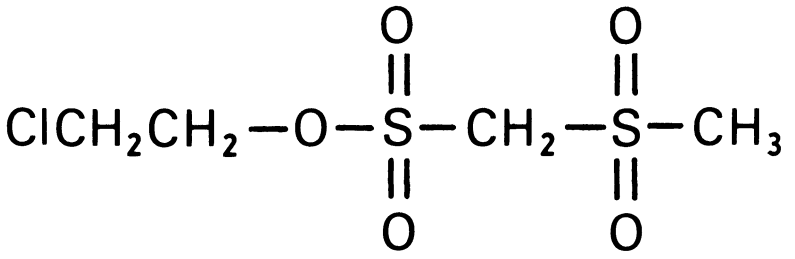


Figure 5. Structure of 2-chloroethyl (methanesulfonyl) methanesulfonate (clomesone).

ethylnitrosoureas against a variety of experimental tumor systems in tests conducted by the National Cancer Institute (J. Plowman, personal communication).

The chemical structure of this compound (figure 5) excludes the possibility of carbamoylating activity, because there is no way in which isocyanate-like products could be formed. The production of hydroxyethylations seemed unlikely, but could not be excluded from the structure alone. Therefore, an analytic study was conducted of guanine adducts produced by the reaction of clomesone, labeled with ^{14}C in the chloroethyl groups, with purified DNA [40]. 7-chloroethylguanine was identified as a product, but no 7-hydroxyethylguanine was detected. By contrast, chloroethylnitrosoureas produced more 7-hydroxyethylguanine than 7-chloroethylguanine. Thus the absence of hydroxyethylation by clomesone was confirmed.

When tested against human cells in culture, clomesone exhibited a degree of selectivity for killing guanine-06-alkyltransferase-deficient (Mer^-) human cells that was even greater than had been observed with noncarbamoylating chloroethylnitrosoureas (figure 6). Also, DNA filter elution experiments showed DNA damage phenomena similar to and even more definitive than those produced by noncarbamoylating chloroethylnitrosoureas. Interstrand crosslinking occurred after a delay of 6–12 hours in transferase-deficient (Mer^-) cells, but was almost undetectable at any time in transferase-competent (Mer^+) cells (figure 7). Also similarly to the chloroethylnitrosoureas, clomesone produced DNA-protein crosslinks in both cell types, and these lesions appeared promptly and were partially repaired in a 24 hour period (figure 8).

Thus clomesone produces only a single class of addition reactions and has maximum effectiveness against a wide variety of animal tumor systems. Chloroethylation, however, affects a variety of sites on the DNA bases. Further selectivity might be obtained by substitutions at the methanesulfonylmethanesulfonate group.

Diaziridinylbenzoquinone (diaziquone, NSC 182986, AZQ)

As the second example of DNA filter elution studies of the mechanisms of action of anticancer drugs, we consider diaziridinylbenzoquinone (AZQ).

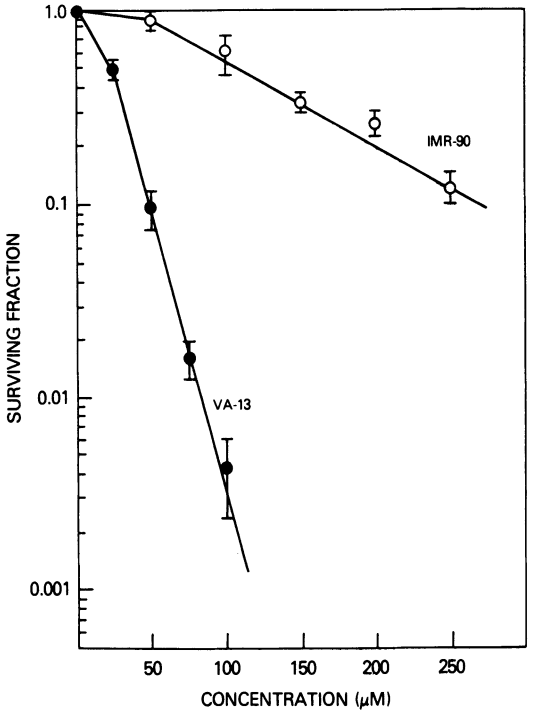


Figure 6. Survival of colony-forming ability of normal (IMR-90, Mer^+) and transformed (VA-13, Mer^-) human embryo cells after treatment for two hours with various concentrations of clomesone [40].

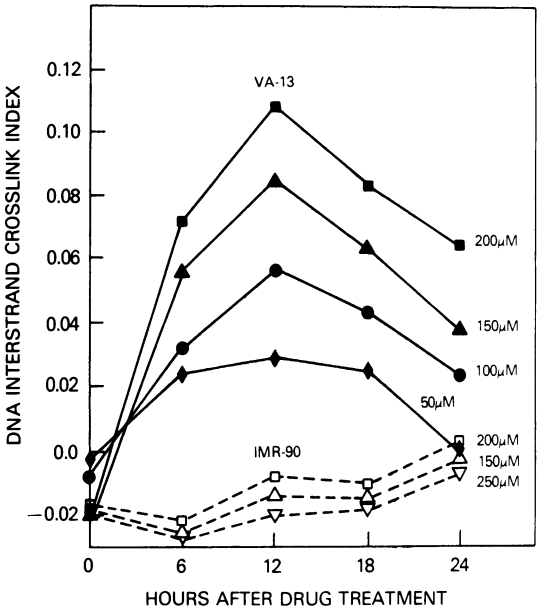


Figure 7. DNA interstrand crosslinking produced by clomesone in normal (IMR-90, filled symbols) and transformed (VA-13, open symbols) human embryo cells. IMR-90 cells are proficient (Mer^+) and VA-13 cells are deficient (Mer^-) in DNA repair by guanine-06-alkyltransferase. Cells were treated with drug for two hours and then incubated in the absence of drug for various times [40].

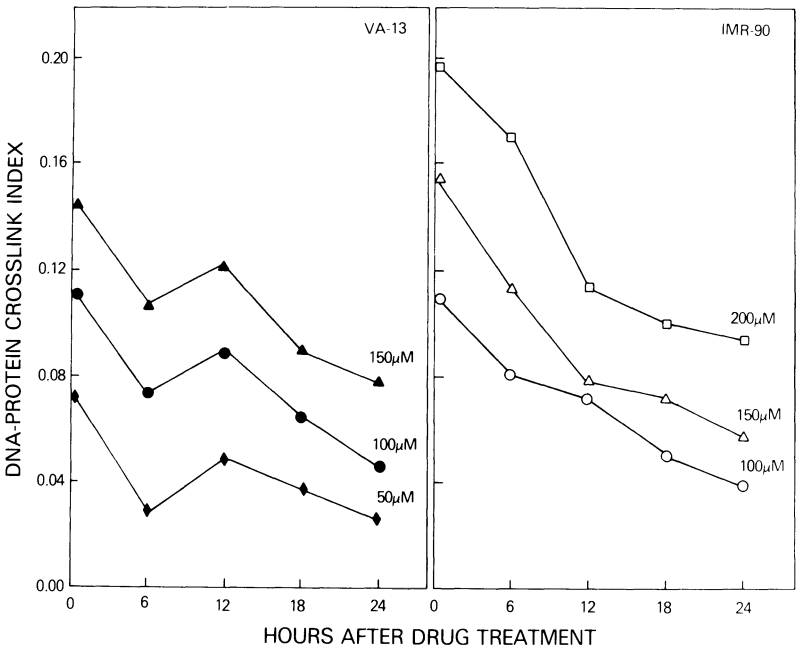


Figure 8. DNA-protein crosslinking in the same experiments as figure 7. Left, VA-13 cells; rights, IMR-90 cells [40].

AZQ is effective against several experimental tumor systems [41] and has clinical activity against primary brain tumors and lymphomas [42, 43]. The chemical structure of the drug suggested that it may engage in two types of chemical reactions (figure 9). First, as a quinone it may undergo oxidation-reduction reactions generating free radicals that may react with DNA to produce strand breaks. Secondly, the molecule has two aziridine groups that may alkylate DNA to form various types of crosslinks. DNA filter elution studies have been conducted to determine which of these mechanisms may occur and which may be related to cell killing [44, 45, 46].

DNA lesions associated with cell killing

AZQ treatment of Chinese hamster ovary cells and mouse leukemia L1210 cells produced interstrand and DNA-protein crosslinks, as well as single-strand breaks [44, 45, 46]. The strand breaks formed promptly and seemed to disappear within 1–2 hours. The interstrand crosslinks, on the other hand, increased in frequency for about 6 hours and then were removed over the next 24 hours.

Studies of various cell lines having widely different sensitivities to AZQ disclosed marked variations in the extents of DNA strand scission and interstrand crosslinking produced by the drug (figure 10 and 11). Furthermore, there was a striking dissociation between these two types of DNA

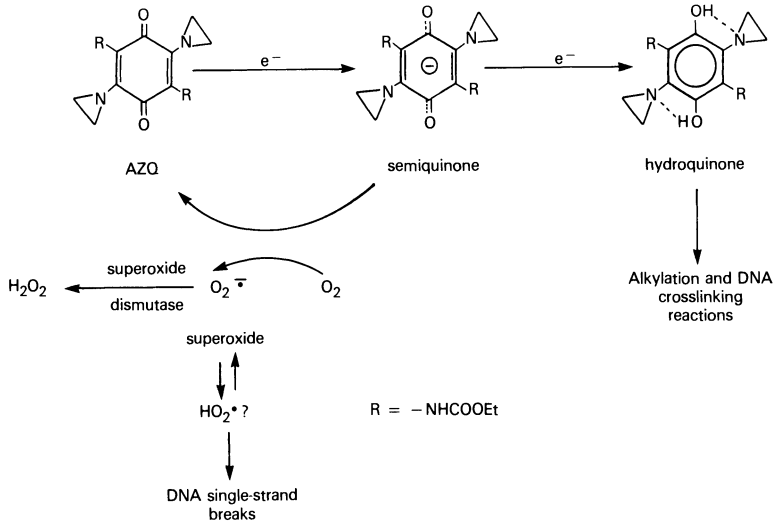


Figure 9. Possible oxidation-reduction and alkylation reactions of AZQ.

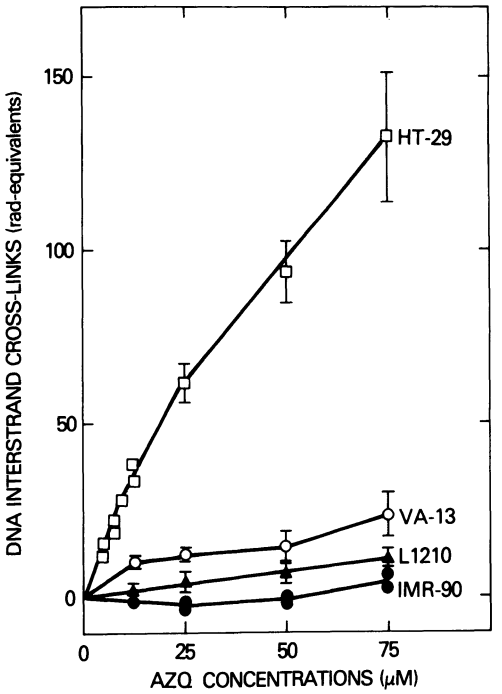


Figure 10. DNA interstrand crosslinking produced by AZQ in various cell lines. The cells were exposed to drug for one hour and then incubated in the absence of drug for four hours in order to allow the completion of delayed crosslink formation. HT-29, human colon carcinoma cells; IMR-90, normal human embryo cells; VA-13, transformed human embyro cells; L1210, mouse leukemia cells [44].

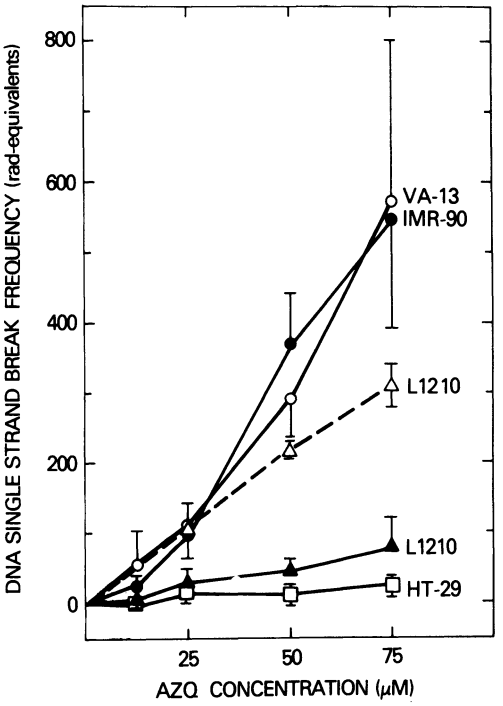


Figure 11. DNA stand breaks produced by one hour exposure of the indicated cell lines to AZQ. L1210 cells were treated either in Eagle's medium (△), which was used for all of the other cell lines, or in RPMI 1630 medium (△) which was otherwise used only to culture L1210 cells [44].

lesions in different cell types. For example, HT-29 human carcinoma cells exhibited large numbers of interstrand crosslinks but no detectable strand breaks (a small amount of strand breakage may have been obscured by the high crosslink frequency). On the other hand, IMR-90 and VA-13 human embryo cells exhibited high frequencies of strand breaks and relatively low (VA-13) or undetectable (IMR-90) interstrand crosslinking.

Cell killing by AZQ, measured by colony survival assays in the various cell lines, correlated well with interstrand crosslinking and not at all with the extent of strand scission (figure 12). These results suggest that DNA filter elution assays of interstrand crosslinking may be predictive of the sensitivity of tumor cell lines to AZQ.

Mechanisms of DNA lesion production studied in isolated nuclei

The mechanisms of AZQ-induced DNA strand scission and interstrand crosslinking were further investigated in isolated nuclei [45], in which membrane permeability limitations are avoided and the composition of the sol-

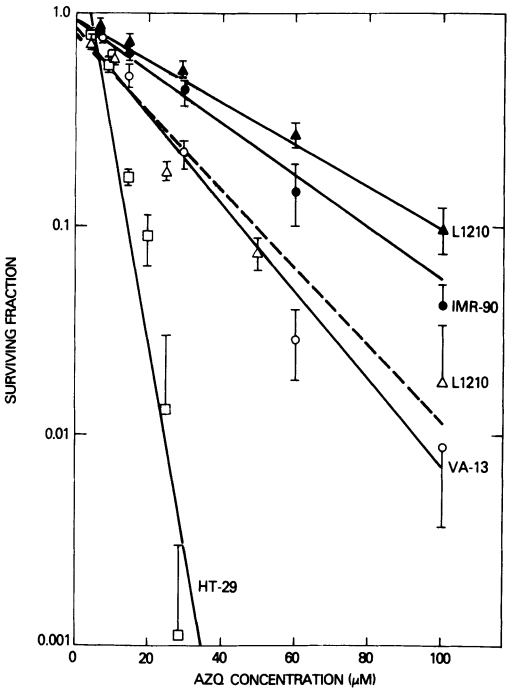


Figure 12. Survival of colony forming ability of the indicated cell lines after one hour treatment with various concentrations of AZQ. (L1210 cells were treated in two different media, as in figure 11.)

vent in contact with the DNA can be controlled. Isolated cell nuclei were incubated with drug under various solvent conditions and then assayed by DNA filter elution. AZQ by itself produced very little strand breakage or crosslinking in isolated nuclei. When a reducing agent such as NADPH was added together with AZQ, however, large numbers of DNA strand breaks appeared. The production of strand breaks was not enzyme mediated and was completely preventable by the addition of superoxide dismutase. Hence the strand breaks must be mediated by superoxide (O_2^-), generated by the transfer of an electron from a reduced form of AZQ (probably the semiquinone) to molecular oxygen (figure 9).

The lack of interstrand crosslinking by AZQ alone in isolated nuclei was unexpected. AZQ did, however, produce interstrand crosslinks in isolated nuclei when both a reducing agent and superoxide dismutase were added. These studies, as well as studies using purified DNA [45,47] indicated that reduced AZQ is a more active alkylating and crosslinking agent than AZQ itself. Thus AZQ and its derivatives emerge unexpectedly as possible bioreductive alkylating agents that may have increased cell killing activity under hypoxic conditions.

Platinum complexes

Platinum complexes have been a frequent subject of investigation by means of alkaline elution, because these clinically promising drugs produce DNA interstrand and DNA-protein crosslinks which may be important to their mechanism of action. One of the clearest and still incompletely understood generalizations about platinum complexes is the requirement for two dissociable ligands (such as chloride) in *cis* configuration in the planar Pt(II) structure. The corresponding *trans* isomers are much less cytotoxic and are devoid of antitumor activity. It was therefore puzzling that the *trans* complexes were at least as effective as the *cis* complexes in producing interstrand crosslinks in purified DNA [48, 49]. Filter elution assays, however, showed that in intact cells cisplatin produced clearly demonstrable interstrand crosslinking, while the *trans* complex produced little, if any, of these lesions [50]. Both the *cis* and the *trans* complexes produced prominent DNA-protein crosslinking. The *trans*-Pt(II) complex, in fact, is an excellent means of producing high frequencies of DNA-protein crosslinks, and cells can repair these lesions effectively. The low cytotoxicity of *trans*-Pt(II) argues against DNA-protein crosslinks per se as the major source of cisplatin cytotoxicity.

Cisplatin cytotoxicity has been found in several, but not all, studies to correlate with interstrand crosslinking. Although cisplatin interstrand crosslinks may be potentially lethal, crosslinks between neighboring guanines in the same strand are more frequent cisplatin products and may be the more prominent contributors to cell killing [51]. Nevertheless, the production of the two types of DNA-DNA crosslinks in cells may be governed by common factors, so that the available method of measurement of interstrand crosslinking may give an estimate of the formation of both types.

Relationship between cytotoxicity and DNA crosslinking

In a study of human cell lines, most of them derived from various tumors, Laurent et al. [52] found significant correlations between cytotoxicity and either interstrand or DNA-protein crosslinking. The correlation with interstrand crosslinking, which was better than with DNA-protein crosslinking, was excellent except for one deviant cell line (figure 13, open circle). The deviant line, derived from an ovarian carcinoma, exhibited unusually high cytotoxicity at relatively low crosslinking levels.

A possible reason for deviations between interstrand crosslinking and cytotoxicity is that some cell types may be unusually sensitive to a given frequency of DNA lesions and may undergo autolysis before the slow repair of the DNA lesions can be completed. Such behavior was observed by Ducore et al. [53] in a study of three Burkitt's lymphoma cell lines [figure 14A, table 1). Line BHM was about three times less sensitive to cisplatin than was line W-1, both in regard to cytotoxicity (cell proliferation or colony assays) and in regard to crosslinking (interstrand or total). Line BHM was

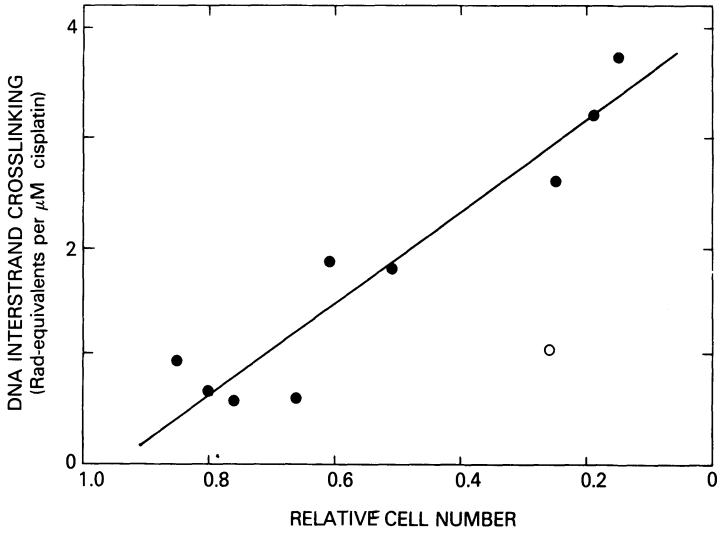


Figure 13. Correlation between DNA interstrand crosslinking and inhibition of cell proliferation in human cell lines treated with 10 or 12.5 μM cisplatin for two hours. Each point represents a different cell line. Interstrand crosslinking was determined six hours after treatment, with 10 or 20 μM cisplatin; results at the two doses were calculated as rad-equivalents per μM and averaged. Cell number relative to untreated cells was determined after three days incubation following treatment with 12.5 μM cisplatin [52].

also three to four times less sensitive than another line, WS, in regard to cytotoxicity, but there was little difference in crosslinking. Line WS was unusual in that the cells began to lyse 6–12 hours after treatment and began to spill DNA and protein into the medium. Evidence of this autolysis was obtained in DNA filter elution assays by the increased elution of fragmented DNA in the pH 10 detergent solution (figure 14B). Neither of the other cell lines showed this behavior, even at higher extents of cytotoxicity and crosslinking. Similar results were obtained with L-phenylalanine mustard, indicating that the WS cells have an increased tendency to lyse in response to chemically different types of DNA damage. Thus cells can have two types of vulnerability to DNA damaging drugs: (1) vulnerable cells may sustain an increased amount of damage and/or be deficient in the repair of damage; (2) vulnerable cells may be unable to maintain metabolic integrity in the presence of DNA damage for the time required for the damage to be repaired.

Relation to DNA repair

An important question regarding the mechanism of the antitumor action of cisplatin is whether some human tumor cell lines may be especially vulnerable to the drug on the basis of deficient repair of DNA crosslinks. Plooy et al. [54] recently reported that certain DNA repair-deficient human fibroblasts from Fanconi's anemia patients have an increased sensitivity to killing

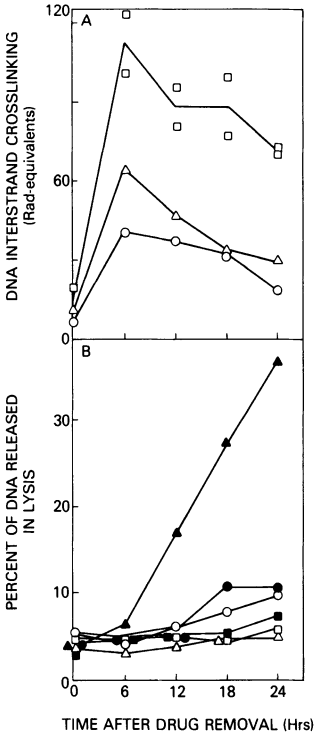


Figure 14. DNA filter elution comparisons among three lines of Burkitts lymphoma cells in response to treatment with 12 μM cisplatin for one hour. Cell lines: ○, ●, BHM; □, ■, W-1; △, ▲, WS. (All were negative for Epstein-Barr virus production or nuclear antigen.) A) Crosslinking at various times after drug removal. B) Release of fragmented DNA in the sodium dodecyl sulfate lysis solution. *Open symbols*, untreated cells. *Filled symbols*, cisplatin-treated cells [53].

Table 1. Relative effects of cisplatin on different lines of Burkitt's lymphoma cells.

	Cytotoxicity		DNA Crosslinking	
Cell Pair	Proliferation Rate	Colony Formation	Total	Interstrand
BHM/W-1	2.8	3.5	3.6 (2.8–4.4)	2.9 (2.4–3.6)
BHM/WS	2.6	4.1	1.5 (1.2–2.1)	1.3 (1.0–1.6)

The values are cisplatin concentration ratios for equal effects on a pair of cell lines being compared. The DNA crosslinking values are shown as the reciprocal in order to facilitate comparison with the cytotoxicity values. DNA crosslinking was determined 6, 12, 18, and 24 hours after treatment for one hr with 12 μM cisplatin (the values shown are mean and range of these determinations). (From data of Ducore et al. [53].)

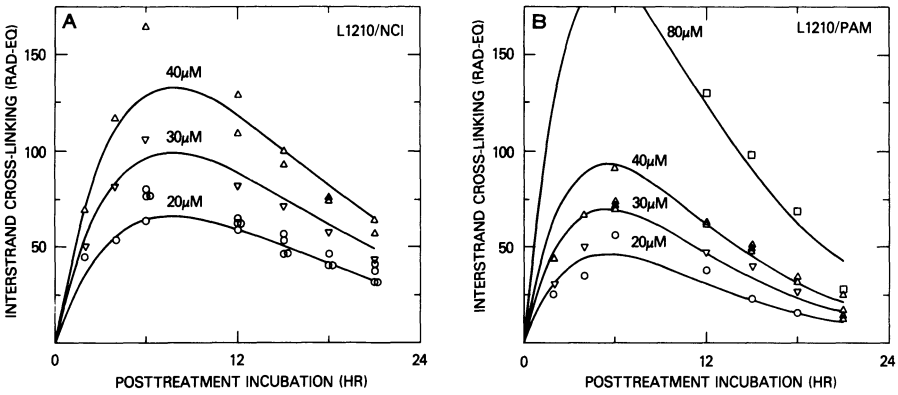


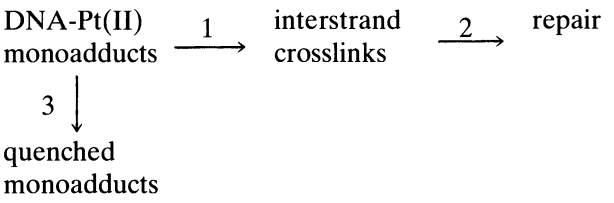
Figure 15. Kinetics of DNA interstrand crosslink formation and removal in parental (L1210/NCI) and resistant (L1210/PAM) cells treated with the indicated concentrations of cisplatin for one hour. Curves were computed for best fit to an equation based on model (A) which assumes that the resistant line quenches monoadducts at an increased rate [57].

by cisplatin and appear to be incapable of removing cisplatin interstrand crosslinks.

In a study of cisplatin-resistant mouse leukemia L1210 cells, Strandberg et al. [55] could not completely account for the degree of resistance on the basis of reduced interstrand or DNA-protein crosslinking and proposed a longer persistence of crosslinks in the more sensitive cells.

Although more studies are needed on human cells, evidence for rodent cells has shown an association between crosslinking and cytotoxicity. Meyn et al. [56] studied a mutant line of Chinese hamster cells that had been selected for increased sensitivity to ultraviolet light. The mutant cells exhibited a remarkable 70 fold increase in sensitivity to cisplatin. The mutant and parental cells formed crosslinks at the same rates during one hour of exposure to cisplatin. (The nonproteinased filter elution assay was used, which measures the combination of interstrand and DNA-protein crosslinks.) Upon further incubation in the absence of drug for up to 24 hours, the parental cells removed most of the crosslinking, whereas the mutant cells removed very little. Similar results were obtained with mitomycin C; hence the repair mechanism normally can handle chemically different crosslinks.

A different mechanism was reported by Micetich et al. [57] in a study of a cisplatin-resistant line of mouse leukemia L1210 cells that seemed to have a reduced rate of interstrand crosslink repair. The resistance could not be entirely explained on the basis of altered extent of crosslinking. The kinetics of formation and removal of interstrand crosslinks (figure 15) were therefore analysed on the basis of the following kinetic model which portrays changes in interstrand crosslink level as the net effect of new crosslink formation (reaction 1) and crosslink removal (reaction 2).



The rate constant for interstrand crosslink repair was measured after inhibition of the conversion monoadducts to interstrand crosslinks by means of thiourea and did not differ significantly between the two cell lines. The observed kinetics (figure 15) could be explained on the basis of an assumed enhanced ability of the resistant cells to inactivate or 'quench' cisplatin-DNA monoadduct (reaction 3) before their conversion to interstrand crosslinks. Monoadducts could be quenched by reaction with sulfhydryl compounds such as glutathione, which was found to be elevated in this resistant cell line [58, 59].

Topoisomerases

Introduction: The concept of protein associated DNA strand breaks (PASB)

DNA filter elution experiments provided the first clues to DNA topoisomerases as possible targets of anticancer drug action. In testing the effect of adriamycin on mouse leukemia L1210 cells, Ross et al. [60] noted a puzzling absence of increased DNA alkaline elution under conditions in which the drug had produced DNA strand breaks as measured by the alkaline sucrose gradient sedimentation technique. The absence of increased DNA alkaline elution was puzzling because all previously studied agents that produced DNA strand breaks in cells, as indicated by alkaline sedimentation (including ionizing radiation, bleomycin, ultraviolet light, nitrosoureas, and related compounds), had exhibited large increases in DNA alkaline elution (reviewed in Kohn [2]). However, the polyvinyl chloride filters that were commonly used at that time in the alkaline elution assays were known to absorb proteins and to retain DNA that is covalently linked to proteins [61]. Such protein-linked DNA could be released from the filters by means of proteinase K [10], and the frequency of the DNA-protein crosslinks could be quantitated [11]. When the proteinase K assay was applied in the adriamycin experiments, large dose-dependent increases in DNA alkaline elution were observed [60]. It was concluded, therefore, that the drug produced a large number of DNA strand breaks, but that the breaks were not observed at first because of simultaneously produced DNA-protein crosslinks that prevented the DNA fragments from eluting from protein-absorbing filters.

One possible explanation was that the strand breaks and DNA-protein

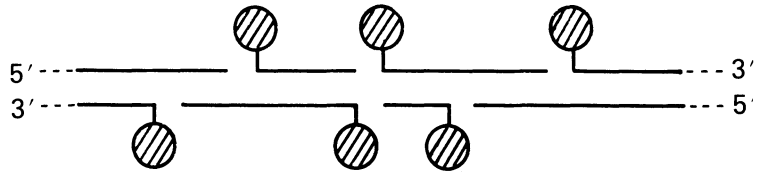


Figure 16. The protein-associated DNA strand break (PASB) model. The model accounts for the evidence that all DNA single-strand fragments in the treated cells are covalently linked to protein and that the number of single-strand breaks equals the number of DNA-protein links. The horizontal lines represent a pair of DNA single-strands in a double helix. Three breaks are shown in each strand, with protein molecules (circles) linked to the 5′ strand termini. The central breaks are depicted as a double-strand break; in this case the two linked proteins could actually be parts of the same protein molecule.

crosslinks were the result of two independent drug effects and were independently distributed along the DNA. If this were so, then the DNA-protein crosslinks would have to be in large excess over the strand breaks. Otherwise some DNA fragments should by chance have escaped protein crosslinking and, therefore, should have eluted even without the use of proteinase K. Upon quantitation of the frequencies of the single-strand breaks and DNA-protein crosslinks, however, the two lesions were found in approximately equal quantities [62]. Similar results were obtained with several structurally dissimilar DNA intercalating agents.

The only simple way to account for these results was to assume that the strand breaks and DNA-protein crosslinks were associated in some way, so that there was one linked protein between every pair of successive single-strand breaks [62]. The simplest way for this to occur would be for a protein to become linked at each strand break, consistently either to the 3′ or to the 5′ termini (figure 16). The protein could then be an enzyme that produces the strand break and becomes covalently linked to one terminus of the break. Such behavior was known to occur for DNA nicking-closing enzymes, now called DNA topoisomerases. Based on this reasoning it was proposed that the protein-associated DNA strand breaks induced by intercalating agents involved the action of a nicking-closing enzyme or DNA topoisomerase [62].

Recent work has supported this idea and has indicated that the assay of protein-associated strand breaks (PASB) by the DNA filter elution technique is a measure of altered topoisomerase II function in cells treated with intercalating agents and epipodophyllotoxins [63–70]. In addition, recent results suggest that a similar action is produced by camptothecin on topoisomerase I.

Kinetics

DNA filter elution experiments provided support for the hypothesis that protein-associated strand breaks (PASB) are produced as a consequence of

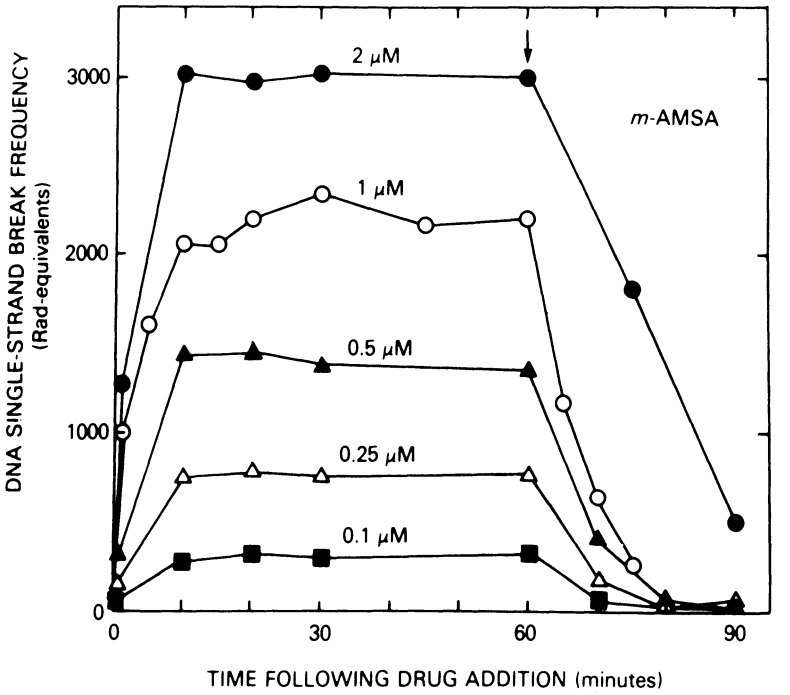


Figure 17. kinetics of the formation and reversal of PASB in L1210 cells exposed to various concentrations of m-AMSA for 60 minutes at 37° [71]. The data shown are for assays of DNA signle-strand breaks (alkaline elution at pH 12.1 using proteinase K). Similar kinetics were observed in assays of DNA-protein crosslinks and of double-strand breaks. Drug was washed away after 60 min (arrow).

the action of a nuclear enzyme. The experimental technique allowed several lines of evidence to be obtained, the earliest of which came from studies of the kinetics and temperature dependence of the formation and reversal of PASB in cells. The first detailed studies were of the action of m-AMSA on mouse leukemia L1210 cells [71]. After addition of m-AMSA to a culture of L1210 cells at 37°, PASB increased in number for 10 minutes and then remained at a constant level for at least one hour (figure 17). This was not due to loss of potency or sequestering of the drug, because when the drug was washed away the PASB declined and essentially disappeared in 20 minutes. The plateau level of PASB increased in proportion to drug concentration, except that at high concentrations there appeared to be a saturation effect, as if a maximum attainable PASB level was being approached. This maximum number was estimated as 6×10^4 PASB per cell and might represent a limit in the number of available molecules of an essential protein or enzyme.

Similar kinetic results were obtained with the anthracycline, 5-iminodaunorubicin (5-ID) and with 2-methyl-9-hydroxyellipticinium (2-Me-9-OH-E+) [72,73]. These are DNA intercalating agents that are structurally

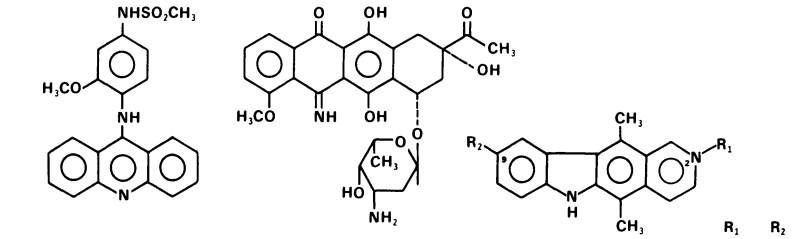


Figure 18. Structure of DNA intercalators that produce protein-associated strand breaks (PASB) in mammalian cells. These compounds were selected for study because of the reversibility of their effects on DNA cells.

dissimilar from each other and from m-AMSA (figure 18). Adriamycin, also an intercalator, on the other hand, exhibited much slower kinetics of PASB formation and reversal due to limited rates of the transport of this drug into and out of the cells [71].

Double-strand breaks

In all cases so far studied, at least some of the PASB are in the form of double-strand breaks [71, 72, 73]. The kinetics of the formation and reversal of the double-strand breaks appear to be identical to the kinetics for single-strand breaks. The ratio of single-to double-strand breaks, however, varies greatly among different drugs (table 2). m-AMSA was found to produce mainly single-strand breaks, whereas 5-iminodaunorubicin and the ellipticines produced mainly double-strand breaks.

The DNA filter elution measurements give single- and double-strand break frequencies relative to calibration assays using x-irradiated cells. The estimates of absolute (as opposed to relative) break frequencies depend upon available break frequency data for x-irradiated cells, obtained by sucrose gradient sedimentation measurement. The ratio of intrinsic single to double-strand breaks can be estimated from DNA filter elution data by the formula

$$\frac{s}{d} = \frac{k_{RS}}{k_{RB}} \frac{[SSB]}{[DSB]} - 2$$

where [SSB] and [DSB] are the measured break frequencies in rad-equivalents (i.e., in terms of the x-ray doses that produced, in intact L1210 cells used as internal standards, the same increases in DNA elution rate as did the drug treatment in the single- and double-strand break assays, respectively). The ratio, k_{RS}/k_{RB}, is the number of single-strand breaks per double-strand break produced by x-ray and is derived from published sedimentation data. The calculated value, s/d, is the ratio of intrinsic single- to

Table 2. Ratio of intrinsic single to double-strand breaks (s/d) produced by DNA intercalating drugs in L1210 cells or isolated nuclei.

Drug	Concentrations[a]	Systems	[SSB/DSB][b]	s/d		Reference
				k′ = 10	k′ = 40[c]	
	(μM)					
m-AMSA	0.5–5.0	cells	0.53 ± 0.09(20)[e]	3.3 ± 0.9	19.2 ± 3.6	Zwelling et al. [71]
m-AMSA	1.0–2.0	nuclei	0.36 ± 0.14(10)	1.6 ± 1.4	12.4 ± 5.6	Pommier et al. [74]
Adriamycin	2.0–8.0	cells	0.20 ± 0.04(19)	0.0 ± 0.4	6.0 ± 1.6	Zwelling et al. [71]
5-ID[d]	0.5–2.5	cells	0.12 ± 0.03(12)	−0.8 ± 0.3	2.8 ± 1.2	Zwelling et al. [73]
5-ID	2.5–5.0	nuclei	0.059 ± 0.005(7)	−1.41 ± 0.05	0.36 ± 0.20	Pommier et al. [74]
2-Me-9-OH-E$^+$	5–20	cells	0.11 ± 0.03(14)	−0.9 ± 0.3	2.4 ± 1.2	Zwelling et al. [73]
Ellipticine	5–20	cells	0.10 ± 0.05(12)	−1.0 ± 0.5	2.0 ± 2.0	Zwelling et al. [73]
Ellipticine	2.5–10.0	nuclei	0.047 ± 0.012(10)	−1.53 ± 0.12	−0.12 ± 0.48	Pommier et al. [75]

[a] Treatments were for 0.5–1.0 hours at 37° C.

[b] [SSB], radiation dose giving equivalent elution in the DNA filter elution assay of single-strand breaks; [DSB], similarly for the double-strand break assay.

[c] $k' = k_{RS}/k_{RD}$, the ratio of single-to double-strand breaks produced by ionizing radiation, is used in the formula given in the text to calculate the ratio of intrinsic single-to double-strand breaks (s/d). The value of k′ is not well established; experimental values have been reported ranging from k′ = 10 to k′ = 40. The values for s/d using k′ = 40 are more likely to be near the correct values than are those using k′ = 10, because of the negative values obtained using the latter.

[d] Abbreviations: 5-ID, 5-iminodaunorubicin; 2-Me-9-OH-E+, 2-methyl-9-hydroxyellipticinium.

[e] Mean ± SD (number of determinations).

double-strand breaks (the two single-strand breaks which make up each double-strand break are excluded from the estimate of intrinsic single-strand break frequency, *s*).

DNA filter elution assays applied to isolated nuclei

In studies of drug effects on isolated nuclei, it is possible to control the composition of the solvent to which the DNA is exposed and thus to avoid membrane permeability limitations and the uncertainties of intracellular fluid composition. Experimental conditions to permit DNA filter elution studies of isolated nuclei without excessive DNA breakdown were first devised by Filipski et al. [63, 76] and further developed by Pommier et al. [74, 75]. The effects of intercalating agents in producing PASB in intact cells were reproduced in isolated nuclei. Hence the essential components for the production of this effect reside in the nucleus.

The production of PASB in response to DNA intercalating agents was found to require the presence of Mg^{++}, to be inhibited by 0.4 M NaCl or pH above 7.0, and to be stimulated by ATP as well as by nonhydrolysable ATP analogs. The latter finding indicated that the responsible enzyme has an ATP site which must be occupied for maximum PASB production, but that ATP hydrolysis is not required [74, 75]. In addition, PASB production was found to be partially inhibited by inhibitors of prokaryotic topoisomerase II: novobiocin, nalidixic acid, and norfloxacin.

Self-inhibitory drug effects

Aside from the differences among intercalators noted above in the single-to double-strand break ratios, there are marked differences in concentration dependence. The differences in the dependence on drug concentration were studied in isolated nuclei so that the concentration of drug in contact with the target was known. It was found that some intercalators, notably 2-methyl-9-hydroxyellipticinium, have an inhibitory as well as a stimulatory phase of PASB action (figure 19). At low concentrations this drug stimulated the production of PASB, but at high concentrations, this effect was inhibited. The inhibitory concentrations of this drug also inhibited PASB production by m-AMSA, which by itself did not show an inhibitory phase. Furthermore, the inhibitory effect of the ellipticinium compound could be reversed by adding high concentrations of m-AMSA. This behavior suggests that two types of drug-enzyme-DNA complexes can be formed and reversibly interconverted depending on the type and concentration of drug. At low drug concentrations, the PASB complexes may contain a single drug molecule. At high concentrations, some drugs may form a different type of complex containing two or more drug molecules and not involving a PASB structure. The function of the enzyme may be inhibited in either state.

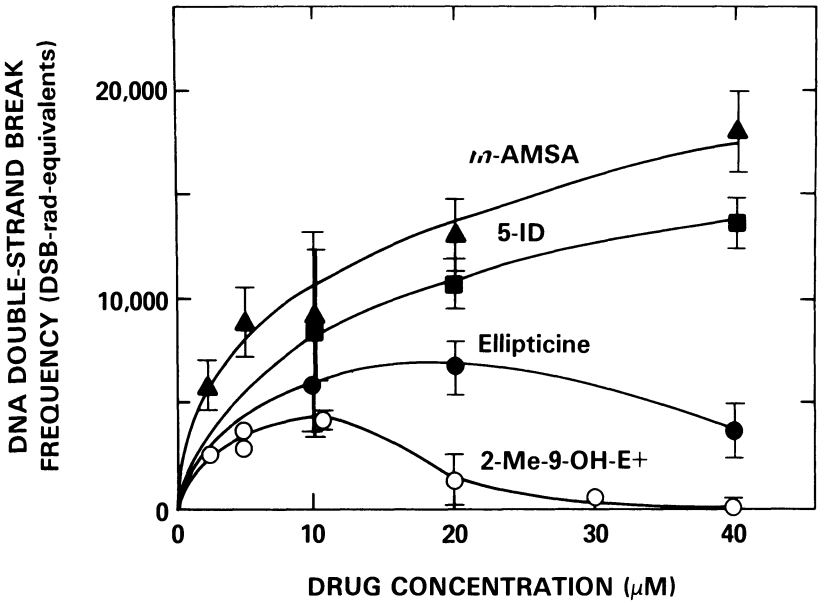


Figure 19. Concentration-dependance of protein-associated double-strand breaks produced by various DNA intercalators in isolated L1210 cell nuclei. Isolated nuclei were exposed to drug for 30 minutes at 37° [74].

Relation of PASB to effects on topoisomerases

The equivalence between single-strand breaks and DNA-protein crosslink frequencies and the apparent localization between the breaks and crosslinks consistently observed with a variety of structurally different DNA intercalating drugs led to the proposal that these effects could be the result of the action of DNA topoisomerase enzymes [62]. It was at first thought that the drug-induced single-strand breaks were derived from the action of a topoisomerase I, whereas the double-strand breaks were derived from the action of a topoisomerase II [71]. It was supposed that different drugs would affect the two enzymes differently, thereby accounting for the observed differences among drugs in the induced single- to double-strand breaks ratios. Subsequent work with the purified mammalian enzymes, however, has shown that DNA intercalators act on toposiomerase II (but not topoisomerase I) to produce protein-linked complexes and that the DNA in the complex can have either a single- or a double-strand break [65, 66, 67]. Thus the single- and double-strand breaks appear to be different forms of trapped topoisomerase II complexes, and their ratio may depend on the geometry of the intercalation.

Epipodophyllotoxins

Early studies had suggested that the PASB response was a characteristic of DNA intercalating agents [62]. It is, therefore, of great interest that an entirely different class of drugs, the epipodophyllotoxins, etoposide (VP-16) and teniposide (VM-26), which do not bind to DNA, produce the same characteristic PASB effects in mammalian cells and isolated nuclei [68, 77, 78]. These compounds, like the intercalators, have been found to produce protein-linked DNA strand breaks in a purified system of DNA and topoisomerase II [70]. It seems likely that the PASB induced by epipodophyllotoxins result from binding to specific enzyme sites, whereas the effects of intercalators may be due largely to DNA binding.

Relation of PASB to cell killing

A striking structure-activity dependence for cell killing is seen in the isomers, m-AMSA and o-AMSA. These two compound differ only in the *meta* or *ortho* location of a methoxy group and have equal abilities to bind intercalatively to pure DNA [79]. The *ortho* isomer, however, is much less cytotoxic and has no antitumor activity. In accord with this difference, o-AMSA is much less effective in producing PASB In cultured cells [71]. o-AMSA also differs from m-AMSA in not trapping protein-linked strand break complexes of DNA with purified topoisomerase II [65, 66]. Thus for these two intercalating isomers, the action on the purified enzyme and formation of PASB in intact cells correlate strikingly with each other and with the different biological effectiveness.

In order to determine whether PASB production could quantitatively account for cell killing, three structurally different intercalators were compared (figure 18, 20). The compounds utilized — m-AMSA, 5-iminodaunorubicin, and 2-methyl-9-hydroxyellipticinium — showed reversible PASB, and hence permitted valid comparison between one hour drug treatments and the prolonged assay of colony formation. If cell killing were simply a function of number of PASB (either of the single- or double-strand type), then a coincidence of curves should have been observed in one or the other panel of figure 20. The discrepancies between the curves in both panels, however, indicate that such a simple correspondence does not exist, at least for compounds of widely different structural types.

A recent study of a multidrug-resistant line of Chinese hamster cells, however, indicates a relationship between cell killing and PASB [80]. This highly resistant line exhibited reduced uptake of several drugs, although not of m-AMSA. In order to obviate the difficulties in interpretation due to uncertain drug uptake, the studies were carried out using isolated nuclei. Nuclei from resistant cells exhibited fewer PASB than did the parental cells in response to m-AMSA, 2-methyl-9-hydroxyellipticinium, and etoposide

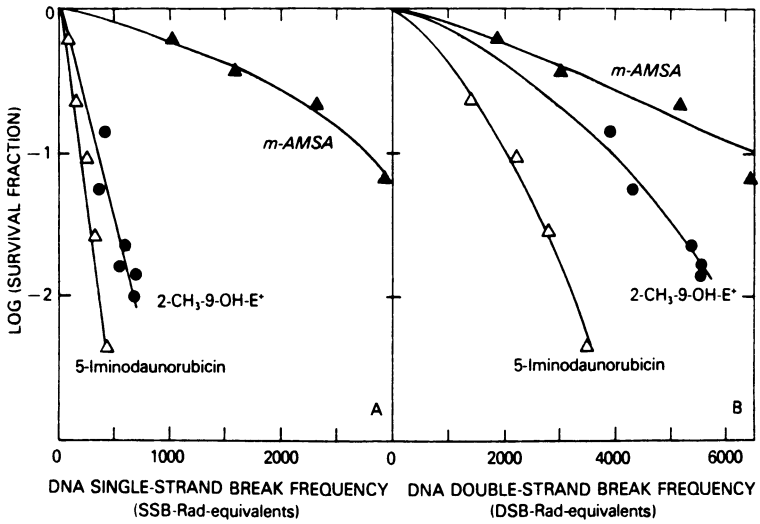


Figure 20. Relationship between cell killing and the production of either single-strand (left) or double-strand (right) protein-associated DNA breaks by structurally different intercalators. L1210 cells was treated for one hour with various concentrations of m-AMSA (▲), 5-iminodaunorubicin (△) or 2-methyl-9-hydroxyellipticinium (●). DNA single and double-strand breaks were assayed by filter elution at pH 12.1 or pH 9.6, respectively, using proteinase-K to eliminate DNA-linked proteins. Cell survival was assayed by colony formation in soft agar [73].

(VP-16). The results indicate that an alteration in PASB, probably reflecting an altered topoisomerase response, can contribute to drug resistance.

Conclusion

The DNA filter elution techniques can be used to measure several types of DNA damage in mammalian cells, including single- and double-strand breaks, alkali-labile sites, interstrand crosslinks, and DNA-protein cross-links. The assays are often sensitive enough to measure the effects of DNA-damaging anticancer drugs in the pharmacologic dose range. One can then evaluate the significance of particular types of DNA lesions in the production of biological effects such as cell killing and mutagenesis. By comparing effects on several cell strains having different drug sensitivities, evidence can be obtained on the possible importance of particular types of DNA lesions in selective cell killing and on the biochemical mechanisms that determine selective cell killing.

In studies of chloroethylnitrosoureas and other chloroethylating agents, it was found that the sensitivity of different human tumor cell strains correlates with the production of DNA interstrand crosslinking, the extent of which is greatly influenced by differences in the activity of a specific DNA

repair enzyme, guanine-06-alkyltransferase. Thus it may be possible to predict the sensitivity of individual tumors to these drugs by assay of the activity of this enzyme in biopsy specimens. Chloroethylnitrosoureas probably should be used only in the treatment of tumors that are deficient in this enzyme activity and thereby potentially vulnerable to these highly toxic drugs. The further development of DNA chloroethylating drugs should be aimed at optimizing their effectiveness against tumors that have the characteristic (e.g., low guanine-06-alkyltransferase activity) that would make them vulnerable to these drugs.

The carbamoylating activity of the clinically used nitrosoureas, BCNU and CCNU, was found to inhibit the repair of DNA strand breaks. This action is unrelated to the antitumor activity, because chloroethylnitrosoureas that lack carbamoylating activity and do not inhibit DNA repair still retain undiminished antitumor activity in preclinical systems, and they retain the selective DNA crosslinking effects on guanine-06-alkyltransferase deficient cells. It seems highly desirable to eliminate unnecessary carbamoylating activity from drugs in clinical use, especially when these drugs are combined with other DNA damaging drugs.

Studies of diaziridinylbenzoquinone (diaziquone, AZQ) showed that the drug produced two independent types of DNA damage: crosslinks and strand breaks. The production of the two types of DNA damage varied greatly in different human cell lines. One line exhibited predominantly or exclusively crosslinks. Another exhibited mainly strand breaks. Cell killing correlated with crosslinking and not with strand breakage. DNA filter elution studies of isolated cell nuclei indicated that the production of strand breaks, but not of crosslinks, was mediated by superoxide generated in redox reactions of the quinone. Crosslinks, presumably formed by bifunctional reactions of the two aziridine groups, however, were enhanced by reduction of the quinone, suggesting that this class of drugs could be subject to bioreductive activation. Since the strand breakage does not seem to be required for cell killing, it would be prudent in further development of this class of drugs to aim for compounds that produce crosslinks but not strand breaks.

Studies of the effects of cisplatin indicated that the sensitivity of cell lines often correlates with DNA crosslink production. In some cases increased sensitivity was associated with a reduced rate of repair of interstrand crosslinks or DNA-protein crosslinks. There are exceptions however. One such exception, a cell line that was more sensitive than expected from the amount of DNA crosslinking, differed from related cell lines in that the cells tended to undergo lysis before DNA crosslinks could be repaired. It may be that a tendency to early cell lysis is a distinct drug sensitivity characteristic that would make cells vulnerable to treatment with DNA damaging drugs.

DNA filter elution studies of the effects of DNA intercalating agents led to the identification of DNA topoisomerase enzymes as possible targets of anticancer drug action. The work of W.E. Ross, B. Long, L. Liu, and their

coworkers has led to the discovery that epipodophyllotoxins, as well as DNA intercalating agents, act on topoisomerase II and, very recently, that camptothecin acts on topoisomerase I. The DNA filter elution measurement of protein-associated DNA strand breaks provides an assay of drug effects on topoisomerases in mammalian cells. Recent studies of resistant cell lines indicate that alterations in topoisomerases can affect drug sensitivity.

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2. Intraperitoneal chemotherapy: Principles and results of clinical trials

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While the intraperitoneal administration of chemotherapeutic agents for their cytotoxic rather than sclerosing properties is not a new idea [1,2], recent modelling studies, suggesting a pharmacokinetic rationale for this method of drug delivery to tumors principally confined to the peritoneal cavity, has renewed interest in this idea [3]. In this review the basic principles of intraperitoneal chemotherapy will be presented along with a discussion of the theoretical and practical problems associated with the technique. Finally, a summary of recent single agent and combination intraperitoneal chemotherapy trials will be presented as well as a brief discussion of other innovative approaches utilizing the intraperitoneal route.

Pharmacologic principles of intraperitoneal therapy

Conceptually, it is perhaps easiest to view the body of a patient with a tumor confined to the peritoneal cavity as consisting of the cavity itself and the remainder of the body (two compartment model) [3]. If the drug is placed into the peritoneal cavity it will exit the cavity at a rate defined by various characteristics of the drug and of the cavity itself. Similarly, the drug will be cleared from the systemic circulation by several mechanisms, principally urinary excretion and hepatic metabolism. The more slowly a drug leaves the peritoneal cavity and the more rapidly it is cleared from the plasma, the greater will be the difference in exposure of the cavity to active drug compared to that of the systemic circulation. This difference can be expressed as the ratio of the area-under-the-concentration-versus-time curve (AUC) for the two compartments. If it were possible to manipulate the characteristics of the drug such that egress from the cavity were slowed or systemic clearance enhanced, then the ratio of the AUCs would increase further.

An important observation specifically related to the mechanism of clearance of drugs from the peritoneal cavity provides one of the strongest rationales in support of the intraperitoneal approach to the treatment of malignant disease confined to the abdominal cavity. Drugs instilled into the peritoneal cavity can theoretically enter the systemic circulation by one of

three routes, uptake into the capillaries of the parietal peritoneum leading directly into the systemic circulation, entry into the portal circulation with access to the systemic circulation following passage through the liver, or uptake into the peripheral lymphatics with entry into the peripheral venous circulation via the thoracic duct. Limited experimental evaluation of this important issue, as well as knowledge of the anatomy of the peritoneal cavity, would suggest that a major portion of drug instilled into the peritoneal cavity will leave the cavity by way of the portal circulation [4–7]. Thus, it is possible that agents which are metabolized in the liver might be converted into nontoxic metabolites prior to entry into the systemic circulation. For a chemotherapeutic agent like 5-fluorouracil or cytarabine, a major pharmacokinetic advantage for exposure of the peritoneal cavity compared to that of the systemic circulation might result from direct intraperitoneal drug administration [3].

Unfortunately, there is limited experimental data available on the factors which control absorption of drugs from the peritoneal cavity. Not surprisingly, it has been demonstrated that water soluble compounds and molecules with high molecular weights are absorbed more slowly than lipid soluble and smaller molecules [8]. Investigators at the National Cancer Institute have examined the absorption of several chemotherapeutic agents instilled into the peritoneal cavity in an animal model [9]. The percentage of drug leaving the cavity varied widely from as low as 9% for L-asparaginase to as high as 92% for hexamethylmelamine. A second observation in this study was that the type of fluid that the chemotherapeutic agent was administered in influenced the rate of absorption. This finding has potential relevance for clinical studies of intraperitoneal therapy, where several readily available choices for solutions to deliver the therapy currently exist. In the only study in man to examine the issue of the route of drug clearance from the peritoneal cavity, investigators at the National Cancer Institute found that from 29% to 100% of intraperitoneally delivered 5-fluorouracil left the cavity by way of the portal circulation [10]. One must be cautious in over-interpreting this report, however, as only four patients were evaluated in this difficult and detailed pharmacokinetic study.

A final theoretically important advantage of intraperitoneal chemotherapy is the opportunity to use systemically delivered neutralizing agents concurrently with intraperitoneal instillation of cytotoxic drugs. If one could select a neutralizing agent which would interact with the cytotoxic drug upon entry into the systemic circulation and thus prevent toxicity, it might be possible to further increase the amount of the antineoplastic drug delivered into the peritoneal cavity. Unfortunately, any of the neutralizing agent which enters the peritoneal cavity will also be capable of inactivating the cytotoxic drug in the cavity. It would therefore be extremely important, if one wishes to design an intracavitary program employing a specific neutralizing drug, to select an agent whose ability to inactivate the cytotoxic agent was highly concentration-dependent. Thus, while the cytotoxic drug entering

the systemic circulation would be inactivated by the higher concentration of the neutralizing drug, the antagonist leaking into the cavity would only neutralize a limited quantity of the cytotoxic drug present in the cavity, where the latter agent will be present in significantly higher concentrations. The safety and efficacy of this therapeutic approach has been demonstrated for two cytotoxic-neutralizing agent pairs (methotrexate-folinic acid [11, 12] and cisplatin-thiosulfate [13, 14]). Details of several treatment programs employing these drug combinations will be presented in a subsequent section.

Theoretical and practical problems associated with intraperitoneal therapy

Perhaps the major theoretical issue raised by intraperitoneal therapy is that of the ability of chemotherapeutic agents to penetrate into tumor nodules present in the abdominal cavity. If the intraperitoneal approach to the treatment of tumors confined to the peritoneal cavity is to be superior to the systemic administration of chemotherapy (and delivery of drug to tumor by capillary flow), then the added benefit will be secondary to free surface diffusion into tumor and direct uptake of the drugs into the malignant cells. Unfortunately, the experimental data currently available would suggest that the ability of chemotherapeutic agents to penetrate into tumor spheroids is quite limited [15, 16]. While technical considerations make this most important issue difficult to approach, investigators at the National Cancer Institute examining the distribution of intraperitoneally administered doxorubicin in a transplantable tumor model in the mouse made a most interesting observation which might be quite relevant to the clinical application of intraperitoneal chemotherapy [17–19]. As doxorubicin possesses intrinsic flourescence, it is possible to analyze tissue distribution and depth of penetration of the drug following therapy [20, 21]. With intravenous treatment faint doxorubicin-specific fluorescence was found throughout the body of the animal as well as in tumor tissue. Following intraperitoneal therapy a dense pattern was demonstrated only in the uppermost five to six cell layers of the tumor. Despite the apparent limited penetration of doxorubicin, 70% of mice treated by the intraperitoneal route were long-term survivors, while all of the mice treated intravenously died from the implanted tumor [18].

It is important to note that while the major therapeutic benefit to be achieved by the intraperitoneal treatment approach will be from free surface diffusion of the drug into the tumor, chemotherapeutic agents delivered into the abdominal cavity will enter the systemic circulation and reach the tumor by capillary flow. In fact, if dose-limiting toxicity of a particular agent is systemic rather than local (chemical peritonitis), then the amount of drug administered intraperitoneally can be escalated to the point where systemic toxicity will prevent further increases in dose. To produce this toxicity, as much drug will have reached the systemic circulation (and tumor) as if the

agent had been administered intravenously. Drug whose dose-limiting toxicity is local must rely more on free surface diffusion for their therapeutic effects, as the amount of drug reaching the plasma following intraperitoneal therapy will be less than if the drug were delivered systemically.

A second major issue in intraperitoneal therapy is that of drug distribution. Delivering drugs into the peritoneal cavity does not by itself insure that the tumor will be exposed to the agent. There are several reasons why patients with intra-abdominally localized malignancies may have difficulty in achieving adequate drug distribution when chemotherapeutic agents are delivered directly into the peritoneal cavity. Most patients considered for this treatment approach will have undergone one or more laparotomies with resultant adhesion formation. Similarly, the presence of tumor itself can elicit an intense fibrous tissue reaction, and inflammation secondary to the intraperitoneal therapy can further increase adhesion formation. Finally, tumor masses can also prevent access to small or large portions of the abdominal cavity. Experience with the intraperitoneal administration of radioisotopes in small treatment volumes has confirmed the difficulty of achieving adequate distribution in patients with intra-abdominal malignancies [22–24].

A major principle in the current use of intraperitoneal chemotherapy is that of the administration of drug in *large* treatment volumes. While first being demonstrated in an animal model [25], the ability of large volumes to improve the distribution of intraperitoneally delivered fluid has now been shown in man where approximately 70% to 80% of patients with even bulky intra-abdominal tumors are able to achieve adequate drug distribution when a two liter treatment volume is employed [13, 26].

An essential component of a successful intraperitoneal chemotherapy program is the development of a safe and effective treatment delivery system. There are two basic approaches to establishing access to the abdominal cavity. First, patients can be treated by the percutaneous placement of a peritoneal dialysis catheter or temporary device with each treatment course. This technique allows the patient to avoid placement of a more permanent delivery system. In addition, patients will not be constantly reminded of the tumor and therapy by the presence of an implanted device and will not have the associated infectious risks from the presence of a chronically indwelling foreign body. Unfortunately, blind catheter placement in patients who have undergone previous laparotomies and who may have significant adhesions is potentially associated with major morbidity from bowel perforation [27]. In individuals who have bulky intra-abdominal tumor, or where it is believed that adhesions might present a problem, percutaneous catheter insertion under ultrasonic guidance may be associated with less risk to the patient. An additional problem with temporary catheter placement with each treatment cycle is that with any given insertion a loculated pocket may be entered and one would theoretically need to evaluate the adequacy of drug distribution prior to each treatment course.

As an alternative to percutaneous catheter placement, it is possible to surgically implant a semipermanent indwelling catheter to deliver the intraperitoneal treatment [28–31]. The indwelling systems are of two general types. The standard Tenckhoff catheter used for peritoneal dialysis is brought out through the skin, while the more recently available portal systems are completely subcutaneous with access provided by the placement of a needle (Huber-type) with each catheter manipulation. The major practical advantage of the portal system is that catheter manipulation is reduced, and there is the theoretical advantage of decreased risk of infection with the subcutaneous localization of the entire delivery system. Finally, patient acceptance of the portal systems is, in our experience, superior to that of the standard Tenckhoff catheter. Following catheter placement, the distribution of fluid administered through the catheter should be evaluated to be certain the catheter is not confined to a loculated pocket.

The development of infectious peritonitis is a major potential complication of intraperitoneal chemotherapy [27]. While the risk of bowel perforation is less when treatment is administered through a surgically-placed indwelling catheter, the presence of this foreign body in a patient who requires frequent catheter manipulations will place the patient at risk for an infectious event. This fact is emphasized by our experience administering cytarabine by dialysis exchange every six hours for five days (total of 20 exchanges) [32]. Nine episodes of bacterial peritonitis developed in the ten patients participating in this trial. The incidence of infection is considerably reduced when less frequent catheter manipulations are required (treatment on a weekly or monthly schedule). Also as greater experience is gained in applying this innovative therapeutic technique, the incidence of infection should decrease.

In our experience the most common organisms responsible for infection are *Staphylococcus epidermidis* (70%) followed by *Staphylococcus aureus* (20%). This experience is similar to that reported for patients receiving peritoneal dialysis for chronic renal failure and supports the hypothesis that infection is introduced by contamination from the skin [33–36]. A cephalosporin active against *Staphylococcus epidermidis* should be employed in cases of suspected catheter-related bacterial peritonitis. However, if bowel perforation is suspected, broader spectrum coverage is indicated. Vancomycin can also be employed, as it is extremely active against *Staphylococcus epidermidis*, but this agent is quite expensive [35]. If a patient has persistent positive cultures or fever, or if an obvious tunnel infection is present, the catheter must be removed [27].

Peritonitis can also be due to chemicals and be quite severe. Inflammation secondary to drugs can be clinically silent and lead to fibrous tissue deposition and adhesion formation, or can be so serious as to cause severe pain or a profound ileus. Also, while certain drugs, such as doxorubicin, can be delivered by the intraperitoneal route in small doses, dose-limiting toxicity is clearly the development of an intense chemical peritonitis [37].

Table 1. Pharmacokinetic advantage of intraperitoneal chemotherapy.

Agent	Mean Peak Peritoneum/Plasma Concentration Ratio	Mean Peritoneum/Plasma AUC Ratio	Reference Number
Cisplatin	20	12	13
Melphalan	93	65	79
Doxorubicin	474	—	37
Methotrexate	92	—	11
Cytarabine	664	474	32
5-fluorouracil	298	376	10, 47

As mentioned, while inflammation may not cause acute symptoms, adhesion formation can be quite significant, leading to bowel obstruction. It is often difficult to evaluate the contribution of therapy to the development of this serious clinical condition, as many patients experiencing bowel obstruction will have undergone several surgeries or will have significant tumor burdens, which in and of themselves might have been the cause of the obstruction. In a series of 115 patients treated with a total of 435 courses of intraperitoneal therapy at the UCSD Cancer Center, with a total population follow-up of 1,103 patient-months, seven patients experienced episodes of partial small bowel obstruction, of which two were definitely (no tumor seen at laparotomy) and five possibly, related to the intraperitoneal treatments [38]. Recent reports have suggested limited success with several techniques in reducing the severity of postsurgical adhesion formation [39, 40]. A determination of a possible role for such therapy in reducing the inflammation and fibrous tissue reaction following intraperitoneal therapy will require the performance of carefully designed clinical trials.

Clinical trials of intraperitoneal therapy (Table 1)

Doxorubicin and mitoxantrone

As previously mentioned, the observation of the efficacy of intraperitoneal doxorubicin in a mouse model makes this agent a most important drug to investigate for intraperitoneal delivery in man [17, 18]. In addition, doxorubicin is active in ovarian cancer, a tumor which remains confined to the peritoneal cavity for much of its natural history [41]. In a clinical trial conducted at the National Cancer Institute, patients with refractory ovarian carcinoma were treated with doxorubicin delivered by the intraperitoneal route [37]. While a major pharmacokinetic advantage for exposure of the abdominal cavity to this agent compared to that of the plasma was demonstrated, and antineoplastic activity was shown, dose-limiting toxicity was abdominal pain at a concentration of 36 μM (40 mg/2L). Emesis and myelo-

suppression were not major problems in this trial. Activity for this agent delivered by the intraperitoneal route has also been noted by other investigators [42].

Mitoxantrone, a closely related nonsclerosing anthracycline, has recently been examined for intraperitoneal drug delivery in a phase I trial [43]. To date, while antineoplastic activity has been observed, there has been little local toxicity. In addition, in an analysis of the potential utility of several chemotherapeutic agents when administered by the intraperitoneal route based on achievable AUCs and in vitro activity against ovarian carcinoma, Alberts has found that mitroxantrone is the theoretically most attractive agent tested to date for intraperitoneal drug administration. Further evaluation of the intraperitoneal delivery of mitroxantrone in phase II trials in ovarian cancer appears indicated.

5-Fluorouracil

5-fluorouracil has a long history of intraperitoneal use for the control of malignant ascites [44–46]. Investigators at the National Cancer Institute have recently performed a detailed pharmacokinetic analysis of the intraperitoneal administration of this agent in large treatment volumes [47]. A major pharmacokinetic advantage for the exposure of the peritoneal cavity compared to the plasma following intraperitoneal therapy has been demonstrated with dose-limiting toxicity developing at a 5-fluorouracil concentration of 4.5 to 5 μM. Side effects were both local (abdominal pain and bacterial peritonitis) and systemic (myelosuppression and mucositis). Two additional studies have confirmed the pharmacokinetic advantage of the intraperitoneal administration of 5-fluorouracil [48, 49]. Unfortunately, while occasional objective clinical responses had been observed following intraperitoneal 5-fluorouracil, a recent phase II trial of intraperitoneal 5-fluorouracil in patients with refractory ovarian carcinoma demonstrated a response rate of only 7% [50]. However, the majority of these patients had previously received systemically delivered 5-fluorouracil, and the one responding patient in this trial achieved a surgically-defined complete remission.

Methotrexate

The intraperitoneal administration of methotrexate has been investigated at both the National Cancer Institute and the UCSD Cancer Center [11, 12, 51, 52]. There has been considerable interest in the use of this agent, when delivered by the intracavitary route, as methotrexate has a known safe and effective antagonist (folinic acid), which can be delivered intravenously to prevent serious systemic toxicity when the chemotherapeutic agent is delivered into the peritoneal cavity. At the National Cancer Institute patients with advanced intra-abdominal malignancies were treated with

methotrexate by intraperitoneal dialysis exchange for 48 hours followed by a continuous intravenous folinic acid infusion from 40 to 56 hours following the beginning of the methotrexate instillation [12]. Therapy was repeated weekly for six weeks, and while no definite clinical responses were observed, the toxicity of the regimen was mild in severity.

In the National Cancer Institute study the folinic acid was used in a *rescue mode*, and the duration of the methotrexate instillation had to be limited because of the potential for systemic toxicity. In the USCD study, on the other hand, the folinic acid was delivered as a continuous infusion *during* the methotrexate instillation [11]. When used in this way, as a *neutralizing agent*, the duration of exposure of the contents of the peritoneal cavity to methotrexate could be escalated from six to 120 hours. Toxicity included thrombocytopenia and a chemical serositis with infusions lasting longer than 96 hours. Several patients exhibited objective antitumor responses. While the exposure to methotrexate, a cell-cycle phase-specific agent, was prolonged when the folinic acid was delivered as an intravenous infusion during the methotrexate administration, it is unknown how much of the cytotoxic activity of this drug was lost by leakage of the folinic acid into the peritoneal cavity.

In both the National Cancer Institute and UCSD trials a major pharmacokinetic advantage for the intraperitoneal administration of methotrexate was demonstrated. In view of the limited toxicity experienced with this agent and the unique potential utility of a cytotoxic drug-neutralizing agent combination regimen in intraperitoneal therapy, further clinical investigation of this regimen is warranted.

Cisplatin

Cisplatin is an interesting agent to explore for its potential applicability for intraperitoneal therapy. Cisplatin is one of the most active drugs in ovarian carcinoma and is an ideal agent to consider for a combination chemotherapy regimen, as its principal toxicity is not myelosuppression [53]. Investigators at the Memorial Sloan-Kettering Cancer Center and UCLA have demonstrated the safety, pharmacokinetic advantage, and clinical utility of this drug when administered by the intraperitoneal route [54, 55]. In the UCLA study, the cisplatin was removed from the peritoneal cavity 20 minutes following drug instillation, and approximately 75% of the agent was recovered [55]. In this way, extremely high local concentrations of cisplatin were able to be maintained for a short period of time. While several patients treated on this protocol demonstrated objective evidence of tumor regression, one patient developed a significant decrease in creatinine clearance.

A somewhat different approach to the use of intraperitoneal cisplatin has been undertaken at the UCSD Cancer Center. Sodium thiosulfate, an agent used in man as an antidote for cyanide toxicity [56, 57], had been demonstrated in vitro to neutralize cisplatin and in vivo to protect against

certain toxic effects of cisplatin, principally nephrotoxicity [58, 59]. In a trial conducted at the UCSD Cancer Center, cisplatin was administered intraperitoneally up to a dose of 270 mg/m^2, with the simultaneous intravenous administration of sodium thiosulfate, with minimal nephrotoxicity [13]. Nausea and vomiting were quite severe despite an aggressive antiemetic regimen. Several antitumor responses were observed in patients with advanced intra-abdominal malignancies. Pharmacokinetic evaluation revealed that at a cisplatin dose of 270 mg/m^2, the peak concentration of active cisplatin (drug that had not reacted with thiosulfate) in the peritoneal cavity was 21 times higher than the peak plasma level, with the ratio of the AUCs between the two cavities being 12. Somewhat surprisingly, however, it was found that the AUC for the plasma following an intraperitoneal dose of cisplatin of 270 mg/m^2 was twice that following a 100 mg/m^2 intravenous dose of the same agent [60, 61]. One possible explanation for this finding is that while the concentration of the thiosulfate in the plasma is insufficient to rapidly inactivate the cisplatin, the concentration of the thiosulfate in the kidney is sufficient to inactivate the antineoplastic agent before it is able to produce damage. This hypothesis is supported by the observation that urinary concentrations of thiosulfate are 25 times higher than plasma concentrations [62]. Thus, in this clinical trial, we were able to demonstrate that the intraperitoneal delivery of cisplatin with intravenous thiosulfate resulted in significantly higher concentrations of drug in contact with the contents of the peritoneal cavity compared to the plasma while at the same time not compromising delivery of drug to tumor by capillary flow.

We have recently analyzed our experience with clinically relevant nephrotoxicity in patients treated on one of several cisplatin-based intraperitoneal or intrapleural programs at the UCSD Medical Center [63]. All patients received simultaneous intravenous thiosulfate with the instillation of cisplatin. Fewer than 3% of more than 450 courses of therapy in 131 patients administered cisplatin at a dose of either 100 mg/m^2 or 200 mg/m^2 were complicated by a serum creatinine rise to greater than 1.5 mg% (the upper limit of normal in our laboratory). In addition, only a single episode of serious nephrotoxicity (serum creatinine 8.0) developed despite the fact that more than 50% of the patients treated on these trials had been heavily pretreated with intravenous cisplatin.

It has recently been reported that high-dose intravenous cisplatin regimens can be associated with a high incidence of serious neurotoxicity [64]. In a retrospective analysis of patients treated on our cisplatin-based intracavitary chemotherapy trials, we have found a lower than anticipated incidence of neurological complications of therapy [65]. It is possible that this observation is explained by the difference in peak cisplatin levels or the shape of the AUC curves between the intravenous and intraperitoneal routes of drug delivery. It is equally possible, however, that the thiosulfate is, to some degree, protective of peripheral nerves. Further evaluation of this observation in an existing model of cisplatin-induced neuropathy or in a

carefully designed prospective clinical trial would be of considerable interest [66].

Investigators at the Netherlands Cancer Institute have recently reported on a clinical trial of intraperitoneal cisplatin (with or without thiosulfate protection) in a group of patients with residual ovarian carcinoma, following systemic therapy with a cisplatin-based therapeutic regimen [67]. Approximately 30% of patients with minimal residual disease at the time of the initiation of the intraperitoneal program achieved a surgically-defined (third look laparotomy) complete remission. While several patients have relapsed, responses have persisted in several patients for longer than two years.

As unexpected clinical activity was observed in the early UCSD Cancer Center trial of intraperitoneal chemotherapy in a patient with mesothelioma [13], a phase II trial of intraperitoneal cisplatin was conducted in this disease [68]. The dose of intraperitoneal cisplatin was 90 or 100 mg/m^2, which was delivered weekly for three weeks. Following a three week break an additional three weekly treatments were administered. Thiosulfate was given simultaneously intravenously with each treatment course. Responding patients received a total of six cycles (18 courses). To date, a total of 13 patients with mesothelioma principally confined to the peritoneal cavity have been treated with this regimen. Three patients have demonstrated surgically-documented evidence of a major response to the treatment program, including one complete remission. Six additional patients had near complete or complete disappearance of ascites. The median duration of responses was nine months (range: 2–18 months). Further evaluation of this treatment approach in mesothelioma, a tumor which remains clinically confined to the pleural or peritoneal cavities for most of its natural history, appears indicated [69]. This is particularly important, as the reported response rate of mesothelioma to intravenous cisplatin is only 10% and the response rate to the most active agent in this disease (doxorubicin) is only 20% to 40% [70, 71].

Cytarabine

Cytarabine was one of the drugs initially modelled by Dedrick and his colleagues which suggested a sound pharmacokinetic rationale for intraperitoneal drug delivery [3]. The drug has an extremely short half-life because of deamination in the liver and would therefore be predicted to demonstrate a major pharmacokinetic advantage when delivered by the intraperitoneal route [72, 73]. Unfortunately, cytarabine, the single most active agent in acute myelocytic leukemia [74, 75], had previously demonstrated essentially no clinical activity in solid tumors in limited clinical evaluation [76]. However, in an in vitro clonogenic assay at our institution, we have demonstrated that cytarabine is cytotoxic to fresh tumor cells from patients with refractory ovarian carcinoma in a concentration-dependent manner [32]. In addition, in several patients' tumors significant cytotoxicity

(less than 30% survival) was achieved at concentrations of cytarabine not clinically attainable in man with intravenous administration, but possibly approachable for some finite period of time in the abdominal cavity following intraperitoneal drug administration. In a recently completed pharmacokinetic evaluation of the intraperitoneal delivery of cytarabine, it was demonstrated that the ratio of the AUCs for the peritoneal cavity compared to that of the plasma ranged from 320 to 1,000 [32]. In addition, a clinical trial of cytarabine administered by the intraperitoneal route by dialysis exchange every six hours for five days in ten patients with refractory ovarian carcinoma revealed definite activity for this agent in this clinical setting. Two patients with minimal residual disease achieved a clinically-defined complete remission which has persisted for longer than two years.

Perhaps the most interesting potential use of intraperitoneal cytarabine would be to expose slowly growing intraperitoneally localized solid tumors for long periods of time to this cell-cycle phase-specific agent [77]. As the drug is metabolized in the liver prior to its entry into the systemic circulation, it might be possible to choose a dose of cytarabine which could bathe the peritoneal cavity for extended periods while having nontoxic quantities reach the systemic circulation. If the practical problems associated with delivery of large treatment volumes over many weeks could be solved, this approach might be the optimal way to employ cell-cycle phase-specific agents against solid tumors confined to the peritoneal cavity.

Melphalan

The intraperitoneal delivery of melphalan, one of the most active agents in ovarian carcinoma, has been examined in two clinical trials [78, 79]. Limited clinical activity has been demonstrated with a major pharmacokinetic advantage for exposure of the peritoneal cavity to the drug compared to the plasma. Dose-limiting toxicity appears to be systemic (myelosuppression). In view of the erratic absorption of melphalan when administered orally and the high local and plasma levels achieved when the drug is delivered directly into the peritoneal cavity, it is reasonable to suggest that intraperitoneal instillation of this agent may be the optimal method of delivery in patients with ovarian carcinoma.

Mitomycin

There has been limited clinical investigation of the intraperitoneal administration of mitomycin C [80, 81]. Dose-limiting toxicity is local pain when more than 10 mg of the drug is administered in a concentrated solution into the peritoneal cavity. However, a major pharmacokinetic advantage for intraperitoneal therapy has been demonstrated, and it is possible that delivering a lower concentration of drug in a larger treatment volume might decrease the severity of local side effects.

Etoposide

Etoposide has been examined for intracavitary drug administration both intraperitoneally and intrapleurally [82, 83]. In a small phase I trial, a major pharmacokinetic advantage for intraperitoneal drug administration has been suggested [82]. Local toxicity was not reported. This is an important point, as there is animal data to suggest that the intraperitoneal delivery of etoposide can result in serious local toxicity (sclerosis) [84].

Streptozotocin

A single patient with ovarian carcinoma and a low peritoneal cavity glucose who received intraperitoneally administered streptozotocin has been reported [85]. Toxicity was acceptable with a significant pharmacokinetic advantage for peritoneal cavity exposure being demonstrated. In view of the reported activity of streptozotocin in carcinoid tumors and colon carcinoma, it would be of interest to investigate this agent further for its applicability for intraperitoneal drug delivery.

Combination intraperitoneal therapy

The administration of several chemotherapeutic agents in combination has become a major therapeutic strategy of the medical oncologist. Theoretically, this approach allows for the delivery of drugs with different mechanisms of activity and nonoverlapping toxicities [86, 87]. In addition, it might be possible to administer two or more drugs which are synergistic with each other in producing tumor cell kill.

The delivery of combination intraperitoneal chemotherapy is a logical extension of the single agent work previously discussed. One caveat which must be mentioned, however, is the concern that by combining two or more drugs which by themselves do not cause excessive local side effects, one might create a very toxic treatment regimen because of changes in the structure of the individual drug components. Thus, each combination considered for clinical trails must be carefully evaluated in vitro before being administered to man, and the initial patients must be carefully monitored for unexpected toxicity. The experience with the serious toxicity encountered with intraperitoneal vinblastine emphasizes this point [88].

In theory, combination intraperitoneal chemotherapy has one major advantage over such therapy delivered systemically. Because of the extremely high local concentrations which can be achieved during intraperitoneal therapy, it is possible that cytotoxic synergy demonstrated in vitro, which is concentration-dependent, might be clinically relevant at the concentrations present in the abdominal cavity following intraperitoneal therapy but not following intravenous treatments. For example, in one in vitro system

(LoVo colon carcinoma cell line) it has been demonstrated that cytarabine markedly enhances the cell kill produced by cisplatin in a concentration-dependent manner [89]. At the highest concentration of cytarabine tested (4×10^{-2} M), the cell kill produced by the combination of cisplatin and cytarabine was 1600 fold greater than that produced by cisplatin alone. While this concentration of cytarabine is highly unlikely to be achieved by the intravenous administration of this agent, it is at least approachable during intraperitoneal drug delivery.

Other combinations which are of interest to examine for potential utility during intraperitoneal therapy include cisplatin/5-fluorouracil, cisplatin/bleomycin, and cisplatin/etoposide. The cisplatin/5-fluorouracil combination has shown some promise when delivered in combination systemically for colon carcinoma [90], while a regimen including cisplatin/bleomycin has been highly effective in curing testicular carcinoma [91, 92]. In addition, recent data has suggested an interaction between cisplatin and bleomycin at the level of DNA-drug binding [93], and bleomycin has been shown to possess definite activity against ovarian carcinoma both in vitro and in vivo [94–96]. The combination of cisplatin/etoposide has been very active in both testicular carcinoma and small cell lung carcinoma, suggesting possible clinically relevant synergy [97–99]. This observation is supported by in vitro data which has shown clear synergy between the two agents in experimental systems [100, 101]. Finally, all of the agents mentioned above have demonstrated a significant pharmacokinetic advantage when delivered by the intraperitoneal route [13, 47–49, 54, 55, 82, 88, 94].

Several combination intraperitoneal chemotherapy trials have been conducted at the UCSD Medical Center. The first trial employed cisplatin ($100-200\,mg/m^2$), cytarabine ($10^{-4}-10^{-3}$ M) and doxorubicin ($18\,\mu M - 2\,\mu M$) delivered in a two liter treatment volume [102]. While objective responses were observed and several patients experienced major palliation of recurring ascites, doxorubicin-induced abdominal pain was excessive at the $18\,\mu M$ dose level. In the second trial, the dose of cytarabine was escalated fourfold (4×10^{-3} M) while doxorubicin was eliminated from the treatment program [103]. Objective antitumor responses, including several surgically-defined complete remissions in patients with minimal residual ovarian carcinoma following front-line cisplatin-based intravenous therapy, were demonstrated. Follow-up of 61 patients with refractory ovarian carcinoma treated on one or both of these trials revealed a median survival of 6.5 months (range: 1 to 32 months) for patients with bulky intra-abdominal disease (a single lesion greater than two centimeters in diameter), while the median survival for the 15 patients with minimal or microscopic disease (no single lesion greater than two centimeters) has not been reached but will exceed two years. Seven patients (15%) with bulky disease are currently alive with only two surviving for more than two years. Thirteen patients (87%) with minimal residual disease are alive with the longest follow-up out to 32+ months.

In our most recent trial, bleomycin (15 units/m^2 or 2 units/m^2) has been added to the treatment program of cisplatin and cytarabine [104]. Again, while responses have been observed, abdominal pain with the 15 units/m^2 dose of bleomycin was excessive. With the lower dose of this agent (2 units/m^2) significantly less abdominal pain has been noted.

Additional intraperitoneal treatment programs

Several investigators have attempted to take advantage of the intraperitoneal route of drug administration by developing innovative approaches to the treatment of tumors confined to the abdominal cavity. Based on the theory that heat will increase the sensitivity of tumors to antineoplastic agents, a patient has been treated with intraperitoneal administration of methotrexate and thiotepa in a hyperthermic delivery system which heated the peritoneal cavity to 42° centigrade [105]. Although a therapeutic response was not observed, the treatment program was well tolerated.

Hypoxic radiosensitizers (misonidazole and demethylmisonidazole) have been delivered directly into the peritoneal cavity in an effort to increase local concentrations and decrease systemic side effects (neurotoxicity) [106]. In addition to demonstrating the safety of this approach, a significant pharmacokinetic advantage for exposure of the peritoneal cavity compared to the plasma has been shown. Clinical interest in these agents is heightened by the fact that synergy has been demonstrated in vitro between the radiosensitizers and certain chemotherapeutic agents, including cisplatin [107].

The intraperitoneal administration of immunostimulant and immunomodulating agents has recently begun to be explored in man. Several surgically-defined antitumor responses have been observed in a group of patients with refractory ovarian carcinoma treated with the nonspecific immunostimulant, *Corynebacterium parvum* [108, 109]. Similarly, a group of Japanese investigators have noted a reduction of ascites and improvement of several immune parameters when patients with advanced intra-abdominal malignancies were treated with the intraperitoneal administration of a streptococcal preparation (OK-432) [110, 111].

The most intriguing data on the intraperitoneal delivery of immunomodulating agents is that of Berek and his colleagues at the UCLA Medical Center [112]. Alpha-recombinant interferon was delivered intraperitoneally to a group of 14 patients with refractory ovarian carcinoma. While systemic toxicity, including fever and general malaise, were observed, local toxicity was acceptable and several patients with minimal (less than five millimeter) disease achieved surgically-defined complete remissions. Similarly, in a recent report, beta interferon was delivered to eight patients with refractory ovarian carcinoma, and while mass lesions did not shrink, four of seven patients with effusions demonstrated major responses [113]. In addition,

natural killer cell activity was increased in several patients in the peritoneal cavity while being unchanged in the peripheral blood.

In preclinical evaluation two interesting therapeutic approaches employing the intraperitoneal route have recently been reported. A murine embryonal ovarian carcinoma has been successfully treated by the intraperitoneal administration of a hematoporphyrin derivative followed two hours later by the intraperitoneal application of 514 nm laser light [114]. While all control animals died following tumor delivery, six of 16 mice receiving two treatment courses were long term survivors (>60 days).

The intraperitoneal administration of hexamethylmelamine (an agent active in ovarian carcinoma and only minimally soluble in aqueous solutions) in intralipid has been examined in a murine system [115]. A major pharmacokinetic advantage for exposure of the peritoneal cavity to this drug compared to delivery in saline has been demonstrated. Before hexamethylmelamine can be considered for intraperitoneal delivery in patients with ovarian carcinoma, it will be important to determine if the drug is directly cytotoxic to tumor cells or whether activation in the liver or other tissues is required.

Conclusion

In this review we have attempted to present a rationale for the intraperitoneal approach to the treatment of malignant disease confined to the peritoneal cavity, as well as the problems associated with this innovative treatment technique. Clinical data is presently available to suggest that patients with minimal residual refractory ovarian carcinoma can achieve surgically-defined responses, including complete remissions, when treated with a cisplatin-based intraperitoneal regimen.

Much work remains to be done in the area of intraperitoneal chemotherapy. Optimal drug concentrations remain to be defined. Efforts also need to be directed to developing methods to decrease the severity of local toxicity, to insure adequate drug distribution, and to decrease the incidence of infection. Finally, if techniques can be developed which allow for the safe delivery of large treatment volumes into the peritoneal cavity on an ambulatory and continuous basis, it might be possible to develop a treatment program which optimally utilizes those cell-cycle phase-specific agents which are metabolized during passage through the liver.

What eventual role intraperitoneal therapy will play in the treatment of ovarian carcinoma or other tumors principally confined to the peritoneal cavity will not be known until randomized controlled clinical trials comparing this innovative treatment approach to standard therapy have been completed. It will only be then that we will be able to determine if the pharmacokinetic advantage achieved during intraperitoneal therapy can be

translated into improved response rates and survival for patients with tumors confined to the peritoneal cavity.

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II

New Drugs and New Clinical Investigation

3. Metabolites of tiazofurin as mediators of its biochemical and pharmacologic effects

Gurpreet S. Ahluwalia, Hiremagalur N. Jayaram, and David A. Cooney

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) was synthesized by the medicinal chemists at ICN as one of a series of potential antiviral compounds [1,2]. In fact, the agent does exhibit a modest to moderate degree of activity against several pathogenic viruses [2,3]. However, at the time, this activity apparently was not judged sufficient to warrant development of the drug towards clinical trials. With the advent of managerial changes at ICN, Dr. Kenneth Paull of the National Cancer Institute made arrangements with Dr. Roland Robins, the chemist who had designed tiazofurin, to screen that compound and a number of related nucleosides for antineoplastic activity. These studies revealed that tiazofurin was effective against the L1210 and P388 leukemias and prompted a broader examination of the compound's oncolytic potential. Surprisingly, tiazofurin proved to be effective against the subcutaneous Lewis lung carcinoma, a transplantable tumor resistant to the vast majority of standard and experimental chemotherapeutic drugs. Moreover, cures were achieved over a broad range of doses of the compound [4]. It was these studies, then, that prompted the development of tiazofurin toward clinical trials as an antitumor drug.

Mechanistic studies

In parallel with the usual preclinical toxicologic and pharmacological studies of tiazofurin, several laboratories embarked on studies into its mechanism of action. These investigations soon established that exposure of murine lymphoblasts to the drug promptly arrested DNA synthesis. Because tiazofurin is a nucleoside (albeit an unnatural 'C' nucleoside), the next logical step was to determine whether any naturally occurring nucleosides could overcome its inhibition of DNA synthesis. These experiments established that guanine and guanosine were both effective antidotes to the biochemical effects of tiazofurin [5–7]. Subsequent studies established that guanosine also could overcome the cytoxicity of tiazofurin in select cases and also nullify the in vivo therapeutic activity of the drug against the P388 leukemia [8]. When it was observed that guanylate pools were strongly diminished in cells and

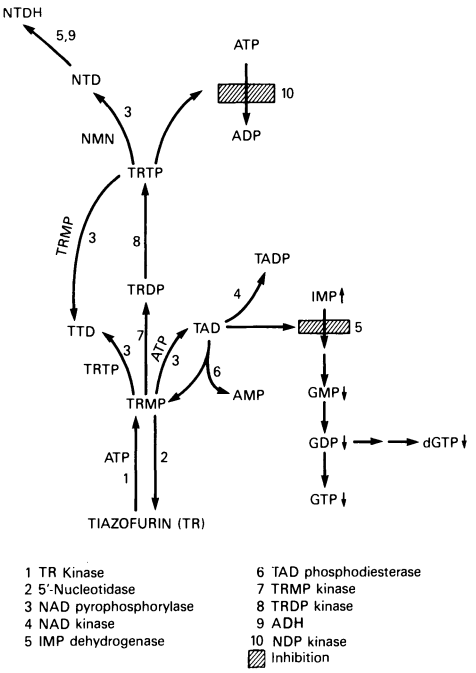

Figure 1. Metabolic fates of tiazofurin: Interaction with cellular enzyme systems.

tumors treated with tiazofurin, the drug was established as an antagonist of that purine [5].

Enzymologic studies, using extracts of treated cells, next suggested that exposure to tiazofurin specifically arrested guanosine-5′-monophosphate (GMP) synthesis by interrupting the activity of inosinic acid dehydrogenase (EC 1.2.1.14) [5,6,9]. Ultimately, the proximate inhibitory species was identified as thiazole-4-carboxamide adenine dinuleoctide (TAD) a phosphodiester analog of NAD, the ordinary cofactor in the inosinic acid dehydrogenase (IMPD) reaction [10,11]. These relationships and the other metabolic fates experienced by tiazofurin are illustrated in figure 1.

In the sections to follow, tiazofurin and its principal metabolites (tiazofurin-5′-monophosphate and TAD) will be discussed first, while the minor metabolites (tiazofurin-5′-diphosphate, tiazofurin-5′-triphosphate, et al.) will be treated together in a subsequent section. The structures of these metabolites are presented in figure 2.

Tiazofurin and its primary metabolites

Tiazofurin

As discussed in the opening section of this overview, exposure to tiazofurin results in inhibition of IMP dehydrogenase and profound depletion of

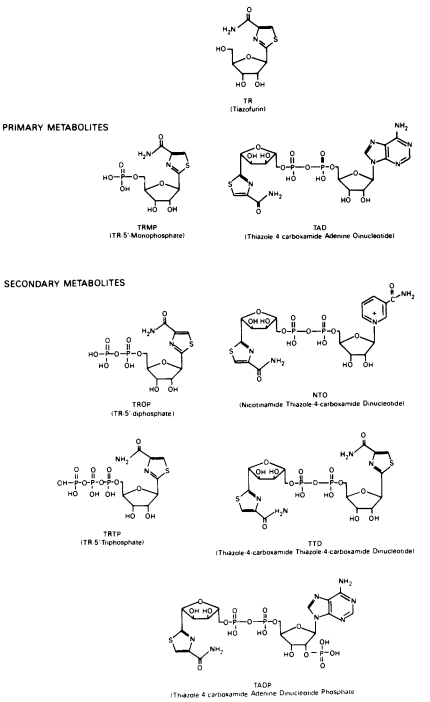

Figure 2. Metabolites of tiazofurin.

guanine ribonucleotide and deoxyribonucleotide pools [7][1]. However, whereas 5 μM of drug suffices to inhibit IMP dehydrogenase activity in cultured P388 lymphoblasts, a concentration greater than 1,000 times this is required to inhibit a partially purified preparation of the enzyme from the same source [5]. This study strongly suggested that it was a metabolite of tiazofurin which was likely to be responsible for its principal toxic actions. Further support for this suggestion was provided by our observation that the concentration of tiazofurin in neoplastic cell lines does not correlate with their sensitivity or resistance to the drug (table 1).

Nevertheless, under select experimental conditions, it is possible to demonstrate that tiazofurin *itself* can produce pharmacologically relevant effects. For example, the drug promptly and competitively inhibits nucleoside transport in erythrocytes and L1210 cells at concentrations which can be achieved in vivo; thus, 5 μM tiazofurin inhibits inosine transport in L1210 cells by 73% [12]. This direct effect of tiazofurin would of course reinforce its primary locus of action by diminishing the availability for salvage of extracellular purines. (Transport of guanosine was not examined in these studies.)

One other property of tiazofurin seems likely to be associated with the parent molecule, and that is the neuropharmacologic sequelae seen with some promptitude following parenteral administration of the drug. Mice, for

Table 1. Accumulation of primary metabolites of tiazofurin in vivo and in vitro.

Source	Sensitivity to Tiazofurin	Drug or Metabolite (nmoles/g)		
		TR	TRMP	TAD
Murine tumors[a]				
P388	S	152.0	8.6	3.9
Lewis Lung	S	52.0	1.5	19.0
L1210	S	72.0	3.6	11.7
B 16	R	34.0	3.5	1.3
Colon 38	R	22.0	2.3	0.9
M 5076	R	83.0	2.5	0.6
P388/TR[d]	R	196.0	3.1	<0.05
Human tumor cells[b] in culture				
H 82	S	54.0	28.0	120.0
H 417	S	84.0	21.0	222.0
H 23	S	106.0	83.0	257.0
H 146	R	37.0	71.0	4.0
H 125	R	77.0	125.0	43.0
H 249	R	116.0	126.0	55.0
Mouse tissues[c]				
Brain	—	24.7	8.0	0.2
Liver	—	43.8	26.5	4.4
Lungs	—	36.6	12.5	0.4
Heart	—	40.7	17.6	3.0
Spleen	—	64.9	16.6	0.5
Pancreas	—	16.3	5.1	0.5
Stomach	—	10.3	2.3	0.3
Small Intestine	—	16.6	3.6	0.4
Large Intestine	—	25.6	5.3	0.4
Kidney	—	27.9	8.1	0.5
Muscle	—	16.7	10.8	2.2

[a] Data obtained from [22]; drug metabolites were determined in the subcutaneously implanted tumors two hours after treatment with 100 mg of tiazofurin/kg, (10 μCi [5-^{3}H] tiazofurin/mg of drug).

[b] Data obtained from [23]; drug metabolism was determined in cellular pellets after a six hour exposure to 10 μM [5-^{3}H] tiazofurin.

[c] Tissue metabolism of the drug was determined one hour after an intravenous dose of 100 mg tiazofurin/kg, (10 μCi [5-^{3}H] TR/mg of drug, as described in [18].

[d] P388/TR; drug resistant (~ 1,000 ×) variant of P388 lymphoblasts [24].

example, given an intraperitoneal injection of 1,000 mg/kg (a dose well below the LD_{10} [13]) exhibit signs of a kind of waxy immobility, and human recipients of intravenous tiazofurin frequently complain of headache as well as demonstrating personality changes and obtundation [14]. Although there is no direct evidence on this score, it is possible that the thiazole ring of tiazofurin (which, as will be seen, confers on the molecule a kind of purine-like property) enables the drug to interact with the adenosine receptors of the central nervous system.

Tiazofurin-5'-monophosphate (TRMP)

Early studies with radiolabelled tiazofurin established that nearly every tumor and normal tissue (including the erythron, *vide infra*) converted the drug to a more acidic species (denoted TRMP in table 1), whose elution time on high presure liquid chromotography (HPLC) was exactly coincident with authentic, chemically synthesized tiazofurin-5'-monophosphate [8]. Treatment of this anabolite with alkaline phosphatase and 5'-nucleotidase quantitatively reconverted it to the parent drug; 3'-nucleotidase, on the other hand, failed to attack this metabolite, a result which reinforced its identification as the 5'-monophosphate of tiazofurin [8].

Subsequent studies established that high-speed (105,000 g) supernatants from tumors and tissues were also capable of catalyzing the monophosphorylation of tiazofurin in the presence of ATP and magnesium ions (table 2), but the responsible kinase was not identified. Some insight into the nature of this enzyme was provided by the studies of Saunders et al. [15], using Chinese hamster ovary (CHO) cells and various kinase-deficient variants thereof. In these cells, deletion of adenosine kinase (EC 2.7.1.20) or deoxycytidine kinase (EC 2.7.1.74) wholly failed to alter responsiveness to tiazofurin, suggesting that neither of these enzymes was responsible for activating the drug. (A result at variance with this one is presented below.)

To gain some insight into the nature of the enzyme responsible for phosphorylating tiazofurin, we explored the capacity of a panel of naturally occurring nucleosides to inhibit the formation of TRMP in vitro. For this study, enzyme and labeled tiazofurin were incubated with ATP-$MgCl_2$ in the presence or absence of a panel of naturally occurring nucleosides (0.01 M). Phosphorylated product was then separated by paper chromatography and quantified by scintillation spectrometry. As table 2 shows, using a 105,000 g supernatant from the Lewis lung tumor, inosine and deoxyinosine were the most potent inhibitors of TRMP formation, but adenosine and deoxyadenosine also produced significant enzyme inhibition. Using extracts of mouse liver, adenosine and deoxyadenosine — but not inosine — were strongly inhibitory nucleosides, suggesting that, in this latter case, TRMP was formed by the enzyme ordinarily responsible for the synthesis of adenosine and deoxyadenosine-5'monophosphate. Taken together, these results suggested that the first step in the anabolism of tiazofurin might be carried out by more than one enzyme system in different tissues.

Table 2. The capability of select nucleosides to inhibit the phosphorylation of tiazofurin.

Nucleoside	Lewis Lung Carcinoma	Mouse Liver
	% Inhibition	
Nicotinamide riboside	40	ND
Adenosine	73	98
Deoxyadenosine	61	69
Guanosine	37	30
Deoxyguanosine	63	43
Inosine	88	33
Deoxyinosine	80	ND
Thymidine	32	7
Uridine	42	11
Deoxyuridine	34	4
Cytidine	18	0
Deoxycytidine	35	0

0.01 M [5-^{3}H] tiazofurin was incubated with 0.01 M ATP·$MgCl_2$ and either 0.01 M Tris HCl, pH 8.5 or the nucleosides listed in the table in the presence of a 105,000 g supernatant from the tissues designated in the table for 20 minutes at 37°. The reaction was stopped by heating the vessels for two minutes at 95°; 5 µl aliquots of each sample were analyzed by ascending paper chromatography (solvent: 70% ethanol, 30% ammonium acetate) and radioactivity corresponding to authentic TRMP was measured by scintillation spectrometry. ND: Not determined

Some further insight into this problem was subsequently provided by the work of Spindler and colleagues [16]. These investigators chromatographed extracts of CHO cells on DEAE cellulose and demonstrated two peaks of phosphorylating activity. The first was coincident with adenosine kinase. The second, distinct from all known purine or pyrimidine kinases, was not absolutely characterized except for the ability of nicotinamide riboside to inhibit its activity substantially. This result raises the possibility that the enzyme which customarily synthesizes nicotinamide mononucleotide (NMN) may accept tiazofurin as substrate. (It should be noted, however, that in our studies with extracts of the Lewis lung carcinoma, nicotinamide riboside was not an especially good inhibitor of the activation of tiazofurin; table 2.)

Further support for the existence of more than one 'tiazofurin-kinase' was provided by the work of Fridland and Connelly [17], who demonstrated that human lymphoblasts with a deletion of adenosine kinase are three- to eightfold more resistant to tiazofurin than the parental strains, but retain a finite ability to phosphorylate the drug. These authors thus conclude that tiazofurin is converted to TRMP by a combination of two pathways — adenosine kinase and a second unidentified phosphorylating system.

In view of our finding that inosine was a prominent inhibitor of the activation of tiazofurin and of the recent reports that cytoplasmic 5′ nucleotidase can, in addition to its normal hydrolytic activity, also catalyze the

Table 3. Inhibition of IMP dehydrogenase by tiazofurin, its metabolites and constituents.

Compound	Inhibition of IMPD IC_{50}(mM)
Tiazofurin	8.5
Thiazole-4-carboxylic acid riboside	> 10.0
2′deoxy tiazofurin	> 10.0
Thiazole-4-carboxamide	> 10.0
Tiazofurin-5′monophosphate	0.5
Tiazofurin-5′-diphosphate	0.5
Tiazofurin-5′-triphosphate	0.6
TAD	0.0002
TADP	0.002
TTD	0.04

The IMP dehydrogenase used was a partially purified preparation from P388 lymphoblasts. The assay system is described in detail in [20].

synthesis of 5′ monophosphates in the presence of IMP and the appropriate nucleoside, it is tempting to speculate that this enzyme might accept tiazofurin as substrate [18]. In this connection, the results of Keller and colleagues showing the vigorous phosphorylation of acyclovir by this soluble 5′ nucleotidase, with IMP as energy donor, seem especially relevant [19].

As will be discussed below, the monophosphorylation of tiazofurin is an absolute prerequisite to the formation of its metabolites and crucial to the expression of its cytotoxic potential. It follows that a failure to phosphorylate the drug must confer resistance to tiazofurin. Although there are hints in the literature to the contrary, we have not so far identified any cell line with an absolute incapacity to form TRMP (table 1). In the context of the foregoing discussion, this failure might reflect the plural nature of the enzymes involved; joint deletion of these would require a double mutation — an event possibly incompatible with viability.

Little is known of the pharmacologic properties of tiazofurin-5′-monophosphate. Well established is the fact that the compound is less cytotoxic to P388 cells than tiazofurin (IC_{50} ~4 μM compared to 2 μM for the parent compound), but this difference is probably insignificant. The ectonucleotidase on the cell surface and phosphatases present in the serum-containing culture medium almost certainly degrade extracellular TRMP to tiazofurin which, after its entry, is rephosphorylated and further metabolized (discussed in detail later). That TRMP is not itself responsible for engendering a depletion of guanine nucleotides is suggested by its inefficient inhibition of IMPD: the K_i is ~500 μM (table 3), a value substantially stronger than that of tiazofurin itself — but nevertheless of little relevance to the cellular pharmacology of the drug inasmuch as the concentrations of this anabolite achieved in the therapeutic setting are usually lower than this value (table 1).

Thiazole-4-carboxamide adenine dinucleotide (TAD). The search for the proximate cytotoxic anabolite of tiazofurin was intensified when it became clear that the concentration of TRMP achieved in the tumors of treated mice did not, as was said, even approach its K_i versus IMPD. Attention was therefore focused on a second acidic metabolite formed in cells exposed to labelled tiazofurin. This metabolite eluted after TRMP in the general area of several natural phosphodiesters (the UDP-sugars) and was detectable in amounts frequently greater than the 5′-monophosphate. When aliquots of the appropriate chromatographic fractions were neutralized and then admixed with a partially purified preparation of tumoral IMPD, inhibition was seen only in the case of the second, more acidic species. Treatment of this latter peak with alkaline phosphatase failed to alter its chromatographic or enzyme-inhibitory behavior, whereas venom phosphodiesterase and a variety of other pyrophosphatasic enzymes destroyed both properties in parallel, yielding two breakdown products, the first, exactly coincident with AMP, the second with TRMP [20]. These studies led to the tentative identification of the active antimetabolite of tiazofurin as a dinucleotide composed of adenosine and tiazofurin in phosphodiester linkage.

This identification was corroborated by NMR and FAB mass spectroscopy of the anabolite [20]. Finally, chemical synthesis [21] yielded a product with spectroscopic, chromatographic, and enzyme-inhibitory characteristics identical to that of the inhibitor isolated ex vivo. By analogy to the pyridine nucleotides, this compound was named TAD, thiazole-4-carboxamide adenine dinucleotide. In fact, it is structurally identical to NAD, nicotinamide adenine dinucleotide, except for the replacement of the nicotinamide ring by thiazole-4-carboxamide.

With NAD as variable substrate, TAD inhibited IMPD in a manner which was clearly not competitive (probably noncompetitive) in type, the K_i ranging from 0.6 to 3.4×10^{-7} M [10, 11]. Although it is perplexing that the drug-derived dinucleotide does not compete with its natural counterpart, this feature has one important pharmacologic consequence, the high levels of pyridine nucleotides found in tumors will not serve to minimize the inhibition of IMPD by TAD, as would happen in a formally competitive case. Noteworthy too is the finding that, whereas TAD also inhibits IMPD from *E. coli*, it fails to influence the activity of L-glutamate, L-malate, or L-lactate dehydrogenases [20].

Further studies reinforced the notion that it was TAD which, at least in large part, was responsible for inhibiting IMPD and reducing guanylate pools in susceptible cells. Thus, in all cases examined so far, the accumulation of TAD antecedes depletion of guanosine phosphates; a representative example of this chronology is shown in figure 3. To reinforce the contention that TAD is tiazofurin's active metabolite, the concentration of this dinucleotide achieved in a substantial number of tumors naturally sensitive or resistant to the drug was measured [22]. Although there have been exceptions, it can be concluded that responsive tumors will achieve a peak level of

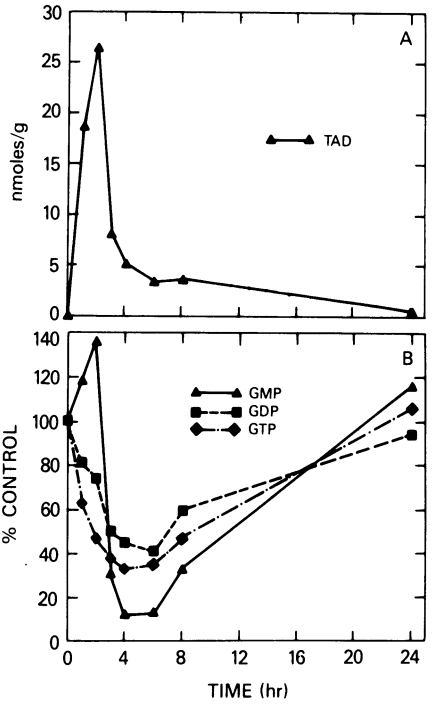

Figure 3. Rate of TAD accumulation and corresponding change in guanine nucleotide levels after tiazofurin treatment. Male BDF_1 mice bearing subcutaneous nodules of the P388 leukemia were injected intraperitoneally with radiolabelled tiazofurin, 200 mg/kg (50 μCi/mouse). At the indicated times, animals were killed, tumors prompty removed, and flash frozen on dry ice. Acid extracts of samples were prepared by homogenization in 10% TCA. Samples were neutralized with trioctylamine in freon and analyzed on a presentandardized Partisil-10 SAX column attached to a Waters HPLC system [10,22]. Each data point represents the mean of four determinations.

the dinucleotide which is between three and 20 times that seen in their resistant counterparts (table 1); the same conclusion applies to cultured human tumor cells [23].

As an illustration of this difference, figure 4 presents documentation of the time course of accumulation and disappearance of TAD in the naturally sensitive subcutaneously grown P388 leukemia and the naturally refractory colon carcinoma 38. Plainly, the concentration of TAD achieved over time in the sensitive tumor greatly exceeds the same parameter in the resistant line. The dual biochemical bases for this difference will be discussed in subsequent sections.

A still more dramatic illustration of the generalization that tumors resistant to tiazofurin accumulate less TAD than their sensitive counterparts was provided by studies of both the P388 and L1210 leukemias rendered solidly resistant (~1,000 x) by cultivation over 50–60 generations in the presence of incremental concentrations of the drug [24–26]. In both cases, the refractory

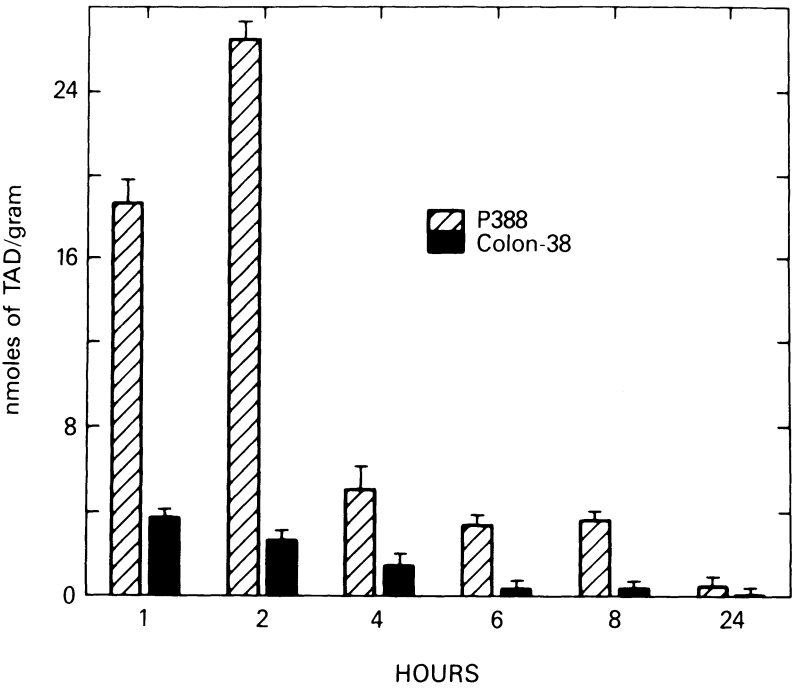

Figure 4. Time course of TAD accumulation in P388 and Colon 38 tumors. Male BDF_1 mice with subcutaneous implants of the P388 leukemia or colon 38 carcinoma were injected intraperitoneally with radiolabelled tiazofurin, 200 mg/kg (50 μCi/mouse). Conditions of drug treatment, sample preparation, and HPLC analysis were the same as given in the legend to figure 3.

lines accumulated little or even no TAD, whereas levels in the parental lines were substantial (table 4).

That this tendency to accumulate little or no TAD is not a universal feature of cells rendered resistant to tiazofurin is illustrated by studies with a rat hepatoma line, whose resistance, although developed by the same general technique as the two mouse leukemias just now described and of the same magnitude, was only accompanied by a 50% loss in the level of TAD accumulated. Indeed, studies with this carcinoma showed its resistance to be related to more than one factor [27] (table 4).

Although the sensitivity or resistance of tiazofurin to human cells has yet to be assessed (phase II trials are ongoing), one recent in vitro study on the synthesis of TAD in normal versus leukemic leukocytes merits mention [28]. In a head-on comparision, under identical conditions of incubation, the malignant cells accumulated 20–30 times more TAD than their normal counterparts (figure 5). Of still greater interest is the observation that guanylate pools fell (by ~50%) only in the leukemic and not in the normal leukocytes following a two hour exposure to 100 μM tiazofurin (figure 5). These results warrant the expectation that tiazofurin will be of value in the treatment of hematologic malignancies.

Table 4. Paramaters associated with induced resistance to tiazofurin.

Markers	Biochemical Alterations Compared to Sensitive Lines[a]		
	Leukemia P388	Leukemia L1210	Hepatoma 3924A
IMP dehydrogenase	No change	No change	Increase
IMP concentration	Decrease	Increase	Increase
Basal guanylate pools	No change	Decrease	Increase
Guanine salvage activity	No change	Increase	Increase
De novo synthesis of IMP	Decrease	Increase	Decrease
Ratio of salvage/de novo activity	Increase	Increase	Increase
NAD pyrophosphorylase	Marked	Marked	Modest
	Decrease	Decrease	Decrease
	Decrease	No change	Decrease
Uptake of tiazofurin[b]	Marked	Marked	Modest
TAD concentration	Decrease	Decrease	Decrease
Stability of resistance	Stable	Stable	Reverts

[a] Obtained from [26].

[b] Uptake was measured in vitro by incubating cells with labeled tiazofurin and, after intervals of 30 or 60 seconds, driving the cells through Versilube silicone oil to terminate entry. In vivo, as documented in table 1, P388 and P388/TR had accumulated nearly equivalent concentrations of free tiazofurin two hours after administration of the drug.

Enzymatic synthesis of TAD. Several lines of evidence strongly suggest that NAD pyrophosphorylase is responsible for the biosynthesis of TAD: (1) Since the earliest studies with cell fractionation, it has been known that NAD pyrophosphorylase is associated with the nucleus [29]; in P388 lymphoblasts, as well as in a panel of cultured lung tumors, the activity which synthesizes TAD is particulate and, under the appropriate conditions, sediments nearly exclusively with the low-speed nuclear fraction [23]; (2) Both NAD pyrophosphorylase and the TAD-synthetic activity require ATP, magnesium ions, and a pyridine-like nucleoside-5′-monophosphate (NMN or TRMP) as substrates; (3) Purified NAD pyrophosphorylase from hog liver efficiently catalyzes the synthesis of TAD as well as NAD, and its affinity for ATP is nearly identical in both reactions (table 5); and (4) The aforementioned murine lymphoblasts rendered resistant to tiazofurin in vitro experience a parallel loss of TAD accumulation and of nuclear NAD pyrophosphorylase [25].

Despite this array of evidence, certain problems beset the proposed identity of TAD-synthetase with NAD pyrophosphorylase. For example, TRMP, at a concentration eight to ten times greater than its putative alternate substrate, NMN (2 mM), fails to inhibit the synthesis of NAD by hog liver NAD pyrophosphorylase (unpublished observation). This failure to engender competitive inhibition may, however, simply be a consequence of the comparatively low affinity of the catalytic center on this enzyme for

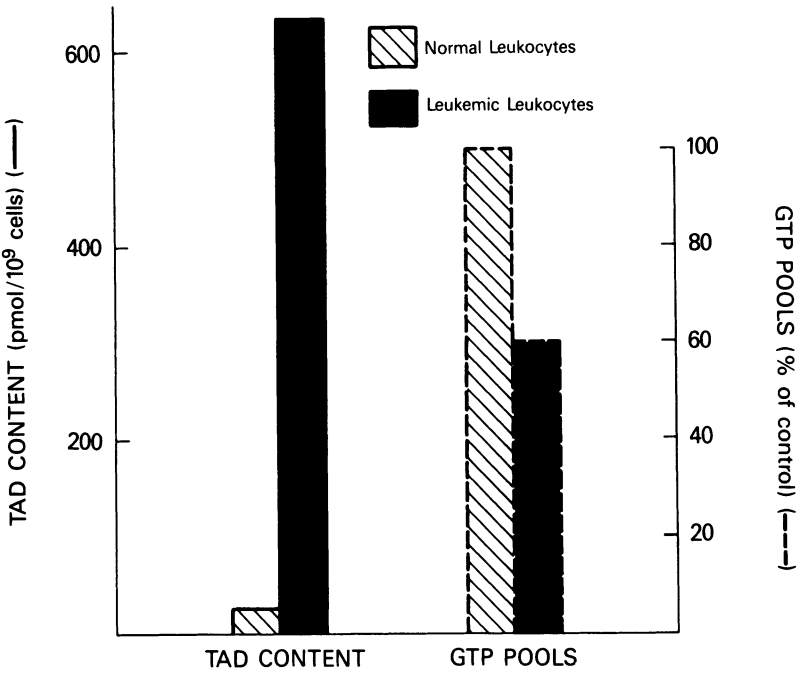

Figure 5. Metabolism of tiazofurin by normal and leukemic leucocytes; guanine nucleotide pool changes. Bone marrow aspirates from normal healthy volunteers or leukemic patients were diluted with RPMI 1640 medium containing 2 mM L-glutamine. For metabolism studies, leukocytes were incubated with 10 μM [5-^{3}H] tiazofurin or saline, and TAD formed by the cells (pmoles/10^9 cells/hr) was determined according to the technique described in [28].

For measurement of the influence of tiazofurin on guanine nucleotides, cells were incubated with 100 μM tiazofurin for two hours at 37°C, after which TCA extracts were prepared for HPLC analysis [28].

The leukemic leukocytes used comprised two groups: a) acute lymphocytic leukemia; and b) acute non-lymphocytic leukemia. The values for these subgroups did not differ significantly from the mean values presented in the figure.

Table 5. Kinetics and NAD pyrophosphorylase: NAD versus TAD synthesis.

Fixed Substrate	Varible Substrate	Product	K_m (mM)
NMN	ATP	NAD	~1.5
TRMP	ATP	TAD	~1.3
ATP	NMN	NAD	~1.3
ATP	TRMP	TAD	~2.6

Kinetic constants (K_ms) were calculated from double reciprocal plots of TAD and NAD synthesis catalyzed by a purified preparation of hog liver NAD pyrophosphorylase at appropriate concentrations of the fixed and variable substrates. The enzyme reactions were carried out in 0.01 M Tris-$MgCl_2$ buffer (pH 7.6) for one hr at 37°C. The reaction was stopped by heating for one minute at 95°C and the products analyzed on HPLC as described in [5] and [22].

Table 6. Influence of tiazofurin and other inhibitors of IMPD on NAD pools of liver; failure of these agents to exhibit synergy with streptozotocin.

Drug	Dose (mg/kg)	nmol NAD/g liver	% of Control
Saline	—	665 ± 43	100
Tiazofurin	400	665 ± 49	100
Mycophenolate	100	621 ± 26	93
Ribavirin	400	595 ± 65	89
Streptozotocin	200	298 ± 102	45
Tiazofurin + Streptozotocin	400, 200	261 ± 21	39
Mycophenolate + Streptozotcin	100, 200	236 ± 48	35
Ribavirin + Streptozotocin	400, 200	328 ± 92	49

Male BDF_1 mice were given intraperitoneal injections of either saline, tiazofurin, mycophenolate, or ribavirin, at the doses listed first in the table. One hour later, streptozotocin was administered by the same route. Two hours later livers were excised, flash frozen, and extracted with 5% perchloric acid. After neutralization with potassium bicarbonate, NAD was measured in the extracts spectrophotometrically using alcohol dehydrogenase [23].

tiazofurin-5′-monophosphate (table 5); NMN, with its lower K_m, does in fact inhibit the synthesis of TAD from an equimolar concentration of TRMP (5 mM) to a pronounced degree (80%).

Additionally problematic is our observation that even massive parenteral doses of tiazofurin (1,000 mg/kg) fail to alter the NAD pools of the subcutaneously grown P388 leukemia — a neoplasm which is known to accumulate substantial concentrations both of TRMP and TAD. Were the same enzymatic machinery required for both TAD and NAD synthesis it would be anticipated that its engagement in the former process would restrict the latter, leading to a fall in NAD pools. In fact, under certain experimental conditions tiazofurin does produce perturbations of the pyridine nucleotides. Thus Berger et al. have reported that prolonged exposure of L1210 cells to cytotoxic concentrations of tiazofurin will deplete NAD pools by interfering with NAD synthesis [30]. Additionally, these authors apparently have demonstrated that tiazofurin, via TAD, can interrupt the salvage of nicotinamide by inhibiting the phosphoribosyl transferase which activates the vitamin in several systems. More to the point, however, are the studies of Liepnieks et al. [31] which documented that administration of tiazofurin to rats bearing the Morris hepatoma 3924A diminished tumoral NAD pools in a dose responsive way without, however, influencing the concentration of that dinucleotide in liver. We have extended these findings by demonstrating that tiazofurin, at doses of 400–1,000 mg/kg, does not materially depress the NAD pools of mouse liver and also fails to potentiate the depression of hepatic NAD produced by streptozotocin, a nitrosourea well known for its ability to reduce pyridine nucleotide concentrations (table 6).

Table 7. Influence of tiazofurin on [^{14}C] nicotinamide metabolism in P388 sensitive and resistant cell lines.

Cell Line	Treatment	[^{14}C] Nicotinamide Incorportation				
		In Vivo[a] (nmoles/g)			In Vitro[b] (nomoles/10^9 cells)	
		NAD	NMN	N	NAD	NMN + N
P388/S	Saline	6.15	0.18	2.62	70.0	30.4
	Tiazofurin	2.42	0.16	2.55	57.5	25.0
P388/TR	Saline	2.81	10.9	2.97	11.0	232.0
	Tiazofurin	1.75	6.16	2.95	10.7	203.0

[a] BDF_1 male mice bearing subcutaneous nodules of P388/S or P388/TR (a line resistant to tiazofurin because of a nearly total deletion or repression of NAD pyrophosphorylase, described in detail in [24] and [25]) were pretreated with 400 mg/kg of tiazofurin or saline. After two hours, an 8 μCi dose of nicotinamide (N) (specific activity = 50 μCi/μmole) was administered intra-peritoneally to each mouse. Fifteen minutes later, tumors were excised and homogenized in 10% TCA (1 + 3, w/v). Each sample was spotted on Whatman 3MM paper, overspotted with cold NAD, NMN, and N and subjected to ascending chromatography (solvent: 70% ammonium acetate, 30% ethanol). The spots corresponding to NAD, NMN, and N were eluted and radioactivity measured by scintillation spectrometry.

[b] P388 sensitive or drug-resistant cell lines were incubated with or without 100 μM tiazofurin for two hours. Following the incubation, cells were pulsed for one hr with 100 μM [^{14}C] Nicotinamide, 15 μCi. At the end of this incubation, cells were harvested and immediately treated with 10% TCA. Acid extracts were neutralized by trioctylamine in freon and analyzed on HPLC [5, 22].

In contrast to the frequent fixity of NAD pools in the face of treatment with tiazofurin are the results of more dynamic experiments entailing the administration of labelled nicotinamide as a direct precursor of NAD. Taking this approach, we have documented that tiazofurin does interrupt the flux of nicotinamide into NAD both in vivo and, to a lesser degree, in vitro (table 7). Moreover, two hours after a dose of 2,000 mg of tiazofurin/kg, this same process was inhibited by approximately 50% and 75% in liver and muscle, respectively (unpublished observation). It remains to be established whether such interruption is mediated by inhibition of nicotinamide phosphoribosyl transferase, NAD glycohydrolase, NAD pyrophosphorylase, or some combination of these enzymes. Clear, though, is the conclusion that the dynamic approach (nicotinamide flux) gives the more coherent picture of the influence of tiazofurin on the metabolism of the pyridine nucleotides[2].

Synthesis of TAD by tissue slices. Obviously, the extent of accumulation of TAD is important in governing the sensitive or resistant nature of any given tumor to tiazofurin. Hampered by the difficulty of conducting in vivo measurements of this parameter on humans without the use of radiolabelled drug, we have attempted to monitor the synthesis of TAD in vitro, using slices of neoplastic nodules incubated with radiolabelled tiazofurin. HPLC analysis of neutralized acid extracts of these slices showed TAD to be

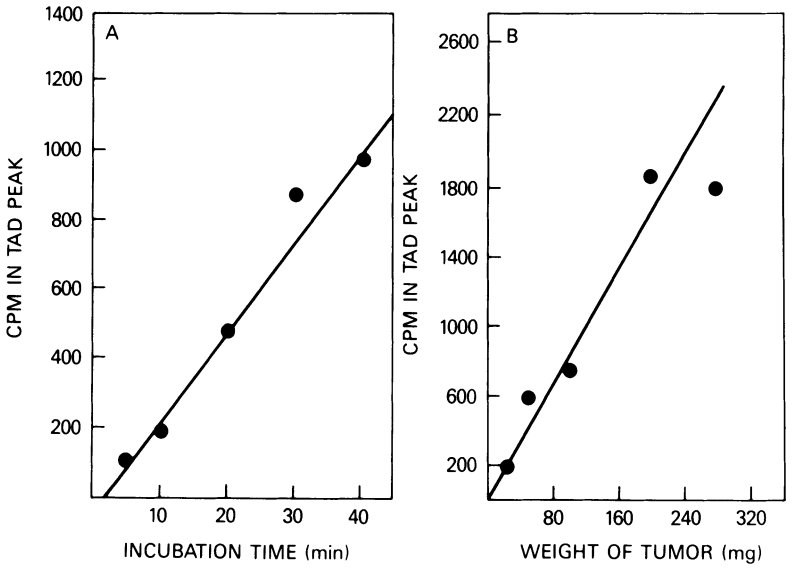

Figure 6. In Vitro synthesis of TAD by slices of P388 leukemia nodules. CDF_1 mice bearing subcutaneous implants of the P388 leukemia were killed by cervical dislocation. Tumors were removed and immediately immersed in chilled Delbecco's PBS medium containing 0.1% glucose. In order to simulate the conditions in which human tumor biopsy samples might be received, tumors were left in the medium on ice for 40–45 minutes before use. Tissue from five tumor samples was sliced and pooled, and the indicated amount of sample was taken randomly from this pool. Either 150 mg or 25–280 mg of sample weight was used for TAD synthesis shown in panel A or B, respectively. Tumor samples were incubated at 37° C for 30 minutes (panel B) or 5–40 minutes (panel A) in 500 μl of the assay mixture containing 7.5 φCi of [5-^{3}H] tiazofurin (final concentration of 10 μM) and 0.1% glucose in Dulbecco's PBS medium. The reaction was stopped by the addition of 10% TCA. Samples were homogenized, centrifuged at 12,000 × g and the supernatants obtained were neutralized with trioctylamine in freon. Neutralized samples were analyzed on HPLC, and radioactivity associated with the TAD was determined.

synthesized at a linear rate for up to 40 minutes of incubation (figure 6a) and with up to 200 mg of sample (figure 6b). (It is perplexing how these rates are maintained when, after incubation, no measurable amount of ATP is detectable on HPLC.) The usefulness of this in vitro method to human tumor biopsy samples and the correlation between TAD synthesized in vitro and the sensitive or resistant nature of a given tumor remain to be determined.

Catalytic versatility of NAD pyrophosphorylase. The forgoing results suggest that the NAD pyrophosphorylase(s) from different tissues may be endowed with different properties. Our further studies with a purified preparation of the enzyme from hog liver also have demonstrated its impressive catalytic versatility. Thus, as table 8 documents, in addition to substituting for the nicotinamide ring of NAD to form TAD, the thiazole-4-carboxamide moiety of tiazofurin could also behave as a quasipurine and substitute for

Table 8. Relative rate of dinucleotide synthesis by hog liver NAD pyrophosphorylase.

First Substrate (2.0 mM)	Second Substrate (3.33 mM)	Putative Product	Specific Activity[a]	% of NAD
NMN	ATP	NAD	5612	100.0
NMN	TRTP	NTD	3717	66.0
NMN	GTP	NGD	511	9.1
NMN	CTP	NCD	ND	0.0
NMN	UTP	NUD	ND	0.0
TRMP	ATP	TAD	3423	61.0
TRMP	TRTP	TTD	141	2.5
TRMP	GTP	TGD	29	0.5
TRMP	CTP	TCD	14	0.2
TRMP	UTP	TUD	ND	0.0

The enzyme assay was carried out using 2 mM concentrations of either NMN or TRMP and 3.3 mM concentrations of the above listed triphosphates in 0.01 M Tris-$MgCl_2$ buffer, pH 8.0. After a one hr incubation with hog liver NAD Pyrophosphorylas (250 μg) at 37° C the reaction was stopped by heating at 95° C for one minute. Reaction products were analyzed on HPLC as described in [5] and [22].

[a] Specific Activity — nmoles of dinucleotide synthesized/hr/mg protein.

ND: Not detectable.

adenine to yield nicotinamide thiazole-4-carboxamide dinucleotide (NTD) or for both adenine and nicotinamide to form thiazole-4-carboxamide thiazole-4-carboxamide dinucleotide (TTD).

The relative rates of synthesis of these dinucleotides are also presented in table 8. As expected, ATP and NMN were the most energetic substrates. Substitution of a single thiazole-4-carboxamide moiety (by the use of either TRMP or TRTP) reduced catalytic efficiency by approximately 40%. However, when tiazofurin replaced both the purine and pyridine moieties, as in the formation of TTD, the rate of synthesis was drastically reduced. (Nevertheless, as will be discussed in a later section, TTD does appear to be formed in vivo, albeit in minor amounts.) Other purine and pyrimidine nucleoside triphosphates were very poor substrates compared to ATP or even TRTP. Additional features of these reactions will be presented in a subsequent section.

Breakdown of TAD. It can be appreciated from figure 4 that the levels of TAD achieved in tumors are not static, but reach an apex several hours after administration of the drug and then decline. Our initial studies had linked this pattern of accumulation of TAD to the enzyme governing its synthesis namely, NAD pyrophosphorylase [25]. Further examination, however, showed that the enzymic degradation of TAD plays an equally important role in determining the net intracellular TAD content over time. The enzyme catalyzing this activity has been assigned the trivial name 'TAD phosphodiesterase' or 'TADase' [32]. Measurements of TAD phosphodies-

Table 9. Correlation of TAD biosynthetic and degradative enzyme activities in murine tumors and human lung cancer cell lines.

Source	Sensitivity to Tiazofurin	NAD Pyrophosphorylase[c] (nmoles/hr/mg protein)	TAD Phosphodiesterase[d] (nmoles/hr/mg protein)	Radio (c/d)
Murine Tumor[a]				
P388	S	75	24	3
Lewis lung	S	70	37	2
L1210	S	90	25	4
B16	R	47	100	<0.5
Colon 38	R	37	140	<0.5
M5076	R	17	67	<0.5
P388/TR	R	13	26	0.5
Human Cell Lines[b]				
H82	S	45	23	2
H417	S	74	13	6
H23	S	90	18	4
H146	R	15	87	<0.5
H125	R	41	85	<0.5
H249	R	5	78	<0.5

[a] Data obtained rom [22].
[b] Data obtained from [23].

terase in a panel of experimental tumors showed that the concentration of this enzyme is an important determinant of sensitivity or resistance to tiazofurin [22, 23]. Moreover, the ratio of NAD pyrophosphorylase to TAD phosphodiesterase in transplantable murine tumors or human cancer cell lines was found to be a still better prognostic variable, with values greater than two being indicative of susceptibility to the drug and values less than 0.5 being indicative of resistance (table 9).

Purification and properties of TAD phophodiesterase. In vitro, TAD was found to be degraded only *via* phosphodiesteratic cleavage yielding TRMP and AMP in equimolar amounts as the exclusive products. Other enzymes tested, namely alkaline phosphatase and NAD glycohydrolase, failed to attack TAD. As the importance of TAD phophodiesterase became evident, a rich source, subcutaneously growing Colon 38 tumor, was selected for its purification and characterization; the enzyme was found to be localized exclusively in the high speed supernatant fraction of tumor homogenates [32]. Following ammonium sulfate precipitation, the activity was purified 200 fold by a single step chromatography on DEAE-Sephadex. Surprisingly, the enzyme did not hydrolyze the most common synthetic substrate for the above class of enzymes (TMP nitrophenylester). It also failed to attack NAD energetically (table 10). Among the other natural phosphodiesters tested, NADH and ADP-ribose were found to be hydrolyzed almost as

Table 10. Degradation of natural phosphodiesters by TAD phosphodiesterase.

Phosphodiester	% of TAD Degradation	Degradation in the Presence of TAD (% of Control)
TAD	100	—
ADP-ribose	111	57
FAD	27	0
GDP-mannose	12	0
UDP glucuronic acid	ND	ND
CDP-choline	ND	ND
Nicotinic acid adenine dinucleotide	28	53
Nicotinamide hypoxanthine dinucleotide	23	6
NADPH	23	30
NADH	84	103
NADP	3	31
NAD	3	82

The enzyme assay was carried out using a 3 mM concentration of the phosphodiesters listed in the table in the presence or absence of 3 mM TAD in Tris-$MgCl_2$ buffer at pH 7.6. The enzyme was a partially purified preparation from the Colon 38 carcinoma [32]. After 30 minutes incubation at 37° C, the reaction products generated were analyzed on anion exchange HPLC [5, 22]. The amount of mononucleotides generated after the enzymatic hydrolysis was determined by comparison to the area under the curve of standard reference nucleotides, using a Waters Data Module® (Waters Associates, Milford, MA). Measurement of the generation of either product was found to be a reproducible index of enzyme activity. The specific activity of TAD degradation under the above conditions was 7.5 μmole/mg protein/hr. The specific activity of degradation of other diesters was compared with TAD and is represented in the table. ND: Non detectable (< 1.0%).

effectively as TAD (table 10). ADP-ribose, in addition to being an excellent substrate for the enzyme, also inhibited the degradation of TAD and vice versa. More detailed kinetic studies are required to evaluate the physiological consequences of TAD accumulation on the degradation of ADP-ribose and ultimately on the metabolism of NAD.

Analogs of TAD. Inasmuch the accumulation of TAD was found to be regulated in part by the intracellular concentration of TAD phosphodiesterase, an attempt was made to synthesize analogs of that dinucleotide which would be less susceptible to enzymatic hydrolysis. Dr. Victor Marquez of the National Cancer Institute has designed and synthesized such analogs embodying the modifications shown diagrammatically in figure 7. These chemically synthesized variants of TAD were then subjected to hydrolysis by TAD phosphodiesterase from Colon 38 and, for purposes of comparison, by venom phosphodiesterase [20, 32] (table 11). In these studies TAD was the most susceptible and its phosphonate analogs the most resistant of the

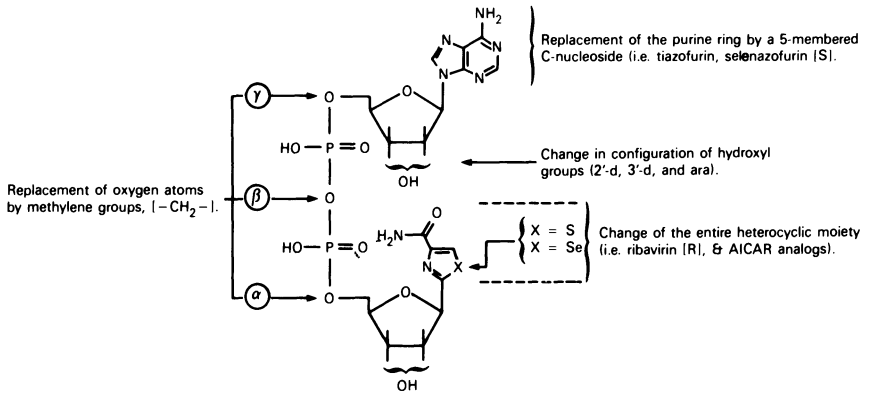

Figure 7. Summary of changes in the TAD molecule. Shown is the structure of the TAD molecule with tiazofurin (T) and adenosine (A) linked together by a pyrophosphate bond forming a dinucleotide (D). Various components of this molecule were chemically changed to give a series of TAD analogs. Replacement of adenine by the base of tiazofurin or selenazofurin (sulfur of tiazofurin replaced by selenium) resulted in 'TTD' or 'SSD' (both bases were replaced by selenazofurin). Replacement of the tiazofurin base of TAD by the base of selenazofurin resulted in 'SAD.' Change in the configuration of hydroxyl groups on the ribose attached to the adenine of TAD resulted in 2'dTAD, 3'dTAD, and araTAD. As indicated one or more oxygen atoms of TAD were replaced by methylene-(CH_2)-groups(s): replacement of 'O' on the adenine end of the molecule resulted in 'γ-methylene TAD'; similarly, replacement on the tiazofurin end resulted in 'α-methylene TAD.' Replacement of the middle oxygen of the TAD molecule by -CH_2-resulted in 'β-methylene TAD'. Replacement of both end 'Os' by -CH_2- in the TAD molecule resulted in 'α, γ-methylene TAD'. Replacement of the base of tiazofurin by the base of ribavirin (R) or AICAR resulted in 'RAD' or 'AICARAD.' The above listed TAD analogs were donated by Dr. Victor Marquez of the National Cancer Institute and were found to be greater than 90% pure on HPLC.

dinucleotides tested for attack by TAD phosphodiesterase. Indeed β-methylene TAD was completely refractory to hydrolysis by this enzyme. The order of degradation of the dinucleotides by venom phosphodiesterase was found to differ from the order observed with the tumoral enzyme, suggesting that the nature of the active site regulating the hydrolysis of these molecules is different on the two proteins.

Cytoxicity studies and inhibition of IMPD by TAD analogs. The oncolytic potential of any TAD analogs would depend on their IMPD inhibitory potency as well as their ability to penetrate cellular membranes intact [33]. When P388 cells growing exponentially in culture were exposed to appropriate concentrations of a panel of chemically synthesized analogs of TAD and growth rate inhibition measured over a 24 hour period, IC_{50} values ranging from 0.6 up to 100 μM were observed (table 12). These analogs also exhibited a wide range of inhibitory potency toward IMP dehydrogenase (table 12), but the two processes (cytoxicity and in vitro enzyme inhibition) did not vary in parallel.

Table 11. Degradation rate of NAD/TAD analogs by TAD phosphodiesterase as compared with snake venom phosphodiesterase.

Dinucleotide	Chemical Name	% of TAD Breakdown	
		TAD Phospho-diesterase	Snake Venom Phospho-diesterase
TAD	Thiazole 4-carboxamide adenine dinucleotide	100.0	100.0
SAD	Selenazole 4-carboxamide adenine dinucleotide	85.4	22.2
RAD	Ribavirin adenine dinucleotide	74.1	57.4
AICARAD	AICAR adenine dinucleotide	36.7	30.6
2′dTAD	Tiazofurin 2′deoxyadenosine[a] dinucleotide	76.2	73.1
3′dTAD	Tiazofurin 3′deoxyadenosine[a] dinucleotide	3.2	85.7
araTAD	Tiazofurin-arabinosyladenosine[a] dinucleotide	59.0	322.0
α-methylene TAD	Thiazole-4-carboxamide α-methylene adenine dinucleotide	10.4	27.2
β-methylene TAD	Thiazole-4-carboxamide β-methylene adenine dinucleotide	0.0	0.0
α, γ-methylene TAD	Thiazole-4-carboxamide α, γ-methylene adenine dinucleotide	1.5	240.0
γ-methylene TAD	Thiazole-4-carboxamide γ-methylene adenine dinucleotide	13.0	37.6
TTD	Thiazole 4-carboxamide thiazole 4-carboxamide dinucleotide	89.0	69.5
SSD	Selenazole 4-carboxamide selenazole 4-carboxamide dinucleotide	39.3	167.2
NAD	Nicotinamide adenine dinucleotide	1.8	3.2

NAD, TAD, or their analogs were subjected to either TAD Phosphodiesterase or snake venom phosphodiesterase degradation, at a substrate concentration of 7.5 mM, in 0.03 M Tris-HCl-$MgCl_2$ buffer, pH 7.6. Both hydrolytic products, (mononucleotides), were quantitated on HPLC using suitable standards, as described in the legend of table 10. Specific activity of TAD degradation by TAD phosphodiesterase and venom phosphodiesterase was 7.02 and 169.60 μmole/hr-mg protein, respectively.

[a] The inconsistencies in notation were adopted only to make unambiguous the location of the changes in ribose moiety.

Table 12. Cytotoxicity and IMPD inhibition of IMP dehydrogenase by TAD and its analogs.

	Cytotoxicity to P388 Cells in Culture	IMPD Inhibition
Dinucleotide[a]	ID_{50}[b] (μM)	IC_{50}[c] (μM)
TAD	7.5	0.5
SAD	9.8	0.05
RAD	40.0	250.0
AICARAD	78.0	>1,000.0
2'dTAD	9.0	2.0
3'dTAD	9.2	30.0
araTAD	10.5	10.0
α-methylene TAD	100.0	2.5
β-methylene TAD	45.0	0.2
α, γ-methylene TAD	56.0	26.0
γ-methylene TAD	2.8	17.0
TTD	4.6	300.0
SSD	0.6	200.0

[a] Abbreviated dinucleotides are described in table 11 and in the legend to figure 7.

[b] ID_{50}: Concentration required to inhibit growth rate by 50% over a 24 hour continuous drug exposure to P388 cells in culture. Growth inhibition studies were conducted as described in [24].

[c] IC_{50}: Concentration of inhibitor for 50% inhibition of partially purified tumoral IMP dehydrogenase activity. Procedural details were as described in [20].

It is important to note that the diesterase-resistant phosphonate analogs, although less potent than TAD, showed in vitro IMPD inhibition in the low micro-molar range. While it is unlikely that any of these dinucleotides will be able to pass the membranes of tumor cells with facility, it is reasonable to anticipate that by appropriate lipophilic modification, or even encapsulation in liposomes, they should acquire the capacity to enter cells. It is the focus of our present research endeavor to fashion just such modifications. If success can be achieved in this endeavor using a stable dinucleotide with a potent inhibitory K_i versus IMPD, it ought to be possible to control the synthesis of intracellular guanine nucleotides, even in cells deficient in the capacity to catalyze the de novo synthesis of TAD. Hopefully this development will yield positive therapeutic effects in turn.

Attempts to inhibit TAD phosphodiesterase. As was discussed earlier (figure 3), a reduced level of guanine nucleotides will have to be maintained for a sufficiently long period of time (possibly up to 24 hours) to ensure cell death. This result can only be achieved if the intracellular concentration of TAD is maintained above its K_i value for IMP dehydrogenase over this timespan. In order to increase the intracellular half-life of TAD, we have attempted to inhibit the enzyme responsible for degrading it. Towards this end, a wide range of pharmacological agents were studied including com-

Table 13. Inhibition of TAD phosphodiesterase.

Chemical Agent	% Inhibition (at 1 mM)	IC_{50}
Sodium fluoride	100	0.125 mM
Rose bengal	100	0.250 mM
Phloxine B	100	0.250 mM
Direct yellow-50	62	—
Chicago sky blue	47	—
Brilliant yellow	43	—
Erythrosin B	37	—
Acid yellow-38	35	—
Eosin Y	30	—
Evans blue	29	—
Eosin B	28	—
Trypan blue	23	—
Bromsulfophthalein	23	—

Non inhibitors of TAD phosphodiesterase	
Potassium pyroantimonate	Acid orange
Sodium arsenate	Methyl orange
Sodium diethyldithiocarbamate	Orange G
Sodium metavanadate	Fluorescein
Disodium ethylene diaminetetraacetate	Congo red
Sodium pyrophosphate	Neutral red
Papavarine-HCl	Indocyanin green
Theobromine	Janus green D
Methylene diphosphate	Bismark brown
Direct yellow-62	Acid yellow-40
Acid yellow-65	Methylene blue
Biliverdin	Bilirubin

A radiometric TAD phosphodiesterase assay was performed, as described in [23], in the presence or absence of 1 mM concentration of the agents listed above. The enzyme was a partially purified preparation from Colon 38 carcinoma [32]. Agents listed under 'Noninhibitors of TAD phosphodiesterase' showed less than 10% inhibition of the enzyme at 1 mM concentration.

pounds known to inhibit other phosphodiesterases, to interact with disulfide bonds, and to accomodate the dinucleotide fold of various dehydrogenases [34]. Among the agents tested, sodium fluoride was the simplest molecule to produce substantial inhibition of the enzyme, with an IC_{50} value of 0.125 mM (table 13). The classical cyclic nucleotide phosphodiesterase inhibitors, papavarine and theobromine, were inactive toward TAD phosphodiesterase. Among the various dehydrogenase-binding dyes tested, rose bengal and phloxine B were the most potent, with IC_{50}s of ~0.25 mM. Some other dyes also showed moderate inhibition. It would appear that none of these agents is of sufficient potency to warrant in vivo testing. Nevertheless, on conceptual grounds, the notion of prolonging the half-life of TAD by

Table 14. Metabolism of tiazofurin by mouse tissues after an intravenous dose.

	Concentration of Tiazofurin Nucleotides (nmoles/g tissue)		
Tissue	TRMP	TRDP	TRTP
Brain	8.0 ± 2.4	0.3 ± 0.1	0.4 ± 0.0
Liver	26.5 ± 7.0	3.3 ± 0.8	3.6 ± 0.8
Lungs	12.5 ± 1.6	0.1 ± 0.4	0.2 ± 0.03
Heart	17.6 ± 0.2	0.6 ± 0.0	0.2 ± 0.1
Spleen	16.6 ± 3.7	0.4 ± 0.01	0.4 ± 0.1
Pancreas	5.1 ± 1.2	0.3 ± 0.04	0.3 ± 0.03
Stomach	2.3 ± 0.2	0.3 ± 0.01	0.3 ± 0.01
Small Intestine	3.6 ± 0.9	0.2 ± 0.1	0.3 ± 0.1
Large Intestine	5.3 ± 1.4	0.2 ± 0.03	0.04 ± 0.01
Kidney	8.1 ± 0.4	0.4 ± 0.1	0.3 ± 0.01
Muscle	10.8 ± 0.5	1.0 ± 0.4	4.6 ± 2.7

Male BDF_1 mice were given an intravenous injection of tritiated tiazofurin, 100 mg/kg, specific radioactivity 10 μCi/mg of tiazofurin. One hour later, animals were killed and tissues processed as described in [18].

retarding its degradation is sufficiently attractive that further attempts to identify inhibitors with activity in the micromolar range seem fully warranted.

Secondary metabolites of tiazofurin

The metabolites of tiazofurin discussed under this catagory are either formed in vivo in minor amounts, relative to the primary metabolites detailed earlier, or have been detected only in vitro, for example, in enzymatic reaction mixtures.

Higher phosphates of tiazofurin; (tiazofurin-5'-diphosphate and tiazofurin-5'-triphosphate)

In common with most naturally occurring nucleoside-5'-monophosphates, tiazofurin 5'-monophosphate is energetically converted in liver, muscle, and other organs to its 5'-diphosphate and 5'-triphosphate in a dose-dependent way (tables 14 and 15). On the contrary, the higher phosphates of tiazofurin are not formed by P388 leukemia cells in vitro nor are they detectable in the subcutaneously grown nodules of this leukemia, in vivo, even after intraperitoneal doses of tiazofurin as high as 2,666 mg/kg, (table 15) [18].

Erythrocytic accumulation of several secondary metabolites of tiazofurin. Although most mammalian erythrocytes are slow to metabolize exogenous pyrimidines to their corresponding nucleotides, they do catalyze the

Table 15. Dose-responsiveness of the accumulation of tiazofurin phosphates in mice bearing subcutaneous P388 tumors.

Dose	Tissue	TRMP	TRDP (nmoles/g tissue)	TRTP
84 mg/kg	tumor	1.4 ± 0.4	0	0
	liver	1.1 ± 7.3	3.3 ± 4.7	4.0 ± 5.6
166 mg/kg	tumor	2.5 ± 1.2	0	0
	liver	24.9 ± 6.7	14.9 ± 4.5	31.1 ± 9.9
500 mg/kg	tumor	10.2 ± 1.4	0	0
	liver	115.9 ± 28.0	145.4 ± 36.5	236.4 ± 108.5
1333 mg/kg	tumor	19.6 ± 9.5	0	0
	liver	1208.0 ± 479.7	877.3 ± 333.6	928.6 ± 427.9
2666 mg/kg	tumor	47.5 ± 19.0	0	0
	liver	2051.0 ± 636.0	1130.4 ± 311.7	2045.4 ± 480.0

Two hours after the intraperitoneal injection of tritiated tiazofurin at the doses indicated, animals were sacrificed, tissue excised, flash-frozen, extracted with 10% trichloroacetic acid, and analyzed by HPLC [5, 8].

anabolism of certain purines at a meaningful rate. Indeed, red cells have been used to trap and phosphorylate the antitumor drug, tubercidin, in vitro prior to its intravenous administration, as a strategy for circumventing the powerful phlebotoxicity of that nucleoside given in free solution [35].

As table 16 documents, tiazofurin is also activity phosphorylated by the red cells of primates and rodents. In this respect again, the drug behaves as a kind of quasipurine. Noteworthy in this table is the additional finding that human erythrocytes accumulated comparatively large amounts of tiazofurin-5′-triphosphate, a finding which probably reflects a rich endowment with one or more nucleotide kinases. It is relevant that purine nucleosides were decisively superior to pyrimidine nucleosides as inhibitors of the phosphorylations of tiazofurin (table 16, note).

Table 17 documents that a single parenteral dose of tiazofurin (750 mg/m^2) was capable of engendering quite substantial levels of intra-erythrocytic nucleotides of the drug, mainly in the form of the 5′-monophosphate. Most important in these data is the finding that none of the species examined proved capable of generating TAD in their erythrocytes. This result is, in turn, an obvious consequence of the anuclear nature of these cells and of the well-known nuclear localization of NAD pyrophosphorylase, the enzyme thought to be principally responsible for the formation of TAD. (In this context, it will be of interest to determine whether the nucleated red cells of birds can convert tiazofurin to the dinucleotide.) Congruent with the observed failure of TAD formation in mammalian erythrocytes was the failure of in vitro exposure to tiazofurin (1 mM, one hr @ 37°) to produce any alteration whatsoever in the nucleotide pools of the red cells of the mouse, rat, monkey, or man (unpublished observations). Since both the rodent species in this study contain substantial IMPD activity (98 and 9 pmole/mg protein/hour in rat and mouse, respectively) and since both

Table 16. Metabolism of tiazofurin by red blood cells in vitro.

Species	Concentration of Drug or Metabolite (nmoles/g of cells)				
	TR	TRMP	TRDP	TRTP	TAD
Mouse	49.5	3.4	0.05	0.1	<0.01
Rat	103.0	8.4	0.05	0.1	<0.01
Monkey	86.0	31.0	0.9	0.8	<0.01
Man	164.0	9.5	2.0	10.5[a]	<0.01

Heparinized blood from mice and rats (male BDF_1 mice weighing between 20 and 25 gm and male Sprague-Dawley rats weighing between 250 and 300 gm) was obtained by cardiac puncture. Fresh blood from Rhesus monkey and human volunteers was obtained by venipuncture and transferred to heparinized tubes. Heparinized blood (5 ml, $5\text{-}8 \times 10^9$ cells/ml) from the four sources was incubated under sterile conditions with 5 ml of RPMI 1640 medium containing 3% L-glutamine and [5-^{3}H] tiazofurin (20 μCi; 10 μmoles) at 37° for 60 minutes. At the end of the incubation period a 1.5 ml aliquot of the reaction mixture was quickly centrifuged at 12,000 g for 0.5 minutes. The cell-pellet was then washed twice (1 ml each) with cold Hank's balanced salt solution, frozen, extracted with 0.5 ml of 5% perchloric acid, and centrifuged at 12,000 × g for 2 minutes. The supernatant was neutralized with KOH and an aliquot of the extract was chromatographed on a HPLC column of Partisil-10 SAX resin as previously described [5, 8].

[a] When fresh human erythrocytes were incubated with 375 μM [^{14}C] tiazofurin in the presence or absence of a panel of naturally occuring nucleosides (5 mM), and the extent of formation of TRTP measured on HPLC, the following percentages of inhibition were determined: deoxyguanosine: 91.3; deoxyadenosine: 88.5, deoxyinosine: 88.3; adenosine: 86.4; guanosine: 60.4; inosine: 40.0; thymidine: 32.9; deoxycytidine: 32.4; deoxyuridine: 23.2; uridine: 20.7; and cytidine: 3.4.

Table 17. Metabolism of tiazofurin by blood cells in vivo.

Species	Concentration of Drug or Metabolite (nmoles/g of cells)				
	TR	TRMP	TRDP	TRTP	TAD
Mouse	5.7	4.2	<0.01	<0.01	<0.01
Rat	32.2	3.0	<0.01	<0.01	<0.01
Monkey	17.0	5.1	0.5	<0.01	<0.01

Groups of three male BDF_1 mice (20–25 gm), Sprague-Dawley rats (300–350 gm), or Rhesus monkeys were injected intravenously with [5-^{3}H] tiazofurin (750 mg/m^2, specific radioactivity 1.042 mCi/mmol). 60 minutes later blood was collected by cardiac puncture in the case of mice and rats and from the femoral artery in the case of the monkeys into heparinized tubes, centrifuged at 1,200 g for 5 minutes, washed twice (one ml each) with cold Hank's balanced salt solution, and processed for HPLC analysis as detailed earlier [5, 8].

formed substantial concentrations of tiazofurin nucleotides, but none of TAD, these negative results underscore the contention that it is TAD, and probably TAD alone, which produces the state of guanylate deprivation.

Therapeutic implications of tiazofurin-5'-phosphates. Inasmuch as muscle, liver, and red cells constitute some of the largest tissue masses in the body, accumulation of the higher phosphates of tiazofurin in these sites might be

expected to provide a depot from which the drug could be slowly rereleased into the bloodstream to reach other target sites [13]. Some evidence for this contention is provided by in vitro studies with cultured P388 cells. For these experiments, human erythrocytes were incubated with 2 mM tiazofurin such that the net intracellular concentration of tiazofurin-5′-phosphates approximated 20 μM. Unmetabolized drug was removed by exhaustive saline washes, and graduated numbers of the "loaded" red cells were added to exponentially growing P388 lymphoblasts in complete RPMI 1640 medium. Erythrocytes processed identically, but never exposed to tiazofurin, served as controls. After 24 hours of incubation, the influence of unloaded and tiazofurin-loaded erythrocytes on the proliferation of the tumor cells was assessed by Coulter counting. These studies are illustrated in figure 8 and provide support for the notion that intracellular phosphates of tiazofurin are in dynamic equilibrium with free tiazofurin which, in turn, can exit the erythrocyte, enter the target lymphoblast, and there be anabolized to a cytotoxic species, presumably TAD.

Additional biochemical properties of the higher phosphates of tiazofurin. The most salient property of tiazofurin-5′-diphosphate and tiazofurin-5′-triphosphate is a negative one: neither compound is an effective inhibitor of IMPD (table 3). Thus, in conformity with the suggestion made in an earlier section, neither of these anabolites is likely to be involved in the principal pharmacologic effects of the drug. Nevertheless, these nucleotides do exhibit certain biochemical properties which may be relevant to the side effects and/or toxicities seen after the administration of tiazofurin.

Ribonucleotide reductase. Although tiazofurin-5′-diphosphate could, in theory, act as a substrate for ribonucleotide reductase, virtually none of the tiazofurin-5′-diphosphate peak on HPLC chromatograms is resistant to periodate-methylamine treatment (unpublished observation) and so is unlikely to include any significant amounts of the reduced (2′-deoxy) species. Nevertheless, direct measurements of deoxyribonucleotide pools in hepatomas treated with tiazofurin reveal that dATP and dCTP both experience significant dose-responsive depressions along with dGTP. dTTP, by contrast, rises to 200% of control after a dose of 200 mg/kg [31]. This kind of bidirectional response on the part of an enzyme known to be subject to complex regulation by its substrates and products [36] is compatible with the contention that administration of tiazofurin can both inhibit and stimulate ribonucleotide reduction; further studies will be needed to establish whether these effects are mediated by TRTP or stem, instead, from the sharply depressed pools of GTP which are the hallmark of this agent [8].

Nucleic acid polymerases. Treatment of tumor cells with tiazofurin produces a rather prompt arrest of both RNA and DNA synthesis, thought to be mediated by a drug-induced paucity of GTP and dGTP. Alternatively or

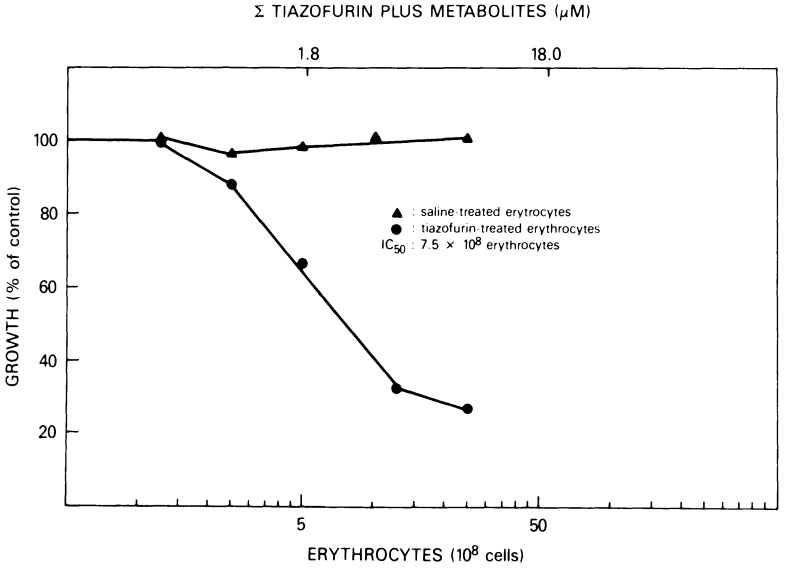

Figure 8. Cytotoxic potential of tiazofurin and its metabolites entrapped in human erythrocytes. Normal human peripheral blood (7 ml) was diluted with RPMI 1640 medium supplemented with 2 mM L-glutamine and 1,000 U sodium heparin. Diluted blood was incubated for four hours with 2 mM [2-^{14}C] tiazofurin (specific radioactivity 0.095 mCi/mmole) or saline at 37° C in an atmosphere of 95% air and 5% CO_2, washed twice with RPMI 1640 medium containing 10% bovine serum and 2 mM L-glutamine, and then suspended in the same buffer at 4° C for 16 hours to allow for the free tiazofurin to diffuse out of the red cells. The cells were again washed twice, suspended in the same medium, and then used for the measurement of metabolites and determination of cytotoxicity. TCA extracts of the red cell suspension obtained after incubation with tiazofurin exhibited a concentration of TR, TRMP, TRDP, and TRTP of 1.9, 1.3, 6.4, and 7.6 nmol/10^9 cells. This yields a net concentration of tiazofurin plus its metabolites of 17.2 nmoles/10^9 cells of which free drug constituted 11%. The IC_{50} (that number of loaded erythrocytes inhibiting cell growth by 50%) was calculated by comparing the growth of P388 cells in the presence of red cells incubated with saline or tiazofurin. For the cytotoxicity measurements, 5 ml of P388 lymphoblasts in the exponential phase of growth (1 to 3×10^5 cells/ml) in RPMI 1640 medium containing 10% donor serum, 2 mM L-glutamine and 5 μM 2-mercaptoethanol were incubated for 24 hours with 25–500 μl aliquots of the red cell preparations described above for 37° C in an atmosphere of 95% air and 5% CO_2. Tumor cells were enumerated on a Coulter counter after stromatolysis of erythrocytes with Zaponin. Under the growth conditions used, the cells exhibited a doubling time of 11–13 hours.

additionally, it is possible that the higher phosphates of tiazofurin could directly inhibit RNA or DNA polymerase. (This cannot be the case in P388 lymphoblasts, since these cells fail to form the higher phosphates of the drug.) Little direct evidence is available on this score with the appropriate mammalian enzyme. We have, however, exposed the reverse transcriptase from avian myloblastosis virus to tiazofurin-5′-triphosphate and observed, at most, 25% inhibition of the enzyme, at a 1 mM concentration of the putative inhibitor (TRTP). Although this degree of inhibition is not impressive in the case of this retrovirus, it is still possible (indeed likely) that the higher

phosphates of tiazofurin will be implicated in the activity of the drug against other viruses towards which it is inhibitory.

NAD pyrophosphorylase. Described in an earlier section was the finding that the flux of labelled nicotinamide into NAD is curtailed in tumor, liver, and muscle after the parenteral administration of tiazofurin. Inasmuch as the latter two of these tissues accumulate substantial concentrations of tiazofurin-5′-triphosphate, we entertained the speculation that this molecule might contribute to the retardation of nicotinamide flux by inhibiting NAD pyrophosphorylase. Direct measurements of this possibility were made with the purified enzyme from hog liver, using concentrations of TRTP ranging from 8 μM to 8 mM, at a fixed ATP-$MgCl_2$ concentration of 1 mM[3]. In view of the fact that TRTP is known to function as an alternate substrate for NAD pyrophosphorylase, it was anticipated that it would inhibit the enzyme. Surprising, though, was the potency of the inhibition seen in these experiments, the IC_{50} being 280 μM. Since the levels of TRTP achieved in vivo can be substantial (tables 14 and 15), it is possible that this metabolite may make a contribution to the inhibition of nicotinamide flux produced by tiazofurin.

Kinases and phosphotransferases. Tiazofurin-5′-triphosphate is equipped with the same high energy phosphate esters as ATP and should, for that reason, be theoretically capable of participating in at least some enzymatic phosphorylations. Indeed, as table 18 documents, all of the kinases examined accepted TRTP as a substrate, although the velocities achieved with this nucleotide proved to be widely variable, ranging from feeble activity in the case of creatine kinase to vigorous relative phosphorylation (compared to ATP) in the case of GMP kinase. A representative depiction of this activity over a wide substrate range (0.06–1 mM) is shown in figure 9.

When the ability of tiazofurin-5′-triphosphate to inhibit this panel of kinases in the presence of an equimolar concentration of ATP was examined, noteworthy inhibition was seen only with nucleoside diphosphate kinase (56%) (table 19). Since tiazofurin-5′-triphosphate is known to accumulate to substantial levels in skeletal muscle (table 14) and since administration of tiazofurin to human produces oftentimes severe myalgia [37], it is tempting to implicate inhibition of this enzyme as a factor contributing to the drug's derangement of energy metabolism and toxicity to muscle.

The failure of the triphosphate of tiazofurin to inhibit GMP kinase establishes that the depression in GDP which follows administration of the drug is attributable solely to a restriction in the availability of its precursor, GMP, and not to a retarded rate of conversion of this nucleotide to its higher phosphates. The observed failure of TRTP to inhibit uridine kinase is also in accord with the results of Karle et al. [12], who failed to observed any inhibition of uridine/cytidine kinase by tiazofurin-5′-triphosphate, de-

Table 18. Comparision of substrate activity of TRTP and ATP towards select kinases.

Enzyme	E.C. Number	Substrate Activity (nmole of substrate converted/hour) TRTP	ATP	Relative Activity (% of TRTP/ATP)
GMP kinase	2.7.4.8	136.1	90.6	150
Nucleoside monophosphate kinase	2.7.4.4	138.5	127.9	108
Hexokinase	2.7.1.1	123.5	132.4	93
Uridine kinase	2.7.1.48	49.2	55.1	89
Acetate kinase	2.7.2.1	89.4	124.5	72
Myokinase	2.7.4.3	48.7	90.4	54
Fructose-6-phosphate kinase	2.7.1.11	334.1	740.0	45
Nucleoside diphosphate kinase	2.7.4.6	23.5	107.2	22
Creatine kinase	2.7.3.2	2.3	90.6	3

The substrate activity of select kinases was examined using ATP-$MgCl_2$ or TRTP-$MgCl_2$(5 mM) as substrates. In addition to ATP or TRTP the reaction mixtures, in a total volume of 25 µl, contained the following:
GMP kinase: 5 µl of 0.1 M triethanolamine, pH 7.6; 5 µl of 10 mM GMP or water; and 5 µl of GMP kinase from hog brain (specific activity 10 U/mg, 20 U/ml).
Nucleoside monophosphate kinase: 5 µl of 0.1 M triethanolamine pH 7.6; 5 µl of 10 mM UMP or water; and 5 µl of NMP kinase from beef liver (specific activity 0.5 U/mg, 20 U/ml).
Hexokinase: 5 µl of 0.5 M Tris buffer, pH 9.0; 5 µl of 1 M dextrose or water; and 5 µl hexokinase from yeast (specific activity 140 U/mg, 2 U/ml).
Uridine kinase: 5 µl of 0.1 M triethanolamine buffer, pH 7.6; 5 µl of 25 mM uridine or water; and 5 µl of crude extract of P388 tumor (specific activity 197 nmol/mg protein/hr).
Acetate kinase: 5 µl of 0.5 M Tris buffer, pH 8.5; 5 µl of 1 M sodium acetate or water; and 5 µl of acetate kinase from *E.coli* (specific activity 200 U/mg, 20 U/ml) from Boehringer Corp., Indianapolis, IN).
Myokinase: 5 µl of 0.1 M triethanolamine, pH 7.6; 5 µl of 10 mM AMP or water; and 5 µl of myokinase from rabbit muscle (Sigma Chemical Co., St. Louis, MO; 5,000 U/mg, 20 U/ml).
Fructose-6-phosphate kinase: 5 µl of 0.5 M Tris buffer, pH 8.5; 5 µl of 50 mM fructose-6-phosphate or water; and 5 µl of fructose-6-phosphate kinase from rabbit muscle (specific activity 130 U/mg protein, 2 U/ml).
Nucleoside diphosphate kinase: 5 µl of 0.1 M triethanolamine, pH 7.6; 5 µl of 10 mM dTDP or water; and 5 µl of NDP kinase from beef liver (specific activity 80 U/mg, 20 U/ml).
Creatine kinase: 5 µl of 0.5 M Tris buffer, pH 8.5, 5 µ of 1 M creatine of water, and 5 µl of creatine kinase from rabbit muscle, (specific activity 800 U/mg, 20 U/ml).
Reaction mixtures were driven together by acceleration to 12,000 × g in an Eppendorf centrifuge, and incubated at 37° C for 30 minutes; the reaction was terminated by heating at 95° C for one minute. The reaction products were then diluted with 120 µl of water and an aliquot of 100 µl was analyzed on the Partisil 10 SAX column of a Waters HPLC as detailed earlier, using an ammonium phosphate buffer system [5,8]. For the computation of results, the substrate and enzyme-dependent appearance of TRDP and ADP were calculated from the areas under their respective peaks.

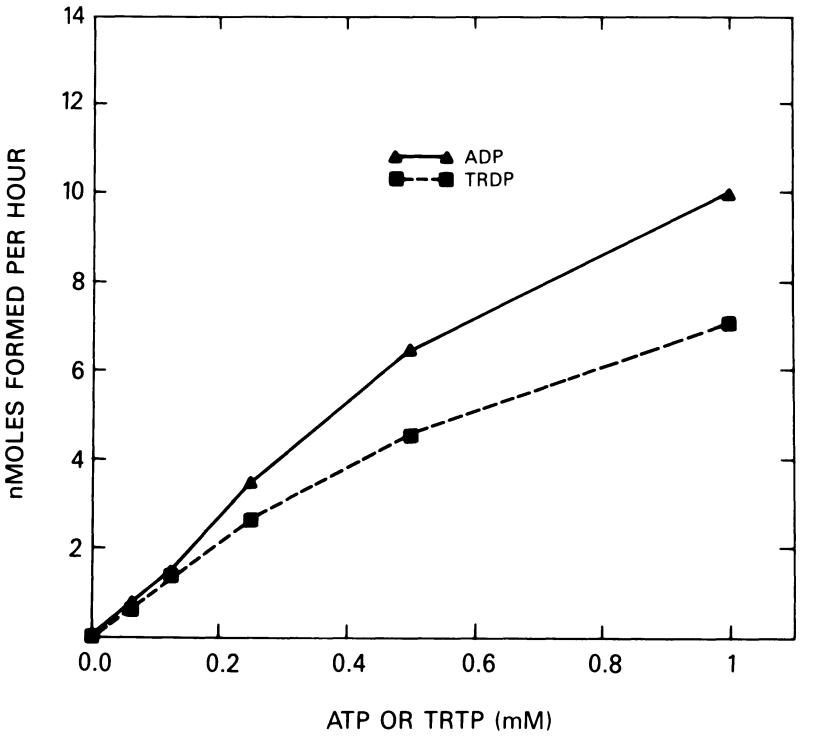

Figure 9. Kinetics of acetate kinase. Enzyme activity was determined by using either ATP or TRTP as substrates at concentrations ranging from 0.063 mM to 1.0 mM in an assay buffer containing 0.04 M triethanolamine (pH 7.4), 0.04 M KCl, 2 mM $MgCl_2$, 0.14 M sodium acetate, and 100 mU of acetate kinase from *E. coli* (specific activity 200 U/mg). After a 45 minute incubation at 37°C, the reaction was stopped by heating for one minute at 95°C. Reaction products were analyzed on anion exchange HPLC as described in [5] and [10]. Concentrations of ADP or TRDP formed were determined by comparing the areas under these peaks to the corresponding reference standards using the automated data module of the Waters HPLC system.

spite the fact that the salvage of uridine was depressed in drug-treated L1210 cells.

Minor dinucleotide metabolites of tiazofurin

In an earlier section, the catalytic versatility of NAD pyrophosphorylase was discussed in some detail and mention was made of the fact that the enzyme can form dinucleotides in which thiazole-4-carboxamide replaces adenine, nicotinamide, or both bases (table 8). For these studies, TRTP replaced ATP, the customary triphosphate in the NAD pyrophosphorylase reaction, and NMN or TRMP were utilized as the complimentary monophosphates. Affinities (Kms) for these alternate substrates were in the 2 mM range. In confirmation of the proposed structures, both reaction products were found

Table 19. Inhibition of select kinases by tiazofurin-5′-triphosphate.

Enzyme	Percent Inhibition Compared to Control Activity With ATP as Substrate
Nucleoside diphosphate kinase	55.8
Fructose-6-phosphate kinase	32.6
Nucleoside monophosphate kinase	28.6
Creatine kinase	27.8
Acetate kinase	22.5
Myokinase	14.8
Uridine kinase	12.5
Hexokinase	12.2
GMP Kinase	6.5

Enzyme activity was measured at an ATP-$MgCl_2$ concentration of 5 mM, in the presence or absence of equimolar TRTP-$MgCl_2$. Other experimental details are given in the legend to table 18.

to be wholly refractory to hydrolysis by alkaline phosphatase from *E. coli*, but were quantitatively decomposed to the anticipated 5′-monophosphates by venom phosphodiesterase. Figure 10 illustrates the susceptibility of NTD to such attack.

Further confirmatory evidence for the postulated dinucleotide structure of NTD was provided by molecular analysis using fast atom bombardment (FAB) mass spectrometry. The mass spectrum of enzymically synthesized NTD is displayed in figure 11. FAB mass spectra of dinucleotides are characterized by cleavage of the phosphate-ester linkages. Viewed in concert, the molecular ions and ion fragments formed on fast atom bombardment of NTD support the conclusion that it is a dinucleotide, composed of tiazofurin and nicotinamide in phosphodiester linkage.

Bearing, as it does, the pyridine ring which normally functions as proton acceptor in dehydrogenase systems, NTD should, at least in theory, be capable of participating in enzymic hydrogen transfer reactions. A direct experimental examination of this possibility established that the fraudulent dinucleotide was functional in both the alcohol dehydrogenase and IMP dehydrogenase reactions (figure 12), as adjudged by its conversion to a strongly acidic species (which eluted in the general area of NADH) in the presence, but not absence, of the appropriate cosubstrate, ethanol or IMP (figure 13). However, the rates of these conversions were, under optimal conditions, no more than 5% of the rates measured with NAD as cofactor. TTD was, as anticipated, inert in both of these systems.

Attempts to identify NTD in the tumor, liver, or muscle of mice given intraperitoneal injections of [^{14}C] nicotinamide along with [^{3}H] tiazofurin have not, so far, met with success. This result probably is a reflection of the failure of NAD pyrophosphorylase to make efficient utilization of TTP in the presence of excess amounts of its natural substrate ATP.

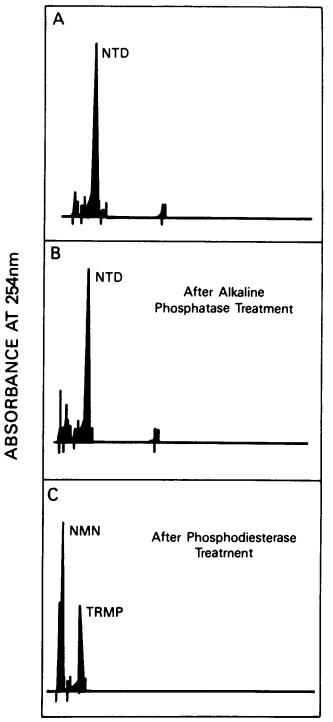

Figure 10. Enzymatic identifiction of purified nicotinamide thiazole-4-carboxamide dinucleotide (NTD). Purified NTD was treated with either alkaline phosphatase in 0.5 M Tris, 5 mM $MgCl_2$ pH 8.4, or with snake venom phosphodiesterase in 0.5 M Tris, 5 mM $MgCl_2$, pH 9.0 for four hours at 37° C. The reaction was stopped by heating for one minute at 95° C and products analyzed on HPLC as described in [5] and [10]. Retention times for NMN, NTD, and TRMP were 2.51, 5.86 and 6.28 minutes, respectively.

In contradistinction to NTD, TTD has been detected in the livers of mice treated with high-dose labelled tiazofurin (1,000 mg/kg). As figure 14, panel A illustrates, this homodinucleotide would have been overlooked if HPLC analyses had been utilized to monitor its presence because of coelution with TAD. However, chromatography on Hamilton HAX4 resin (figure 14B) with a steep gradient of ammonium formate (0-6 M) resolved TTD from TAD and permitted isolation of the former compound in quantities sufficient for identification. Like other members of its family, TTD resisted attack by alkaline phosphatase but was decomposed by venom phosphodiesterase to yield tiazofurin-5′-monophosphate as the sole identifiable product in ~80% yield. Since synthetic TTD (generously provided by Dr. V. Marquez) coeluted with the biosynthetic molecule from mouse liver, it is concluded that this dinucleotide is, in fact, formed in vivo, at least in liver. However, concentrations of TTD in that organ were never found to exceed 1 μM, and even this level was only demonstrable after the administration of the deliberately enormous dose of tiazofurin of 1,000 mg/kg at those times

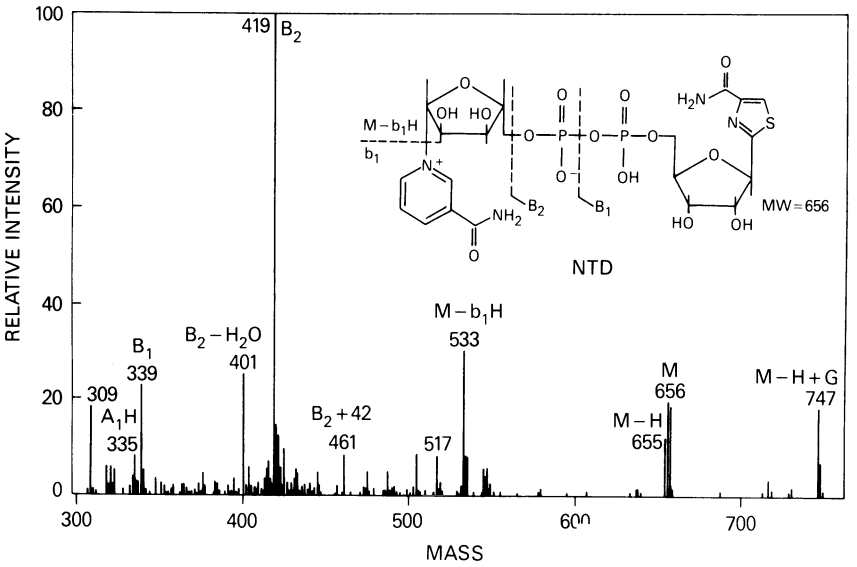

Figure 11. FAB mass spectrum of purified NTD. NTD was synthesized enzymatically by incubating NMN, TRTP, and purified hog liver NAD pyrophosphorylase in 0.03 M Tris-$MgCl_2$ buffer, pH 7.6, for six hours. NTD was isolated from the reaction products using a semi-preparative anion exchange column, as described for the purification of TAD [20]. The FAB mass spectrum was obtained essentially as described in [20].

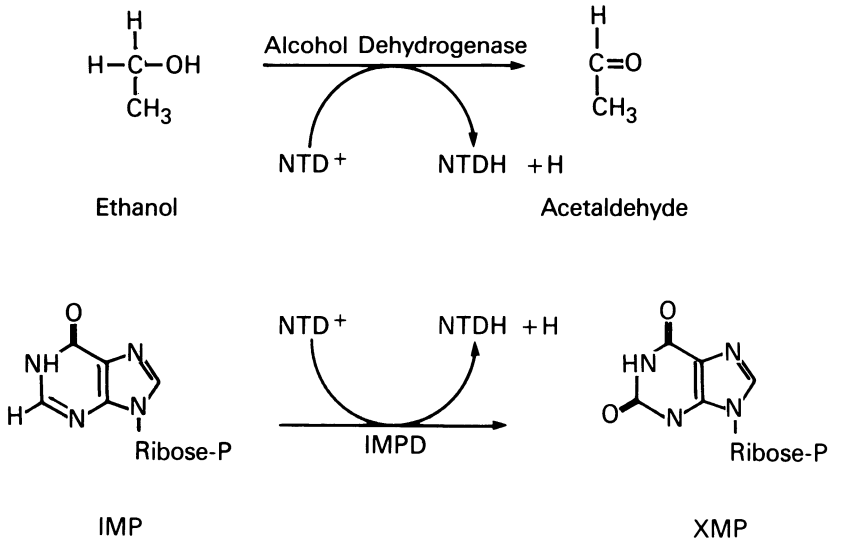

Figure 12. Enzymatic reactions for the conversion of NTD to NTDH.

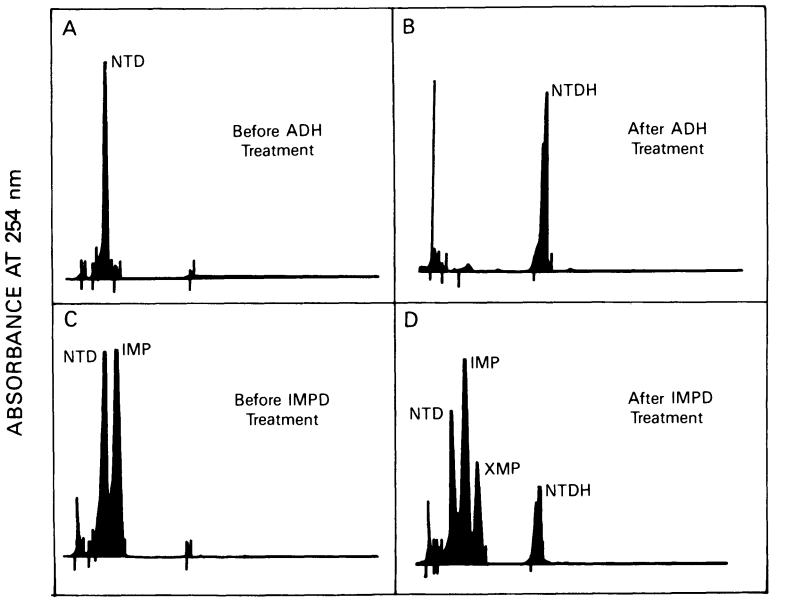

Figure 13. Enzymatic conversion of NTD to NTDH by alcohol dehydrogenase or IMP dehydrogenase. NTD was treated either with IMPD (*Escherichia coli*) in buffer containing 0.05 M Tris, 2 mM DTT, and 0.8 mM IMP, or with ADH (yeast) in buffer containing 100 mM sodium pyrophosphate, pH 8.7, 100 mM semicarbazide, 30 mM glycine, 230 mM ethanol, and approximately 2.5 μg of the enzyme. After incubation for two hours at 37° C, the reaction was stopped by heating for one minute at 95° C. Reaction products were analyzed on anion exchange HPLC as described in [10]. Retention times for NTD, IMP, XMP, and NTDH were 5.88, 7.76, 9.55, and 18.20 minutes under the HPLC conditions used.

when its precursor, TTP, was experiencing its apex. This scarcity and the absence of any known or biochemically important properties make it probable that TTD makes little or no contribution to the pharmacologic effects of tiazofurin.

Thiazole-4-carboxamide adenine dinucleotide-2'-phosphate (TADP)

Since the 2'-hydroxyl of the adenosine moiety of TAD is unesterized, it ought to be susceptible to phosphorylation at this site in a reaction analogous to the conversion of NAD to MADP. As in the case with NAD and NADP, the resulting triphosphorylated dinucleotide (TADP) might be expected to have properties quite distinct from those of TAD. For this reason, we sought to determine whether, in the first place, the phosphorylation of TAD is enzymatically feasible, and, secondly, whether the product of such phosphorylation, TADP, could be identified in extracts of tissues from experimental animals treated with tiazofurin.

Using a partially purified preparation of NAD kinase (NADK) from liver and ATP-Mg^{++} as phosphoryl donor, it was possible to demonstrate that a

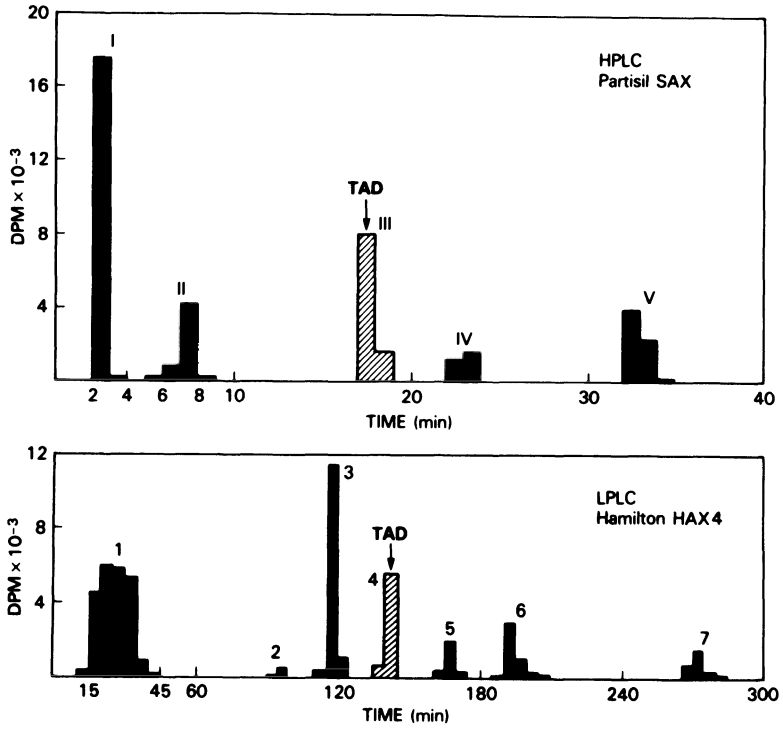

Figure 14. Purification of TTD.

Upper Panel: HPLC analysis of an acid extract of the liver of a mouse given a single intraperitoneal injection of [^{3}H] tiazofurin, (1,000 mg/kg, 100 μCi/mouse), four hours before sacrifice.
I: Tiazofurin, II: TRMP, III: TAD, IV: TRDP, V: TRTP

Lower Panel: LPLC (low pressure liquid chromatography) of the extract described in the upper panel, using Hamilton HAX4 resin and a linear gradient of 0 to 6 M ammonium formate pH 3.9.
1) Tiazofurin, 2) Thiazole-4-carboxylic acid riboside, 3) TRMP 4) TAD, 5) TTD 6) TRDP, 7) TRTP.

novel product could be formed in vitro from [^{14}C] TAD (figure 15). This product was more negatively charged than the parent dinucleotide, as would be expected from the acquisition of a new phosphate ester; treatment with alkaline phosphatase reconverted it to TAD, whereas venom phopshodiesterase decomposed it to yield two major products, one exactly coincident with tiazofurin-5′-monophosphate, the other exactly coincident with adenosine 2′-,5′-diphosphate. Mass spectral analysis of enzymatically synthesized TADP revealed the anticipated molecular ions and many identifiable fragments (figure 16a). These results are compatible with the suggestion that NAD kinase can convert TAD to TADP. Noteworthy is the finding that the rate of TADP synthesis was sluggish (5%) compared to that measured with the natural substrate, NAD. Additionally, the affinity of the enzyme for

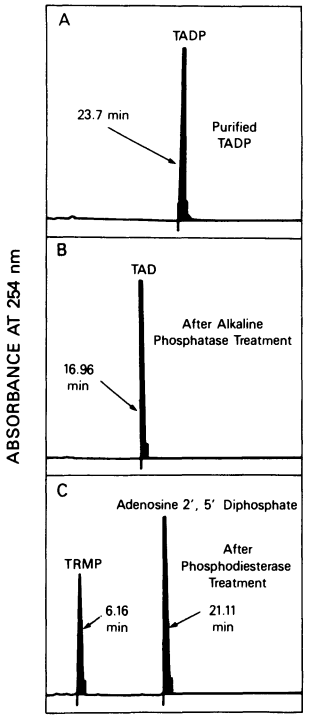

Figure 15. Enzymatic identification of purified TADP. Enzymatic identification of TADP was carried out in essentially the same way as for NTD, described in the legend to figure 10.

TAD was substantially lower than its affinity for the pyridine nucleotide (Kms of 33.3 mM for TAD versus 15.4 mM for NAD).

Like TAD, TADP exhibited the capability to inhibit IMPD (figure 16b); however, its K_i, with NAD as variable substrate, was approximately one order of magnitude less potent than that of TAD. Both dinucleotides exerted noncompetitive inhibition. Versus two other representative dehydrogenases (isocitrate and glutamate), 100 μM TADP exerted only negligible inhibition (<10%). Such specificity is reminiscent of that exhibited by TAD.

Attempts to identify TADP in mouse liver, muscle, erythrocytes, or subcutaneous P388 tumor nodules have, so far, been unsuccessful despite the infusion of massive doses of tritiated drug. This failure is doubtless an indication of the miniscule rate of formation of this dinucleotide in vivo.

Conclusion

Phase I trials with tiazofurin have just been concluded. Although a large number of pharmacokinetic studies were conducted during those trials, little

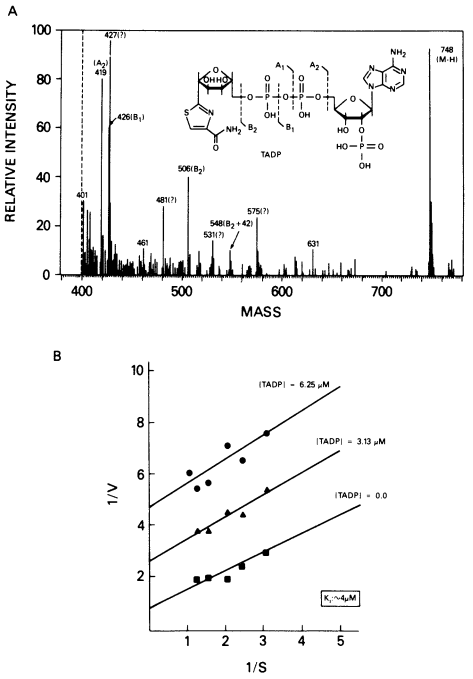

Figure 16. Mass Spectrum of TADP; inhibition of IMPD by TADP. TADP was synthesized from TAD and ATP-$MgCl_2$ by the agency of a partially purified preparation of hepatic NAD kinase. After purification by low-pressure chromatography on Hamilton HAX4 resin and exhaustive desalting, the FAB mass spectrum (A) was obtained essentially as described in [20]. Panel B illustrates the apparently noncompetitive inhibition of IMPD from P388 cells by this dinucleotide with NAD as the variable substrate.

information on the metabolism of the drug was generated, principally because of the need to use radiolabelled compound to detect the metabolites in question and, additionally, because of the habitual difficulty of obtaining relevant, i.e., neoplastic, tissue specimens. Although human cancer cells in continuous culture have provided some information on the general genetically stable capability of tumors from man to synthesize and destroy TAD [23], the use of fresh tissue slices for this purpose, described for the first time in an earlier section of this chapter, may provide a more relevant tool for estimating this parameter. It may also be possible to monitor that TAD levels in biopsies from phase II recipients of tiazofurin, after its conversion to fluorescent ethenoderivatives, by treatment of the appropriate extracts with chloroacetaldehyde [38]. Only until measurements of this kind are made will it be possible to determine whether tiazofurin is being metabolized in man in such a way as to achieve effective tumoricidal depletion of guanine nucleotides.

Acknowledgment

The authors would like to extend their thanks to Dr. David Johns for his encouragement, to Dr. Victor Marquez for providing synthetic standards and substrates, to Dr. James Kelley for FAB mass spectrometric measurements, to Ms. Yvonne Wilson for her preparation of the typescript, and to all of the other collaborators whose hard work made this chapter possible.

Notes

1. Sokoloski et al. [39] have also demonstrated that treatment of Sarcoma 180 cells with tiazofurin (100 μM) brings about a lowering (by 57%) of pools of GDP mannose, an effect which is followed by derangements glycoprotein synthesis and membrane functions.
2. In this connection it is perplexing that in the studies of Berger et al. [30], with cultured L1210 cells, NAD pools fell progressively upon exposure to tiazofurin, whereas the flux of labelled nicotinamide into NAD was not retarded until 24 hours of exposure to the drug had elapsed. In this case, then, the static approach (measurement of NAD pools) was more revealing than the dynamic.
3. In these analyses, [^{14}C] ATP was used, so that any NAD formed was radiolabelled. At the end of incubations, the nucleotides present were quantitatively converted to nucleosides with alkaline phosphatase. [^{14}C] NAD remained intact under these conditions. The labelled dinucleotide was then separated from the [^{14}C] adenosine either by ascending paper chromatography (Whatman 3MM, isopropanol: toluene: water, 320/40/40, v/v/v) or by adsorption onto DEAE discs, followed by exhaustive water washes. This approach to the measurement of NAD pyrophosphorylase accomodates large numbers of samples and is considerably more facile than the spectrophotometric assays [24].

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4. Extragonadal and central nervous system germ cell tumors: A review of diagnostic and therapeutic strategies

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Neoplasms arising in germ cells are relatively uncommon, but are highly curable malignancies. The incidence of primary testicular tumors ranges from approximately 2.1 to 3.1 cases per 100,000 males in the United States and Great Britain [1]. Amongst those cases of germ cell tumors about 1% to 2% have an extragonadal origin. These tumors possess a similar spectrum of pathologies as their primary testicular counterparts [2–6] and include all types of seminomas and nonseminomas. A scheme of the interrelationship of these neoplasms was developed by Teilum [7] and is depicted in figure 1.

Several controversies surround the subject of extragonadal germ cell tumors (EGGCT) including their pathogenesis and etiology, the question of 'burned out' testicular primaries, the necessity for orchiectomy, and the observation that these tumors may be more refractory to the standard chemotherapeutic regimens used in primary testicular cancer. This review will attempt to provide a review of EGGCT's including their origin, clinical syndromes, past therapeutic strategies, and further directions of treatment.

Origin of extragonadal germ cell tumors

Although genetically determined at conception, the testis does not acquire male characteristics until the seventh week of development. In the four week embryo, gonadal or genital ridges form on either side of the midline. They are comprised of coelomic epithelium and condensation of underlying mesenchyme. The primordial germ cells (PGC) arise within the yolk sac (endodermal origin). They migrate along the dorsal mesentery of the hind-gut toward the genital ridges. By the sixth week, the PGCs invade the genital ridges (GR), during which time the GR is proliferating and forming the primitive sex cords [8]. These, in turn, migrate to the hilum of the testis forming testis cords, which eventually form the tunica albuginea and the rete testis. The urogenital ridge extends from the level of the sixth cervical vertebra to the second sacral vertebra. The location of these structures within the embryo and fetus help to elucidate the locations that EGGCTs will appear in later in life.

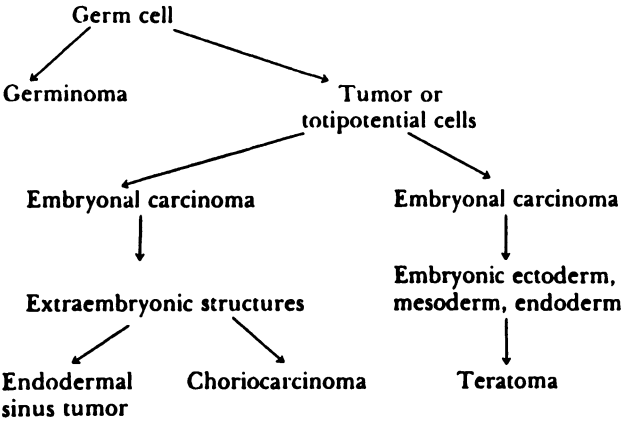

Figure 1. Interrelationship of pathological types of germ cell malignancies (classification according to Teilum). [From: Teilum G: Classification of endodermal sinus tumors (mesoblastoma vitellinum) and so-called embryonal carcinoma of the ovary. *Acta Path Microbiol Scand* (64): 407–427, 1965, with permission.]

Knowledge of the migration patterns of the primordial germ cells is essential in understanding theories of the pathogenesis of EGGCTs. There are two major schools of thought regarding the origin of these tumors. Schlumberger [9] examined 16 teratomas, both benign and malignant, and concluded that these tumors arise from pluripotent primordial germ cells which become located in extragonadal sites during embryogenesis. These PGCs become arrested in primitive stages and do not develop into normal, mature testes, maintaining the potential to develop into teratomata. He also postulated that those teratomata in the anterior mediastinum developed from thymic anlage which also had the ability to differentiate. Pugsley and Carleton [10] support Schlumberger's notion of the derivation of mediastinal tumor from thymic anlage. They studied two cases of mediastinal teratoma and found thymic tissue immediately adjacent to and intimately related to the malignancy. In one tumor a nodule of germinomatous tissue was found, thus supporting the fact that these tumors evolve from primordial germ cells located in the thymus.

Friedman [11] noted that primary testicular seminoma can have nonseminomatous metastases, whereas the converse was not true. He, therefore, proposed a developmental pattern where the germinoma (seminoma) is a precursor of embryonal carcinoma. The embryonal carcinoma, in turn, has biphasic potential and can develop either along trophoblastic lines to choricarcinoma or somatic lines to teratoma. He felt that primordial germ cells are misplaced during fetal development and that these give rise to tumors at the different locations at which they occur. Dixon and Moore [12], however, suggest that seminoma is a sideline of development and not in the direct line of differentiation from primordial germ cells.

Although the above theories provide a framework in which EGGCTs can

develop and account for the midline location of these tumors, there is still dispute in the literature as to whether tumors truly develop in extragonadal sites or have primary testicular lesions which are inapparent.

Prym [13] in 1927 described a patient with widespread choriocarcinoma who at postmortem had a small scar within the testicle. He felt that the scar within the testicle represented the primary lesion, having involuted during the course of the malignancy. Stowell et al. [14] dispute the significance of this scar as have other authors over the years [4].

Rather et al. [15] described six new cases and reviewed 18 other cases with widespread germ cell neoplasms including choriocarcinoma, seminoma, and anaplastic carcinoma. All six of the newly reported cases had testicular scars or fibrosis, with two of the six having areas of active seminoma within the testicular scar. The other four cases had interstitial cell hyperplasia (two cases), hyalinized tubules (one case), or fibrosis (two cases). Similarly, in their review, seven of 18 testes examined had foci of seminoma. They postulated that at one time the testicular primary had rapid growth giving rise to widespread metastases. These primary lesions then matured forming scars, cystic structures, and tubular changes. Additional reports [16] have shown small primary lesions (2 mm in the testis) with widespread systemic metastases. This raised the argument that all patients with EGGCT should have radical orchiectomy with 2 mm section of the testis to rule out a testicular primary.

Azzopardi and his colleagues [17, 18] observed discrete changes in the testes of patients with seminomatous and nonseminomatous tumors. In seminomas, he found that the testis contained lesions consisting of centrally necrotic lesions (aggregated degenerated tumor cells) surrounded by histiocytes and fibroblasts. Immunohistochemical studies showed heavy concentration of phospholipids (a characteristic of seminoma cells) and presence of calcium carbonate, mucopolysaccharide, and lipofuchsin. This was similar to other histologic reports of 'scars' in testicles of patients with extragonadal seminoma. In patients with extragonadal nonseminomas, peculiar hematoxophil deposits were noted within seminiferous tubules and were felt to have formed as a result of necrotic neoplastic tissue which proliferated rapidly and infarcted. Among these cases of non-seminomas, microscopic areas of seminoma and teratomatous elements were noted. These changes were distinct from patients who did not have testicular tumors but had changes within the testes for benign reasons. These pathological changes further supported previous hypotheses that EGGCTs had their origin in the testes and were not truly extragonadal primaries.

In 1965, Abell et al. [19] put together a series of 14 patients who developed retroperitoneal seminomas with normal testicles on clinical examination. Of these 14 patients, three subsequently developed testicular primaries and two had testicular asymmetry, both of whom underwent orchiectomy and had negative pathology. Seven patients had normal testes, one had an undescended testicle, and one was lost to follow-up. As a result

of his evaluation of these 14 patients, he felt that there was truly an entity of extragonadal retroperitoneal seminoma and defined several criteria for making the diagnosis: (1) the presence of nonneoplastic gonadal tissue in the capsule of, or adjacent to the neoplasm; (2) an encapsulated neoplasm without lymph node involvement; and (3) a high retroperitoneal neoplasm with adjacent lymph nodes positive, but without neoplasm in lower aortic, iliac, or pelvic lymph nodes.

Luna and Valenzuela-Tamariz [20] performed an autopsy series of 20 germ cell tumors of the mediastinum. All 20 had serial sectioning of the testes performed. Four patients had tubular atrophy and interstitial cell hyperplasia, one patient had a microscopic focus in the testicle, and one had a fibrous scar in the testicle. They also reviewed a large series of Greiling et al. [21] where 220 cases of primary testicular cancer were examined at autopsy. No case had mediastinal metastases as the only site of metastatic disease. From their data they conclude that mediastinal germ cell tumors were unlikely to have a primary testicular source, but that retroperitoneal primaries were much more likely to have a gonadal source.

Many other cases have been reported in the literature where patients were felt to have a normal clinical exam, but once orchiectomy or postmortem was performed, there were obvious abnormalities or malignancy within the testis (15,19,22–29). Whether or not orchiectomy should be performed once the diagnosis of extragonadal neoplasm has been made remains controversial. Some authors feel that clinical management does not necessarily change if a patient is not orchiectomized and continued careful examination would suffice. For academic purposes, however, most agree that the only way to definitively rule out a testicular primary is by orchiectomy and serial section. Instances of multiply recurrent EGGCT should alert the physician that there may be an occult testicular primary and orchiectomy is warranted [27].

In the last several years, however, new and safe noninvasive techniques have been developed to aid in ruling out testicular primaries in patients with EGGCTs and normal physical examinations. In 1978, Sample et al. [30] used Gray scale ultrasound to evaluate patients with probable testicular pathology. They examined 55 patients, 14 of whom had normal clinical testicular exams. Of these fourteen, there were seven with pain and seven with retroperitoneal or mediastinal masses and no pain. Two occult testicular neoplasms were discovered in the group with extragonadal disease and two nonmalignant lesions were found in the group with pain. This technique was also very accurate in assessing testicular versus extratesticular origin of scrotal masses. Kirchling of the Mayo Clinic [31] used real time ultrasound in assessing six patients with EGGCT and normal physical exam. Four out of six ultrasound examinations showed testicular abnormalities, one of which was an area of fibrosis and calcification which may have represented a burned-out lesion, and the other three all proving to be malignant at orchiectomy.

Another technique that has been used to evaluate testicular pathology is scrotal thermography [32]. In this technique the scrotum hangs in front of an aluminum shield, preventing interference from heat of the thighs. After equilibrating with the environmental temperature, photographic images are made using an infrared scanner and comparison between right and left testicle are made. In 48 normal patients the authors never saw greater than one degree Fahrenheit difference between the two testicles. In 16 patients there was an abnormal infrared emission. The mean difference between left and right was 6.4 degrees Fahrenheit. Seven patients had tumors, with four seminomas and three feminizing interstitial cell tumors. Three of these patients had clinically normal testes. Only one patient (with a virilizing interstitial cell tumor) had a false negative examination. It, therefore, may prove to be a very useful tool in evaluating testicular pathology.

With the increasing use of thee noninvasive techniques, our detection of occult testicular primaries will improve, thereby sparing more and more patients the psychological and physical pain of orchiectomy. It also should help to define populations of patients studied with EGGCTs as these cases have important clinical considerations, as will be discussed later.

Clinical syndromes

Extragonadal germ cell tumors have been shown to originate in many foci throughout the body. Specific signs and symptoms are associated with the location of the primary as well as with the type of tumor. The most common sites of EGGCT include the mediastinum and retroperitoneum. Other sites of occurrence include the vagina [33,34], liver [35], stomach [36,37], presacral region [38,39], and face [40]. Additionally, seminomatous and nonseminomatous tumors are among the most common midline central nervous system malignancies, accounting for greater than 50% of pineal and suprasellar malignancies [41].

Serum tumor markers including alpha-feto protein (AFP) [42–44], beta subunit of human chorionic gonadotrophin (hCG) [45–46], and lactate dehydrogenase (LDH) [47] have all been associated with EGGCTs of all histologic types. A case report by Lee and associates [48] also shows a possible correlation of calcitonin level with the amount of tumor burden in a patient with a seminoma, hypercalcemia, and bony metastases. They have similar prognostic and therapeutic implications with these tumors as with their primary testicular counterparts. They correlate with the regression and growth of disease [24] and, additionally, aid in helping to make the diagnosis of EGGCT when there is no obvious source and equivocal pathology or an undifferentiated carcinoma [49,50]. AFP and hCG have also been shown to correlate with germ cell tumors of the pineal gland both when measured in the serum and cerebrospinal fluid (CSF) [45,51,52]. Assays of the CSF for these respective proteins can oftentimes aid in the diagnosis of tumors in this

region, where tissue is not as accessible for biopsy. These markers may also presage the development of CNS metastases before they are clinically or radiographically apparent [51].

Mediastinal and retroperitoneal presentations

The most common sites of EGGCT include the anterior mediastinum and retroperitoneum. Mediastinal EGGCTs comprise approximately 1% of all tumors in this region [53]. Symptoms of these tumors are similar to other malignancies in the same location, most often presenting with chest pain, of either a pleuritic or substernal nature, dyspnea, hoarseness, cough, hemoptysis (especially with embryonal carcinoma or choriocarcinoma), gynecomastia (with choriocarcinoma), a chest wall mass, or supraclavicular adenopathy. Superior vena cava syndrome has been reported in many large series [54] and has an incidence ranging from as low as 0% to as high as 25% in some series [55, 56]. Occasionally these tumors are asymptomatic and are discovered on routine chest x-rays.

Retroperitoneal tumors, because of their location, usually grow to very large proportions before being detected and have an array of symptoms including back and/or flank pain, abdominal fullness or discomfort, non-specific gastrointestinal complaints such as bloating, nausea, vomiting and constipation, left supraclavicular masses, fevers, sweats, and weight loss.

The mean age of diagnosis in most series is in the middle of the third decade [23–28] and the range of ages is from 14–55. The third and fourth decades have the highest incidence. There is a strong male predominance in all series, although several series report the rare occurrence of these malignancies in females [2, 5, 57, 58]. These reports are quite rare however. In one series of primary thymic germ cell malignancies [59], there were four benign teratomata and seven cancers, including malignant teratoma, embryonal carcinoma, and seminoma. All four benign tumors were seen in females and all malignancies occurred in males. All malignant histologies have been seen in females [5] except for endodermal sinus tumors of the mediastinum, which have been seen exclusively in males.

The literature is replete with case reports [4, 5, 60–62] and, additionally, several large series of EGGCTs have been reported [2, 6, 19, 26, 28, 42, 55–59, 63–74]. Table 1 outlines a summary of many of the largest series of mediastinal and retroperitoneal germ cell tumors. Seminomas are the most common, accounting for 40% to 50% of all EGGCTs. Of the nonseminomas, embryonal carcinoma and teratocarcinoma account for approximately two-thirds of tumors, with choriocarcinoma, endodermal sinus tumors, and mixed tumors comprising the remaining third.

When compared to testicular primaries, EGGCTs of the mediastinum and retroperitoneum exhibit clinical behavior that separates them from primary testicular malignancies. These differences in metastatic patterns and

Table 1. Frequency of different histologies in extragonadal germ cell tumors.

Author [reference]	Pathology of Tumor					
	Seminoma	Embryonal	Chorio*	EST**	Ter***	Mixed
Cox [2]	28	5	2	—	7	3
Woolner et al. [6]	2	—	—	—	1	1
Abell et al. [19]	10	—	—	—	—	—
Johnson and Laneri [26]	6	1	3	—	4	1
Montague [28]	1	3	—	1	—	—
Kuzur et al. [42]	—	—	—	10	—	—
Recondo and Libshitz [55]	5	1	1	—	4	6
Vogelzang et al. [56]	—	—	—	3	4	5
Hurt et al. [57]	17	—	—	—	—	4
Martini et al. [58]	10	4	4	—	10	2
Bergh et al. [59]	2	—	—	—	8	—
Buskirk et al. [63]	12	—	—	—	—	—
Cortes-Funes et al. [64]	4	4	1	—	2	2
Dittler et al. [65]	3	—	—	3	—	—
Feun et al. [66]	4	6	3	—	4	1
Garnick et al. [67]	1	8	—	3	—	2
Hainsworth et al. [68]	6	9	7	—	7	—
Jain et al. [69]	15	—	—	—	—	—
Mendini et al. [70]	8	—	—	—	—	—
Raghaven and Barrett [71]	6	—	—	—	—	—
Schantz et al. [72]	21	—	—	—	—	—
Utz and Buscemi [73]	7	3	2	—	—	5
Lee and Jackson [78]	6	—	—	—	—	—

* Choriocarcinoma
** Endodermal sinus tumor
*** Teratoma

clinical syndromes may play a role in determining the poorer prognosis associated with these tumors. An interesting observation has been the high incidence of bony metastases seen in tumors of these locations. This is especially true with seminomas, but has been noted with all histologies. Luna and Valenzuela-Tamariz [20] reported an autopsy series of 18 truly extragonadal malignancies and found an incidence of nine patients with bony metastases (50%). A similar analysis of primary testicular lesions [75] disclosed only a 13% (12/80) incidence of metastasis to bone. In the several large series that specify patterns of metastatic spread [2, 56, 58, 66–68, 76], the incidence may be as high as 25%. Seminomas appear to metastasize to bone more frequently than nonseminomas [58]. EGGCTs, otherwise, have similar metastatic patterns to primary testicular cancer with involvement of lung, liver, lymph nodes, central nervous system, kidneys (choriocarcinoma), and other sites.

A second important feature of these tumors which determines clinical

behavior is their bulk at presentation. Testicular tumors are felt to be confined by the tunica albuginea and therefore do not grow to very great size in the primary lesion [71]. The location and ease of examination of the testes also accounts for these tumors being discovered in earlier stages. EGGCTs, on the other hand, have no anatomical confining structures to limit growth, and therefore, most invade contiguous structures and are very large before symptoms develop and a diagnosis is made. Very few of these malignancies are encapsulated when discovered, also imparting poorer prognosis. In the earliest series, where good local control was obtained and patients had long disease-free survival without the use of chemotherapy [72], the tumors were smaller, more likely encapsulated, and more amenable to either surgical resection or inclusion within a relatively small radiation port. Even in later series, using radiation predominantly as primary therapy, smaller tumors were more likely to be controlled with radiation alone, while more locally advanced tumors required chemotherapy [78]. Bulk of germ cell tumors was shown to be a poor prognostic feature by Einhorn [79] in a randomized study of patients with primary testicular tumors. Bulk of disease is also one of the factors purported to carry a poor prognosis in an early study examining the treatment of EGGCTs with cisplatin-based chemotherapy [66].

Associated syndromes

Several other clinical syndromes have been associated with mediastinal germ cell tumors. Idiopathic thrombocytopenia has been reported by Garnick and Griffin [80] in three patients with EGGCT (out of a total cohort of 22 patients). These patients all presented with large mediastinal masses and thrombocytopenia before any treatment with chemotherapy. No clear etiology was ever found, and none of the three patients' counts responded to steroid treatment or splenectomy. Counts recovered once the underlying malignancy was treated.

An unusual hematologic malignancy was reported in association with EGGCTs by Nichols et al. [81] (two patients with megakaryocytic leukemia and one patient with a myelodysplastic syndrome with a prominent megakaryocytic component). The patients presented with pancytopenia and bone marrow infiltration with hematopoietic blast cells. Development of these conditions was felt to be unrelated to chemotherapy, as they had an early presentation after therapy was completed and lacked the typical cytogenetic features of treatment-related leukemia. The development of hematologic abnormalities solely in mediastinal germ cell tumors supports the theories of Schlumberger, which postulate the presence of pluripotent stem cells located extragonadally as giving rise to these malignancies. These stem cells may have a predisposition to give rise to hematologic malignancies as well, accounting for the association of two very rare malignancies in a single patient.

Klinefelter's syndrome (47, XXY), has been associated with an increased incidence of malignancies, most notably breast cancer [82]. Only recently, a similar association has been observed with Klinefelter's syndrome and EGGCT. Storm et al. [83] reported the first case of mediastinal choriocarcinoma in a 16-year-old boy with Klinefelter's syndrome (KS). Sogge et al. [84] reviewed the literature of KS and EGGCT and reported two additional cases of their own, one of choriocarcinoma and one of endodermal sinus tumor. At that time (1979), four other testicular primary tumors and four extragonadal tumors had been reported. Interestingly, of the 24 reported cases in the literature of mediastinal choriocarcinoma, two were in patients with KS. The authors postulate several reasons why this association might exist. Firstly, in fibroblasts from patients with an XY/XXY mosaic [85] there is a demonstration of increased in vitro transformation of fibroblasts. Additionally, reports of nuclear sex of testicular malignancies (teratomata and embryonal carcinoma) and mediastinal teratomata show a substantial number of cases having female chromosomes. Since KS has the XXY pattern, it is more likely that their malignancy would contain 46, XX than the chromosomes of a normal male [86]. Since Sogge's review, other cases have been reported, and a more recent review [87] cites 20 cases of EGGCT and KS. Seven of these cases were choriocarcinoma — a very high percentage, considering that as of 1976 there were only a total of 23 cases of mediastinal choriocarcinoma in the literature. Again chromosomal imbalance is postulated as a cause. Duplication of chromosomes with tetraploidy and double X and Y chromosomes have been noted in testicular tumor karyotypes and are felt to occur relatively frequently. There is a possibility the extra X chromosome in the extragonadal germ cells of patients with KS may cause an increased propensity to undergo malignant degeneration. Kaplan [88] in a cytogenetic analysis of EGGCTs found a mediastinal tumor with a 92, XXYY phenotype. Other EGGCTs appearing in infants did not display such abnormalities. Another postulate proposed by Schimke et al. [87] is that patients with KS have dysgenetic testes. Dysgenetic undescended testes have increased malignant potential, and there may be dysgenetic germ cells in the mediastinum, pineal gland, and other locations, increasing the risk of KS patients to develop EGGCT. Because of the high hCG levels associated with choriocarcinoma, prepubescent patients with choriocarcinoma and KS all undergo precocious puberty. (The hCG stimulates the testes to produce increased amounts of testosterone.) Patients who develop EGGCTs, especially choriocarcinoma, should be examined for KS, and conversely, patients with KS should be watched very closely for the development of these malignancies.

Therapy

The therapeutic modalities for EGGCTs, as for primary testicular germ cell tumors, can be divided into precisplatin and postcisplatin eras. Before the

use of cisplatin in the treatment of testicular malignancies, prognosis was very poor, unless there was a tumor which was sensitive to radiation, such as seminoma, in which case there was a chance for long-term disease-free survival. Likewise, in EGGCT, in most early case reports of nonseminomatous disease [4, 60–62] the tumors were unresponsive to chemotherapy and patients died of widespread metastasis. Even in series of radiosensitive tumors, adequate local and distant control could only be obtained if tumors were encapsulated, relatively small [19, 63, 72], and amenable to surgical resection [74]. It became clear that through the late 1970s most series of EGGCTs had long-term survival predominantly in seminomas [2, 28, 55, 58, 70, 73, 89] with a few long-term survivors in nonseminomatous tumors [26, 43, 59, 65].

Despite relatively good survival of primary extragonadal seminomas, careful analysis was performed in several series treated primarily with radiation therapy. Cox [2] showed that all seminomas treated with radiation therapy had good local control regardless of dose (but at least 2,000 rads), but distal spread was only seen in patients receiving less than 4,000 rads (two out of six patients). In nonseminoma, good local control was obtained in two patients with embryonal carcinoma who received doses of at least 4,000 rads. Both patients had long-term disease-free survival. Hurt [57] found that by giving 3,000–3,500 rads, most patients (15/17) with pure seminoma had good control and that distant metastases were the cause of death. He outlined poor prognostic features, including superior vena cava syndrome, hilar involvement, and age over 35 and recommended the use of chemotherapy in those patients, as well as in patients with mixed seminomatous/nonseminomatous histology. Lee et al. [78] treated six mediastinal seminomas primarily with radiation therapy. Although they report six out of six patients with no evidence of disease at 24 months or beyond, one patient with locally advanced disease recieved concomitant cisplatin chemotherapy, and one patient had a bony relapse, which was also treated primarily with radiation, achieving a second remission.

Once cisplatin's efficacy against germ cell tumors was discovered, many patients with EGGCTs were treated with cisplatin containing regimens. Early case reports [90] were encouraging, with two of two patients achieving long-term complete remission (CR) despite being heavily pretreated. However, Feun et al. [66] reported dismal results in 19 patients with EGGCTs treated with combination cisplatin, vinblastine, and bleomycin (PVB). Three of 19 patients achieved CR of less than two months duration; six patients achieved partial response (PR), which were also of short duration; and eleven patients had no response. Median duration of all responses was two months. Several features of the patient population including (a) heavy pretreatment before entry onto study, limiting dose of cisplatin received; (b) bulky disease; and (c) poor performance status, all bode poorly for the patients studied. Garnick et al. [67], likewise, noted a very poor response rate using PVB, especially in patients with mediastinal disease.

They reported a CR rate of 10/15 patients with extragonadal neoplasms, with six relapsing relatively early. Six out of eight mediastinal primaries failed to achieve CR. Retroperitoneal disease with visceral involvement also had a poor prognosis with eight out of eight dying of disease. Only those patients with retroperitoneal disease had a similar response and disease-free survival when compared to similarly treated testicular primaries.

Cortes [64] reported a 46% disease-free survival of greater than six months using PVB therapy in mediastinal germ cell tumors. This was a decreased response when compared to testicular and ovarian patients treated with the same drugs in their hands.

Vogelzang [56], in a series of twelve nonseminomatous tumors of the mediastinum, had a long term disease-free survival of 25% (3/12). In his series all three long-term survivors had surgical resection of residual disease following chemotherapy, prompting him to advocate surgical exploration and debulking following definitive chemotherapy.

Kuzur [42] evaluated a series of ten endodermal sinus tumors, of which one patient was alive without evidence of disease at five years, despite the use of cisplatin-based therapy in seven of the ten. This earmarked this malignancy as one with an especially poor prognosis among EGGCTs. He pointed out that stage for stage, this histology had a worse prognosis than testicular or ovarian tumors with the same histology.

Hainsworth et al. [68] presented the largest series (at that time) of patients with EGGCT treated with PVB or PVB plus adriamycin chemotherapy. They excluded endodermal sinus tumors because of their poor prognosis and reported 21 out of 31 patients achieving a CR, including five of six who had received previous radiation. Eighteen of the 21 continue with no evidence of disease. The other ten patients all had PRs with no long-term survivors. This demonstrated similar survival to testicular patients with advanced stage disease. Choriocarcinoma and undifferentiated carcinoma were the only lesions with a poor prognosis. The therapy given in this study was much more intensive than that used by Feun and the overall performance status was better as well, possibility accounting for the differences in results.

Jain et al. [69] report a series of extragonadal seminomas, again demonstrating the importance of cisplatin based regimens when compared to older forms of treatment. Ten of 11 patients treated with VAB-6 (vinblastine, actinomycin-D, bleomycin, cisplatin, and cyclophosphamide) or PVB were in continued CR, whereas only five of ten patients were without evidence of disease using surgery, radiation therapy, and non-cisplatin-based chemotherapeutic regimens. The importance of cisplatin in extragonadal seminoma was further borne out in a second paper from Memorial Sloan-Kettering [91] which showed a 90% disease-free survival in these tumors treated with VAB-6.

Despite the improved survival with VAB-6 and PVB therapy, a substantial number of patients still were not achieving CR, especially those with choriocarcinoma and endodermal sinus tumor of mediastinal origin.

Table 2. Results of combination chemotherapy in extragonadal germ cell tumors.

Author (reference)	Number (N) of patients	N CR*	N PR**	N SD***	N PD****	N with long-term remission
Kuzur et al. [42]	10	1	5	—	4	1
Vogelzang et al. [56]	12	7	2	—	3	3
Cortes-Funes [64]	14	6	4	—	4	5
Feun et al. [66]	19	3	6		8	0
Garnick et al. [67]	15	10	—	—	5	4
Hainsworth et al. [68]	31	21	10	—	—	18
van Hoesel and Pinedo [90]	2	2	—	—	—	2
Logothetis et al. [92]	49	29	NA	NA	NA	28

* complete response
** partial response
*** stable disease
**** progressive disease

Logothetis et al. [92] report their series of 49 patients treated with a variety of chemotherapeutic regimens. From their results, which include a 57% overall disease-free survival rate, three of five patients with endodermal sinus tumor are alive with no evidence of disease and three of seven patients with choriocarcinoma are alive, free of disease. The patients with mediastinal endodermal sinus tumor who survived were all treated with alternating CISCA (cyclophosphamide, adriamycin, and cisplatin) and VB (vinblastine and continuous infusion bleomycin). Likewise, two of the three patients with choriocarcinoma still alive were treated with CISCA/VB. There were 19 patients with seminoma, 18 of whom are free of disease after treatment with cisplatin-based therapy, and 30 patients with nonseminoma, 12 of whom are alive with no evidence of disease after treatment with cisplatin-based chemotherapy. They, therefore, recommend treatment of poor prognosis extragonadal germ cell tumors with alternating CISCA/VB, and treatment of extragonadal seminomas with cisplatin-based chemotherapy. These data are summarized in table 2.

Garnick [93], in an editorial, also stresses the importance of trying alternating regimens of chemotherapy for the treatment of these poor prognostic tumors. He cites several ongoing studies which are examining such regimens as high-dose cisplatin, etoposide, vinblastine, and bleomycin (National Cancer Institute); alternating cycles of PVB with etoposide, cisplatin, and bleomycin (Dana Farber Cancer Institute); and alternating cycles of VAB-6 with etoposide cisplatin (Memorial Sloan-Kettering Cancer Center).

One other novel approach in the treatment of EGGCTs deserves mention. It involves the use of high-dose etoposide (VP-16-213) with autologous bone marrow reinfusion [94]. In their series, six patients with refractory germ cell tumors were treated. Four PRs and one CR was obtained. Four of the six patients had failed standard dose VP-16 therapy in the past. Although this form of therapy is still highly experimental and should only be

attempted in a center experienced with management of the complications inherent in the procedure, it may one day prove to be a useful addition to the armamentarium against EGGCT.

As mentioned earlier, many case reports and series have cited incidences where a previously unsuspected testicular primary will become apparent [14, 19, 70]. This raises the question as to whether orchiectomy should be performed once the diagnosis of EGGCT is made. In the longest series following treatment with disease-free survival [68], no patient at any time had developed a testicular primary and, therefore, they did not recommend orchiectomy. Griest et al. [95], in a series of 20 patients undergoing orchiectomy after completion of PVB therapy for metastatic testicular cancer with known gonadal primaries, found three cases with residual cancer, six with teratoma, and 11 with atrophy and necrosis. From their findings, they feel that the testis can act as a sanctuary for the primary lesion and that orchiectomy should be performed following therapy if a primary lesion had been noted pretreatment.

Extrapolating this data to patients with EGGCT and no obvious gonadal primary, ultrasound or other noninvasive studies should be performed on all patients suspected of having an extragonadal primary. If a lesion is noted on examination, or if any asymmetry is apparent, orchiectomy should be considered after completion of chemotherapy, as it is not guaranteed to disappear with cytotoxic therapy. If noninvasive examination is unequivocally negative, we would not recommend orchiectomy at this time.

Tumors of the central nervous system

Midline tumors of the central nervous system (CNS) account for one of the most common sites of EGGCTs. These tumors occur primarily along the third ventricle, extending from the pineal area posteriorly to the suprasellar region anteriorly. Originally these tumors were believed to have all arisen from the pineal gland cells, as they resembled the fetal and neonatal pineal body. However, Russell [96] in 1944, described pineal tumors as atypical variants of testicular teratomata, because they resembled the spheroidal cell carcinoma of the testis. They were, therefore, called germinomas since they were felt to be of germ cell origin. A classification of all pineal body tumors is outlined on table 3 [97]. These CNS variants of EGGCT also fit very well into Teilum's schema of germ cell tumors [7]. All histologic variations included in the schema have been noted to occur in the CNS, although germinomas comprise over 70% of all primary CNS germ cell tumors.

Clinical characteristics

Primary pineal tumors account for approximately 0.6% to 1.0% of all CNS primaries in the Western World. For unknown reasons, the incidence is higher in Japan, with pineal tumors accounting for 4% to 5% of all CNS

Table 3. Histologic classification of primary germ cell turmors of the central nervous system.

I. Tumors of germ cell origin
1. Teratomata
2. Germinona ('pinealoma,' atypical pineal teratoma, suprasellar germinoma, 'ectopic pinealoma')
3. Embryonal cell carcinoma
4. Endodermal sinus tumor
5. Choriocarcinoma
II. Tumors of pineal cell origin
1. Pineocytoma
2. Pineoblastoma
III. Other cell origin
1. Glioma
2. Ganglioneuroma and ganglioglioma

malignancies [41]. The mean age of incidence in most series depends on location of the tumor. Suprasellar malignancies almost always occur in patients less than 30 years of age with peak incidence in the late second and early third decade. Neoplasms of non-germ cell origin have higher mean age of incidence and can be seen in patients over 40 years of age. Older patients are much more likely to have non-germ cell tumors of the pineal, especially gliomas, ganglioneuromas, and gangliogliomas. The germ cell tumors of the pineal areas, as in the suprasellar region, have a peak incidence in the late teenage years to the early 20s [98, 99].

Males predominate all series with ratios ranging upwards from 4:1 to as high as 8:1 in pineal primaries [100, 101]. Suprasellar primaries, on the other hand, have a more even distribution.

These tumors, given their strategic location within the CNS, have a multitude of clinical signs and symptoms associated with them. In the pineal regions, most patients demonstrate symptoms of increased intracranial pressure and obstruction of the ventricular system with headache, vomiting, drowsiness, dizziness, fatigue, irritability, and decreased cognition.

Visual changes include blurred vision, diplopia, and decreased visual acuity. Specific neurological symptoms include poor coordination, decreased hearing, or tinnitus. Neurologic exam can disclose gait or limb ataxia, hemiparesis, bitemporal hemianopsia, homonymous hemisensory deficits.

Due to the location of these tumors near the midbrain and pons, many interesting neuro-ophthalmalogic findings have been noted [100] including papilledema, decreased pupillary light and accomodation reflexes, upward and downward gaze paralysis, retractory nystagmus, sixth cranial nerve palsy, vertical and lateral nystagmus, and Collier's sign (retractory nystagmus and lid retraction). A very interesting collection of signs, including impaired upward gaze, defective convergence, and pupils which respond more briskly to accomodation than light (Parinaud's syndrome) [102], are associated with these tumors, secondary to compression of the dorsal-rostral midbrain from above.

Tumors of the suprasellar area are also associated with many nonspecific changes due to increased intracranial pressure as well as changes due to compression of the optic chiasm [101]. In addition, many hormonal changes are seen in association with tumors of this location. Diabetes insipidus is seen in over 80% of patients. Less commonly seen are hypogonadism or precocious puberty, short stature, and panhypopituitarism. In a radioimmunoassay analysis of nine patients with suprasellar germinomas, eight patients were deficient in growth hormone, eight had either too little or too much luteinizing hormone and follicle stimulating hormone, seven had impairment of thyroid function, and two patients had elevations in human chorionic gonadotrophin, associated with precocious puberty. Adrenal function was also noted to be low in those patients in whom it was tested [101].

Hormonal changes are also seen in primary pineal germ cell tumors, but are not nearly as common. Diabetes insipidus has been seen, as has panhypopituitarism, growth retardation, lack of secondary sex characteristics, and precocious puberty [99].

Germ cell tumors of the CNS are associated with the same tumor markers as their non-CNS counterparts. Both hCG [45] and AFP (51, 52) have been demonstrated to be increased with presence of these malignancies in the CNS. Haase and Norgaard-Pedersen [52] demonstrated a decrease in both AFP and hCG in a patient being treated for an endodermal sinus tumor with elements of choriocarcinoma. Allen et al. [51] showed a good correlation between marker and tumor type. He also demonstrated that CSF values correlated well with radiographic and clinical course, that ventricular levels were lower than lumbar levels, and often-times CSF marker values increased long before clinical symptoms arose or radiographs became abnormal. Bagshawe [45] also demonstrated that CSF measurement of hCG was a valuable tool in predicting CNS relapse. The use of tumor markers greatly improved the noninvasive diagnostic potential for these tumors. Before markers were measured in the CNS, a very small minority of patients had definitive changes in the CSF (i.e., positive cytology) which facilitated diagnosis. Other changes in the CNS, namely lymphocytosis and protein elevation, were nonspecific and did not improve diagnostic accuracy [99, 101, 103].

Radiographic changes

Most studies which report radiographic findings of primary germ cell tumors of the CNS were compiled before the advent of computed tomography (CT). Before the use of CT scanning, the most commonly used radiographic techniques included plain skull films, pneumoencephalography, angiography, and ventriculography. Pneumoencephalography typically showed symmetrical hydrocephalus of the lateral and third ventricles with obstruction near the aqueduct and a soft tissue mass in the area of the pineal gland indenting the posterior third ventricle. Pineal gland calcification was commonly seen on plain films (approximately 50% of the time) as was erosion of

the sella turcica. Of the less common signs are those of increased intracranial pressure with separation of the sutures. Angiography usually demonstrated a vascular mass. In pineal tumors there was upward displacement of the internal cerebral vein and enlargement of the posterior medial and lateral choroidal arteries. In suprasellar malignancies there was elevation of segments of the anterior cerebral arteries, stretching of the anterior choroidal arteries, and thalamo perforators [100, 101]. Although most series felt that they could not make specific diagnoses based on these radiographic findings, Takeuchi et al. [101] felt that they could distinguish suprasellar germinoma from teratoma or craniopharyngioma in this area.

A large series was published from the hospital of the University of Pennsylvania [104] and specific CT findings were correlated to tumor type in 32 germ cell tumors of the pineal gland and 28 brain stem gliomas. Using the absence or presence of calcifications; enhanceability; homogeneity or heterogenous density; cystic patterns, especially in correlation with age; sex; and location of the tumors, the authors felt that they could decide on which patients to operate on and which to treat primarily with radiation therapy. They also found that CT follow-up helped to determine the tumor type, as those patients with radiosensitive tumors (germinoma, embryonal carcinoma, and primary pineal tumors) showed an early response to radiation therapy. This response also correlated with improvements in neuroendocrine function as well as decrease in clinical symptoms and signs.

Therapy

Several important issues exist in the literature when considering optimal management of primary germ cell tumors of the CNS. The necessity for surgical intervention, both for treatment and diagnosis; the possibility of spinal seeding and implantation; the exact port and dosage of radiation; and the use of chemotherapy all must be considered carefully. Generally, overall survival is good in this group of neoplasms, with five- and ten-year disease-free survival rates of approximately 50% to 60% [98, 105] and with higher figures for germinomas (70% to 80%).

The older series of primary CNS germ cell tumors had an inordinately high surgical morbidity and mortality, with some series greater than 40% [103]. It was, therefore, felt that a shunting procedure to relieve hydrocephalus was the only surgical procedure warranted, with the use of radiation as primary therapy to the tumor. Since about 70% of these tumors are radiosensitive, this seemed to be a reasonable approach.

Through the mid 1970s, new surgical approaches (occipital transtentorial and supracerebellar infratentorial) as well as new surgical techniques, such as microsurgery with the use of the operating microscope, greatly decreased morbidity and mortality. Chapman [107] reported no complications in eight patients receiving a biopsy or resection of pineal tumors.

Another interesting finding in several large series of these tumors

[98, 105, 108] was equivalent survival in those patients who had had tissue diagnosis and those who had not and were treated with radiation and/or shunting. With the use of CT scanning and the criteria for identification of tumor subtypes and the advent of measuring tumor markers in the CSF, surgical verification may become less of a necessity in the future.

Dosage of radiation, size of the ports used, and use of prophylactic spinal radiation therapy are other important issues in the treatment of these neoplasms. The frequency of seeding of the craniospinal axis depends on the series. Incidence of 0% [108] to as high as 25% [99, 109] is seen, and generally averages about 10% in most series. A major issue in several series is whether or not surgical intervention is responsible for this spinal seeding. Again, depending on the series read, widely varying figures are cited for the incidence of surgical intervention causing spinal seeding [98]. It most likely is not, but the question is clearly not settled at the present time.

Takeuchi et al. [101] used 5,500–6,000 rads to the primary tumor, as well as 3,000 rads of whole brain radiation and 2,000 rads added to the spinal axis. Twelve of 15 patients with suprasellar semionoma treated this way are alive with no evidence of disease. One patient who received spinal radiation developed spinal metastases and died.

Onoyama et al. [105] used 5,000–6,000 rads to the primary tumor in 58 patients, with six receiving additional spinal cord radiation (one who had known spinal metastases before therapy). The five patients with uninvolved spines pretreatment did not have spinal cord relapses. Four of 52 patients who did not receive spinal cord prophylaxis developed spinal cord seeding.

Sung et al. [99] treated 73 patients with 5,000–5,500 rads to the primary tumor (46 patients), 4,000 rads whole brain radiation (27 patients), and 3,500 rads to the spinal cord (4 patients). Relapse-free survival was 60% at five years with midline pineal tumors and 70% for suprasellar germinomas. Suprasellar germinomas had a greater propensity to metastasize and seed the spinal cord and subarachnoid space. Local control of the primary was markedly improved with increased dose and larger ports. It is unclear whether those patients who received prophlyactic spinal cord radiation were the patients who had spinal cord relapse.

Wara et al. [108] treated 19 patients with 4,000–5,500 rads to the primary and ventricular system and no spinal cord prophylaxis. No patients repalsed in the spinal cord and 15/19 are alive with no evidence of disease.

Jenkin et al. [98] treated 31 patients with radiation therapy to the primary only. Nineteen got adjuvant spinal cord radiation. Of those patients who had biopsy-proven germinoma, two of five who did not receive adjuvant spinal cord radiation relapsed in the cord. None out of five patients who had adjuvant spinal cord radiation had relapse in that location. The authors felt that even though they saw this pattern of relapse, large enough ports encompassing the entire ventricular system would be adequate therapy without utilizing prophylactic spinal cord radiation. Local control in this series improved with doses of 5,000 rads or greater delivered to a volume port.

In all large series of pineal germ cell tumors, most histologies are grouped together, with germinoma having the best prognosis and other histologies having poorer long-term survival. Endodermal sinus tumors, although very rare in this location, have been the subject of a number of case reports and reviews [110–117]. These tumors have a universally poor prognosis with only two cases having long-term survival. One was reported by Bestle [112] which was treated with radiation therapy and surgery. The other was treated by Prioleau [117] with partial resection, radiation, and VAC (vincristine, actinomycin-D, and cytoxan) chemotherapy.

This raises an interesting question of whether primary germ cell tumors of the CNS should be treated with chemotherapy. The blood-brain barrier has been shown not to be intact with primary or metastatic brain tumors present [118, 119] and passage of molecules excluded by normal brain has been demonstrated in animal brain tumor models [120]. Additionally, pharmacokinetic studies of blood and CSF levels of bleomycin, cisplatin, and vinblastine following intravenous administration of these drugs has been performed [121].

Bleomycin is cleared rapidly from plasma (alpha half-life is 0.25 hours, beta half-life is 1.32 hours). Peak levels in CSF appear two hours after administration of the drug. The peak CSF level is 40% of simultaneously obtained plasma levels. It is cleared more slowly from CSF than plasma. Cisplatin is cleared slowly from plasma (half-life is 38 hours), and peak levels in CSF appear 30 minutes after administration of drug. The peak CSF level is 25% of the peak free cisplatin level in plasma. Vinblastine has no detectable levels in CSF at times from two minutes to 24 hours following its administration.

Therefore we see that certain chemotherapeutic agents which have known efficacy against germ cell tumors can achieve significant levels in the CSF. However, further studies must be performed before efficacy of systemic chemotherapy is evaluated in primary germ cell tumors of the CNS.

Several conclusions can be made from these studies. (1) It seems that surgical intervention may increase the risk of CNS spread, but that large volume, high dose radiation therapy should be adequate treatment for prevention of this complication. (2) Local control is best obtained with doses over 5,000 rads delivered through generous ports encompassing the entire ventricular system. (3) Spinal cord prophylaxis does not appear to be warranted, as the number of cases where seeding develop are too few to recommend treatment of the cord for all patients. This therapy should be reserved for those patients with positive CSF cytology. (4) Those patients who do relapse in the meninges can be salvaged by additional radiation to the symptomatic area. (5) Germinomas have improved survival with respect to other histologies of pineal tumors, most likely due to their extreme radiosensitivity, while endodermal sinus tumors have a very poor prognosis, raising the question of the use of chemotherapy for refractory tumors of this nature.

Miscellaneous syndromes

In addition to mediastinal, retroperitoneal, and CNS locations of EGGCTs, there have been reports of cases involving other less common sites. It seems that infants less than two-years-old have a propensity for development of EGGTs in characteristic sites, most notably the vagina and presacral area.

Norris et al. [34] reported a series of six cases of endodermal sinus tumor involving the upper vagina. All six cases occurred in infants under 15 months of age and had pathological features which distinguished them from more common adenocarcinomas of the mesonephric type found in children and adults. Two of six had long-term survival with radical surgery and/or chemotherapy or radiation. Other case reports exist in the literature with similar clinical and histological characteristics [34]. The origin of these tumors is still unclear.

Unlike the tumors of the vagina, which appear to have their origin in yolk sac precursors, the sacrococcygeal germ cell tumors appear to have a teratomatous pathology. Although these tumors usually occur in young children and infants, a few reported cases have been seen in adults. In a large series [38] of 32 cases, only four patients were older than two-years-old. Most cases were observed the birth (23 cases) or shortly thereafter (4 cases). An association with sacral agenesis was noted in six patients. This congenital anomaly has also been noted in a family with presacral tumors and reported by Kenefick [39]. The cases reported in infants mostly consisted of mature teratomata (18 cases), although immature teratomata (4 cases), embryonal cell carcinoma (1 case), and teratocarcinoma (5 cases) also occurred in infants. The four adults were noted to have benign cystic teratomata (two) and teratocarcinoma [2]. The single case of embryonal carcinoma and 6/7 of the teratocarcinomas all died of progressive disease. Patients with mature or immature teratomata were all alive or died of unrelated causes. Surgery was the only mode of therapy used in these cases. An interesting observation was that those tumors appearing at birth or shortly thereafter were usually benign, with only one infant dying from recurrent tumor. Those four, which occurred in infancy but well after birth, were all malignant and were the cause of death in all four patients. It is postulated that there is a small area of immature teratoma, in those tumors present at birth, which does not mature, eventually proliferating and causing a malignancy. These tumors should therefore be excised as early as possible in order to prevent this malignant transformation.

Other less common sites of occurrence of EGGCT include endodermal sinus tumor of the face [40], liver [35], vulva [76] and choriocarcinoma of the stomach [36, 37].

Another important syndrome related to EGGCT is that of unrecognized EGGCT or undifferentiated carcinoma. Patients often present with widespread metastatic disease without an obvious primary source. The pathology is usually read as undifferentiated carcinoma which can be a misleading

diagnosis, leading to treatment with inappropriate and ineffective chemotherapeutic regimens. A series of five patients was reported by Fox et al. [49]. The patients were each male and under 40 years of age. Four out of five patients had minimal mediastinal, but widespread pulmonary disease, the fifth had a large mediastinal mass. Each patient had their original pathology read as undifferentiated adenocarcinoma or large cell carcinoma. Markers were positive in one of five patients [hCG). Three of the patients eventually had the diagnosis changed to embryonal carcinoma and received standard (for the time) chemotherapy for germ cell malignancies. The other two patients were initially treated inappropriately, but eventually had their pathology reread as embryonal carcinoma and got appropriate chemotherapy. One of the five remains without evidence of disease, the others dying of progressive malignancy. The authors suggest that undifferentiated carcinoma in young men without an obvious source should be reviewed carefully and strong consideration given to germ cell origin. Immunoperoxidase using AFP and hCG on frozen tissue as well as serum marker studies might aid in the diagnosis.

A second series was reported by Richardson et al. [50] of 20 patients with probable EGGCT, 12 of whom presented with unusual or misleading anatomical locations of disease (axilla, CNS, bone, supraclavicular lymph nodes, and ileum) and a wide array of original pathological diagnoses (poorly differentiated adenocarcinoma, amelanotic melanoma, large cell undifferentiated carcinoma, and mucin-secreting adenocarcinoma). Once referred to Vanderbilt University, however, serum tumor markers and immunohistochemical staining with AFP and hCG aided in correcting the diagnosis in the 12 to that of germ cell origin. Four out of four tumors stained were positive for either AFP or hCG despite negative tumor markers. Eleven of 12 were treated with PVB chemotherapy, and all 11 responded; six with CR and five with PR. Two of the six CRs relapsed, 3/5 of the PRs are alive with disease and 2/5 are dead. Interestingly, in this series were one female and one male aged 61. Nonetheless, these authors concur with Fox's group that poorly differentiated or undifferentiated adenocarcinoma in patients between 20 and 50 years of age should have germ cell origin of their malignancy carefully considered, as their prognosis may not be as bleak as the original pathology would indicate.

Conclusion

1. Extragonadal germ cell tumors are rare malignancies, arising in either totipotent stem cells or misplaced primordial germ cells. They occur in a variety of locations including the mediastinum, retroperitoneum, and pineal gland.
2. There has been controversy regarding the role of orchiectomy in evaluation and treatment of patients with EGGCT. With the advent of non-

invasive studies, such as ultrasound and thermography, orchiectomy can be bypassed as long as clinical examination and examination with these noninvasive techniques is unequivocally negative.

3. EGGCTs express different clinical patterns than those of testicular origin, with an increased incidence of bony metastases, bulky disease at presentation, and interesting clinical syndromes such as thrombocytopenia, megakaryocytic leukemia, and Klinefelter's syndrome.
4. EGGCTs response to chemotherapy differs from testicular primary tumors. Nonseminomas (especially mediastinal tumors, endodermal sinus tumors, and choriocarcinoma) appear to be more refractory to standard cisplatin-based regimens and may require more intensive chemotherapy with alternating combinations of drugs. Seminomas respond well to standard cisplatin-based regimens and should be treated with chemotherapy up-front rather than with primary radiation therapy. However, radiation may be sufficient in carefully selected patients.
5. EGGCT of the CNS can be diagnosed correctly using CT scan, CSF, and serum tumor marker studies. A majority of these tumors are highly sensitive to radiation therapy and generally do not require invasive surgery for tissue diagnosis or treatment. Generous radiation ports and dosages of upwards of 5,000 rads should be employed in treatment of these tumors. Spinal cord prophylaxis should be reserved for those patients with positive CSF cytology.
6. EGGCTs can present as widely metastatic undifferentiated carcinoma, and they should be considered in young patients where this clinical situation arises. Serum tumor marker studies and immunoperoxidase of pathology specimens can aid in facilitating the diagnosis.

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5. Role of preclinical pharmacology in phase I clinical trials: Considerations of schedule-dependence

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Antitumor activity or host toxicity can be increased or decreased by changing the rate of drug delivery. This phenomenon is known as schedule-dependence, and the most familiar examples occur with the use of methotrexate (MTX), fluorodeoxyuridine (FdUrd), and cytosine arabinoside (ara-C). The general motivation for the study of schedule-dependence is to improve the therapeutic index of a drug, i.e., to maximize the ratio of therapeutic effects to toxic effects.

The main purpose of this chapter is to consider the role of schedule-dependence in the development and testing of new anticancer drugs. There are several specific issues of concern:

1. Are potentially useful drugs not discovered because the preclinical screening procedures do not utilize the appropriate schedules of drug exposure?
2. Do the conditions for preclinical drug screening and toxicology adequately mimic clinical delivery conditions?
3. Can substantial clinical resources be saved by tailoring the choice of testing schedules to the pharmacologic properties of individual drugs?
4. Is the maximal clinical activity of a new drug missed due to testing on inappropriate schedules?
5. Can the matching of prelinical data with clinical trial design provide clearly defined advantages?

Types of schedule-dependence

Both toxicity to normal host tissues and antitumor efficacy may be schedule-dependent. Preclinical antitumor models are controversial; thus, the practical approach has been an emphasis on toxicity as a function of schedule. Toxicity studies are more straightforward, since host tissue effects should be primarily independent of the specific tumor type. For preclinical testing, normal animals are preferred for toxicity tests.

An initial test of schedule-dependence is a comparison of toxic effects based upon the same total dose delivered by a bolus injection versus a

continuous infusion. These types of comparisons yield examples of drugs for which the maximum tolerable dose (MTD) for a continuous infusion is greater than for a bolus, smaller than for a bolus, or of the same magnitude. If a drug is much more toxic by one schedule, then further testing (for efficacy) must be done with doses adjusted to equitoxic levels. Improvements in the therapeutic index can only be assessed from these latter experiments. It will be noted in subsequent sections that there are many examples of schedule-dependent toxicity but few examples of schedule-dependent antitumor efficacy.

Moderation of acute toxicity (peak concentration effects)

For some drugs, *much larger doses* are tolerated by continuous exposure than as a single bolus. This type of toxicity profile can be explained by a peak concentration threshold, i.e., a drug concentration which can not be exceeded at any time during therapy without producing adverse effects. The underlying mechanism of such a threshold might be acute cardiovascular collapse, acute neurotoxicity, or generalized shock syndrome. Although relatively few compounds under development have comparative infusional data, this pattern has been recently observed for both hexamethylene bisacetamide (HMBA, NSC95580) and deoxyspergualin (NSC356894).

As illustrated in figure 1 for a drug with a t1/2 of two hours, the size of each bolus dose is limited by its initial peak concentration. If the drug is given once a day for five days, the minimum (nadir) concentration is less than 0.1% of the peak concentration. Continuous infusion provides the capability of maintaining circulating concentrations near the toxicity threshold for the entire duration of the infusion, which eliminates any possibility of subtherapeutic nadir levels and also greatly increases total drug exposure (AUC or CxT). For a comparison of five daily bolus doses with a five-day infusion, the total dose delivered and, thus, the total drug exposure is eightfold larger with continuous infusion. These comparisons are heavily dependent upon the t1/2 of the drug. If the t1/2 is shorter than two hours, the differences will be more pronounced. For a longer t1/2, the differences will be smaller.

Comparison of toxicity between a bolus dose and continuous infusion provides the clearest indication of a peak concentration effect. However, any divided-dose (intermittent bolus) schedule can provide indications that there may be a peak effect. There are several drugs for which the murine LD_{10} from a single dose can also be given on five consecutive days, i.e., the LD_{10} for a Dx5 schedule is actually five times as large (cumulative dose) as for Dx1. In the last two years, a number of drugs under development by NCI have produced this pattern. In addition to HMBA and deoxyspergualin, merbarone (NSC 336628), acodazole (NSC 305884), and trimetrexate (NSC 352122) are recent examples. Even larger cumulative amounts of drug can be given if each daily dose is divided into several fractions, e.g., dosing every three to four hours.

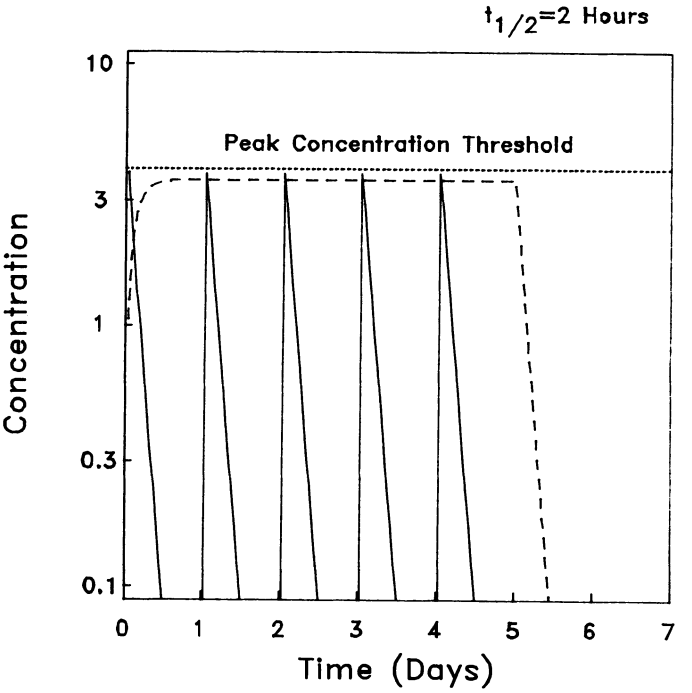

Figure 1. Role of a peak concentration threshold in determining the schedule-dependence of a drug. For this example, it is assumed that the drug half-life is two hours. Daily bolus injections (solid lines) are sized to produce peak concentrations just below the threshold (dotted line). Continuous infusion delivery (dashed line) is able to maintain maximum permissible concentrations for the duration of therapy. The nadir concentration for the daily bolus schedule is 0.1% of the peak concentration. The total drug exposure (AUC or CxT) is eight-fold higher for continuous infusion.

Threshold effects, time-dependence

There are two well-known examples of drugs for which *much smaller doses* can be tolerated by continuous infusion than can be given by single (bolus) doses: MTX and FdUrd. This type of schedule-dependence appears to be due to the requirement for prolonged inhibition of a target (e.g., thymidylate synthetase). Although higher peak levels are produced by the bolus dose, concentrations fall rapidly below the minimum required for inhibition. For continuous infusions, concentrations must be sustained below the threshold imposed by host tissue toxicity, thereby markedly reducing the tolerated dose.

Unlike the situation in figure 1, the toxicity threshold concentration is not fixed, but depends upon the exposure time (variable concentration threshold). As illustrated in figure 2, the concentrations generated by a bolus dose may remain below acute thresholds and produce a very large total drug exposure. Since the peak level for an infusion occurs at the end of the delivery period, the rate of infusion is constrained by the lower threshold

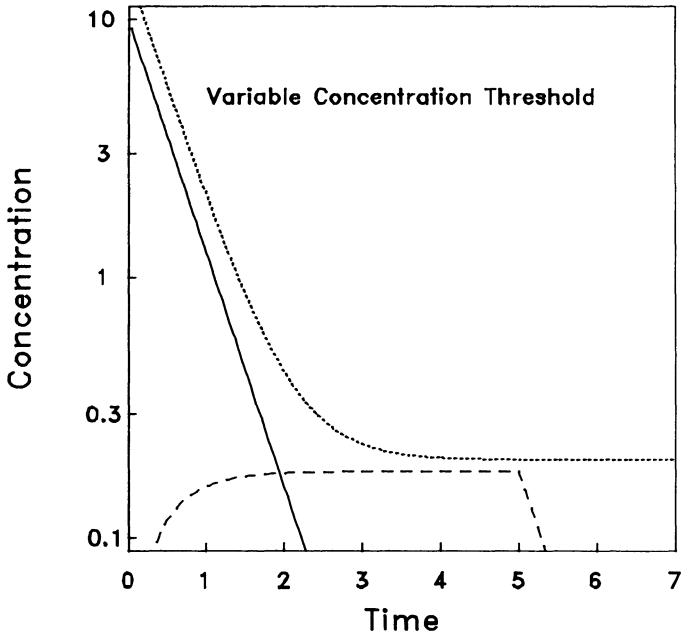

Figure 2. Role of a variable concentration threshold (dotted line) in determining the schedule-dependence of a drug. More drug can be given as a bolus (solid line), since the dose is removed from the body before the time at which the concentration threshold is exceeded. For continuous infusion (dashed line), the opportunity to deliver high doses at early times is lost. The infusion rate must be sized to remain below the long-time threshold for concentration.

imposed at later times. In the particular case illustrated, the total drug exposure following a single bolus dose is more than fivefold greater than the total exposure for continuous infusion.

Schedule-independent toxicity

Certain classes of drugs produce host tissue toxicity which is primarily dependent upon total exposure. Alkylators and DNA binders are common examples. For these drugs, the rate of drug delivery is unimportant, as long as it does not drop below repair rates or lead to development of resistance. The choice of schedule can be left to other determinants, such as the timing of other drugs in a protocol.

Preclinical toxicology

The quantitive preclinical toxicology information for most anticancer drugs consists of the determination in mice of the LD_{10}, LD_{50}, and LD_{90} for a single bolus dose and five daily bolus doses. After lethality studies are completed in mice, toxicity studies are done in rodents and dogs to assess

specific hazards. Although there are some technical barriers to the use of continuous infusion in these species, the difficulties are being steadily overcome.

The toxicology branch at NCI designed and implemented a successful study of continuous administration of HMBA. Portable infusion pumps were strapped to ambulatory dogs who were given continuous intravenous infusions of HMBA for up to 120 hours. Plasma concentrations of HMBA were measured and correlated with toxicity. HMBA was given by continuous infusion in phase I human trials, and these canine toxicology studies predicted both the qualitative and quantitative aspects of human toxicity. Two additional drugs, merbarone and deoxyspergualin, entered phase I testing in late 1985 based upon similar infusion studies in dogs.

Technological advances, such as implantable osmotic pumps, may now permit the continuous delivery of drugs to mice without the labor-intensive (i.e., expensive) external pump arrangements which were required in the past. Current studies on the preclinical toxicology of 5-azacytosine arabinoside (NSC 281272) are the first efforts to utilize this technology prior to clinical testing.

Preclinical screening (efficacy)

The schedules used for preclinical in vivo testing for antitumor efficacy vary greatly, depending upon the growth rate and historical experience for individual tumors in the panel. At the present time, there is no schedule in routine use at NCI which approximates continuous exposure. Due to high labor costs, the q3h $\times$ 8 schedule has been deleted from routine screening. There is always some concern that potentially active agents are missed because the appropriate schedule was not evaluated. Ara-C is the clearest historical example where this possibility nearly happened. Of course, if there are examples of drugs which never made it to the clinic due to inadequate schedule-testing, *we may never know.* Due to renewed interest in the area of schedule-dependence, the q3h $\times$ 8 schedule is now tested for all NCI drugs which are approved for final toxicology and formulation studies, i.e., pass Decision Network 3 [1]. Thus, even though a drug's schedule-dependence may be missed in early testing, information will be available before phase I clinical trials, if the compound progresses that far.

Although there are some screening data for intermittent dosing, preclinical efficacy screening at the NCI has never included use of continuous infusions until the present time. Now that the preclinical profile for many drugs includes considerable information about schedule-dependence, an accurate determination of the therapeutic index requires more focus on screening. Deoxyspergualin, which entered phase I clinical trials in late 1986, was the first of a pilot series of compounds in which efficacy studies using screening by continuous infusion are being evaluated [2], at least in one tumor model. Until there are data available for more compounds, a true

evaluation of the impact of schedule-dependence in drug development can not be obtained. For bleomycin, it has been shown in an animal model that the therapeutic index can be improved by continuous infusion [3]. Not only was toxicity less, but antitumor efficacy was greater. Further studies of established agents would also be helpful, especially in the area of efficacy testing.

Although we have listed many examples of host toxicity which are related to peak drug concentration, we have no clear-cut evidence which relates antitumor efficacy to *peak* concentration. Thus, for the examples cited above (thalicarpine, trimetrexate, HMBA, and deoxyspergualin), the therapeutic index is greatly enhanced by continuous administration (at least in animal systems). Since more drug is delivered to the tumor site at equivalent (or less) host toxicity, the total drug exposure is increased.

Preclinical pharmacokinetics

Based upon the analysis presented thus far, the elements of pharmacokinetics (half-life, clearance, peak concentration, and steady state levels) can play a major role in schedule-dependence. These elements can help to identify the mechanism(s) which underlie schedule-dependence.

In addition, pharmacokinetics can help to sort out differences which are occasionally observed between data obtained from screening and toxicology. Some differences may be due to the route of administration. Preclinical screening normally utilizes IP drug delivery and preclinical toxicology studies normally utilize IV delivery. Determination of drug bioavailability and first-pass metabolism is one type of pharmacokinetic contribution. In addition, even if bioavailability is 100%, peak drug concentrations can be considerably lower following the IP route, due to delayed absorption.

The requirement for timely acquisition of data has hindered the prospective use of pharmacokinetics in drug development. The NCI is currently obtaining pharmacokinetic data from mice on a routine basis for both bolus injections and constant infusions. In addition, dog pharmacology data are being obtained for selected drugs as part of extended preclinical toxicology studies. Both the kinetic data and the analytical methodology are now available at the time initial clinical trials begin. In addition to building a database for analysis of interspecies pharmacology and toxicology, these data may have a substantial role in adjusting the manner in which early clinical trials proceed [4].

Clinical testing

Traditionally, drugs have been tested in phase I trials on three or more schedules (viz., single dose, five daily doses, continuous infusions, and

Table 1. Schedules used for Phase I testing.

Bolus (15 seconds to 2 hours)
d × 1
d × 5
d × 3 (M,T,W)
d × 3 (M,W,F; qOd)
d1, d8
weekly
biweekly
Continuous Infusion (12 hours to 30 days)

others; see table 1) without any particular rationale. The ultimate goal of clinical testing is to determine the therapeutic index of a drug, i.e., both the efficacy and toxicity. If three or more schedules are tested in phase I trials, the toxicity half of the therapeutic index is determined. However, looking back over years of testing reveals an incomplete efficacy evaluation for most agents on even one schedule. As a result, the relationship between therapeutic index and delivery schedule has rarely, if ever, been determined.

Since it is unlikely that all three schedules can be evaluated for efficacy for each and every drug, it does not seem worthwhile to conduct so many phase I trials. Certainly, there is no intention to skip a schedule that has some promise, but criteria are needed for choosing schedules. The preclinical profile generated for each drug contains information which can provide useful guidelines for schedule selection. Ultimately, we need to define the therapeutic index in randomized clinical trials to assess the relevance of preclinical information.

There are many *alternate testing schemes* which require substantially fewer total patient resources. A few are listed for consideration:

Option 1: Individualization of schedules for each drug. If the preclinical screening, toxicology, and pharmacokinetic data are available in suitable form, it should be possible to select one or two schedules for each drug. For drugs which demonstrate exceptional potential based upon preclinical development, two schedules could be tested in the clinic. If the preclinical profile is not as favorable, only one schedule might be tested in the initial clinical trials.

Option 2: Sequential testing of schedules. The current testing procedures evaluate all schedules simultaneously. If there is a rationale for more than one schedule, presumably it would be more efficient to determine the MTD by one schedule, then modify the starting dose and escalation rate for the next schedule based on the results of first trial. The momentum which develops to push a drug into phase II trials has been a deterrent to the use of this type of approach. Although this momentum must be kept in mind in

future planning, it should not deter us from a more rational approach to drug development.

Option 3: Test only one schedule for each drug until phase II is completed. Many investigational drugs are dropped from further consideration after initial phase II trials. For these agents, the detailed knowledge of the MTD by three different schedules is of no value. If a drug has lasting usefulness, its route of administration and schedule can be optimized at a later date. If a drug is dropped from further consideration after evaluation on a single schedule, there is always some possibility that the 'wrong' schedule was chosen. However, with a combination of retrospective studies of clinical experience and improved preclinical data, the likelihood of such an occurrence can be reduced.

Coordination between preclinical and clinical testing

The current preclinical profile for a drug is based upon regulatory requirements, the perceived needs of clinical groups, and the interests of preclinical groups. There is always some competition between the demands for more information versus the cost and time requirements of such studies. On the one hand, all investigators have certain studies which they prefer to be done before clinical trials are initiated. However, even if the desired studies are financially possible, there is usually an unwillingness to delay clinical trials until all possible studies can be finished.

Nonetheless, the content of the preclinical package must be responsive to changes in the philosophy and technical approaches both at the clinical level and in the area of drug screening. The clinical use of continuous exposure schedules is rapidly expanding, due in part to the development of improved pumps, catheters, and access ports. There seems to be an increasing emphasis on clinical studies of continuous exposure both for established drugs and for phase I agents. Preclinical toxicology has responded by exploring schedule-dependence in a set of schedules which more closely mimic human exposures. In drug screening, testing is increasingly based upon in vitro assays which tend to be continuous drug exposures of cell culture systems.

Since initial clinical trials begin at dose levels determined by preclinical toxicology testing, full consideration must be given to the impact of inappropriate matching of human and animal conditions. While clinical overdosing is the most worrisome problem, a far more common problem is underdosing, i.e., starting the trial at too low an initial dose.

One of the clearest examples of this situation occurred with thalicarpine (NSC 68075), which has an acute cardiodepressant effect. As discussed earlier, this type of toxicity can lead to considerable underprediction of human doses given by continuous exposure if pulse doses given to mice are the reference. In the retrospective mouse-man study by the toxicology

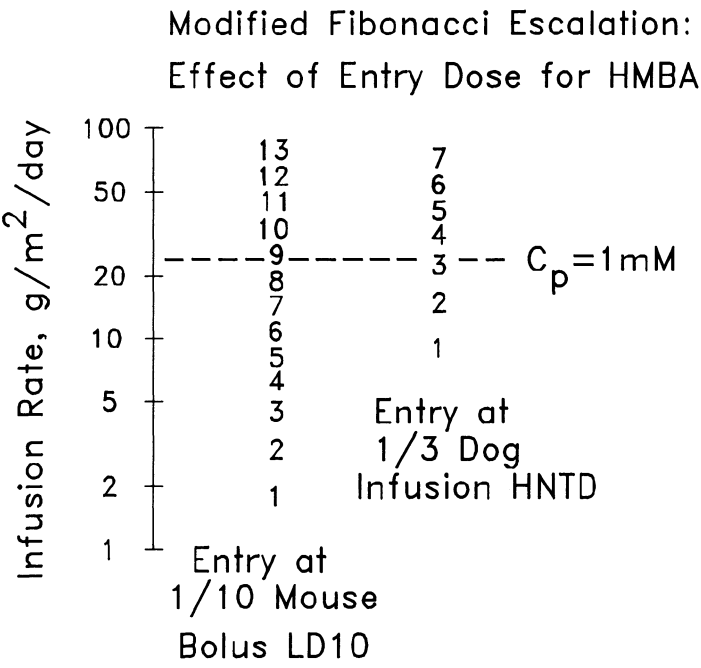

Figure 3. Effect of starting dose on the phase I clinical trial of HMBA. The conventional starting dose is 1/10 of the mouse LD10. Based upon an estimated LD10 from preclinical screening, nine escalation steps would have been required using the modified Fibonacci procedure. Since continuous infusion toxicology data were available from canine studies, only three steps were actually required, due to the higher starting dose. HNTD is the highest nontoxic dose.

branch at the NCI [5], thalicarpine mouse LD_{10} values greatly underpredicted clinical MTD values. However, the clinical 'bolus' schedule had been modified to deliver the dose as a two- to six-hour infusion, to avoid acute cardiac effects. Follow-up studies in animals also demonstrated that the LD_{10} could be increased by giving a two-hour infusion instead of a bolus injection.

A similar situation would have occurred with HMBA but was avoided because continuous infusion technology was used in the preclinical toxicology studies. As shown in figure 3, the starting dose for a clinical trial based on bolus injections in mice would have required nine escalations to reach the MTD. Only three escalations were actually needed, since a more realistic starting dose was obtained from the canine infusion studies. As mentioned above, the same strategy is currently being used with deoxyspergualin and merbarone.

It should be recognized that a long-term infusion (e.g., 120 hours) is not always required to ameliorate acute toxicity caused by peak concentrations. The data available at the time clinical testing begins may strongly suggest such a conclusion, but this deduction may simply reflect the suboptimal

database which is available for decision-making. There is a need to modify the screening and toxicology data for bolus delivery so that some type of short infusion can be evaluated and avoid premature commitment to long-term infusions. When a drug produces acute toxicity by a peak concentration effect, a five-day continuous infusion will always appear to be superior to five daily bolus injections, which will always be superior to a single bolus dose. However, infusions of two to six hours may be adequate to overcome acute toxicities. The terminology tends to be misleading, since a bolus dose to rodents is a very short pulse injection, whereas the clinical equivalent of a 'bolus' is usually an infusion over 30–45 minutes.

Finally, there must always be a recognition that there are inherent differences in pharmacology which must be considered in any interspecies comparison. Metabolic variations may provide an explanation for much of the differences in pharmacology and toxicology among species [4]. Figure 4 illustrates two additional reasons why the current murine tests do not adequately mimic human exposures for cases of schedule-dependence: (1) The peak concentration is tenfold higher in mice, when equivalent doses (mg/m^2) are given as a bolus. Total exposure (CxT) is the same, assuming that total body clearance ($ml/min/m^2$) is the same; and (2) time above a threshold can vary substantially, since the t1/2 in the mouse is often tenfold less than in humans.

Bolus injections in mice can produce levels which exceed thresholds for either a longer or shorter period of time than in humans, depending upon where the threshold is situated. Thus, a comparison of bolus doses can be rather different, even without differences in injection rate. On the other hand, infusion studies in mice adequately mimic the clinical situation, since the differences between species occur only in the time to steady state and post-infusion disappearance. These differences tend to be only a small part of the total exposure profile, unless the half-life of the drug is very long.

Acknowledgment

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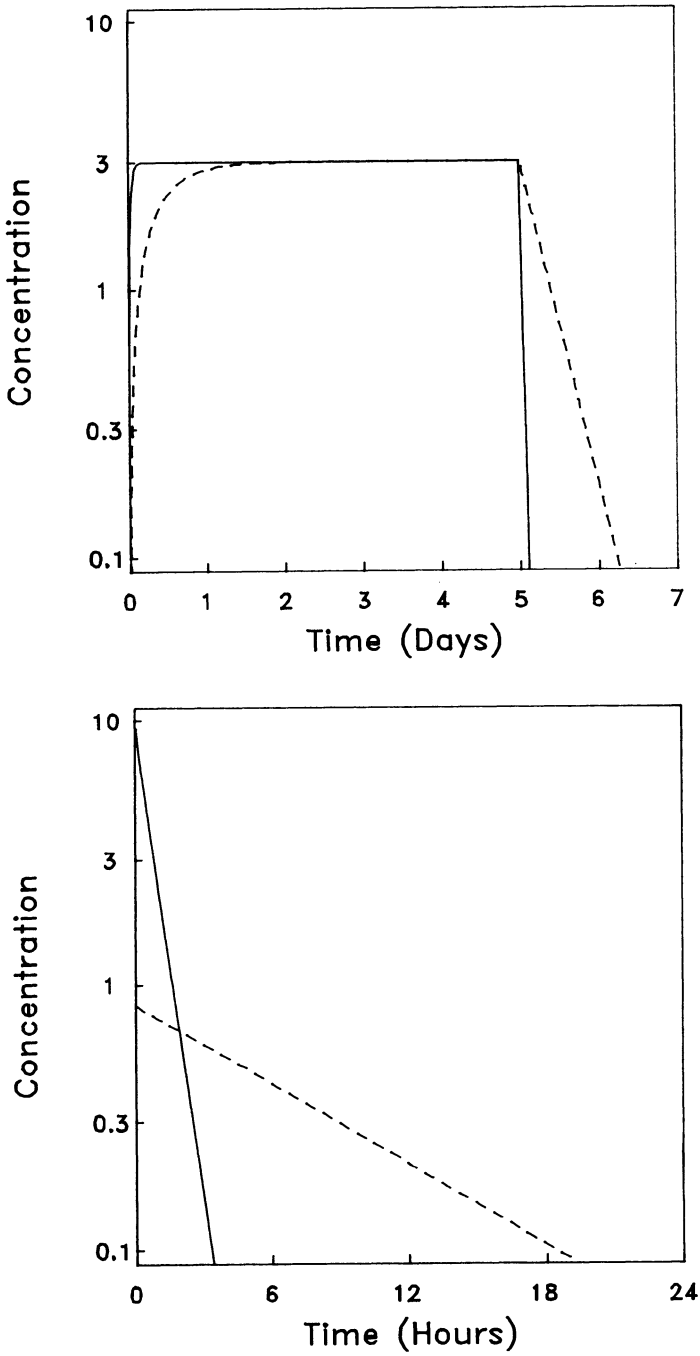

Figure 4. Comparison of drug exposure profiles in mice and humans. It is assumed that the total body clearance (ml/min/m^2) is the same in both species. For these examples, it is assumed that the half-time is 30 minutes in the mouse and six hours in man.
(A) For continuous infusion delivery at the same dose rate (mg/m^2/day), the same steady state levels are achieved in both man (dashed line) and mouse (solid line).
(B) For bolus delivery of the same dose (mg/m^2), the total drug exposure (AUC or CxT) is the same for both man (dashed line) and mouse (solid line), but the time course is rather different.

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6. Interferon: Current concepts of mechanisms of action

Paul P. Trotta and Robert J. Spiegel

Almost 30 years ago Isaacs and Lindenmann [1] discovered that the supernatant obtained from cells incubated with heat-inactivated influenza virus contained a substance capable of preventing the growth of active virus. This substance, named interferon (IFN), was later shown to be composed of a system of structurally related proteins that act directly on the target cells, not on the virus, and are produced by many types of animal cells in response to various external stimuli (e.g., viruses, certain types of double-stranded RNA, antigens, or mitogens). Three classes of IFNs have been described that differ in their amino acid sequences, as well as immunochemical and physicochemical properties: alpha (leukocyte), beta (fibroblast), and gamma (immune) IFN. Alpha and beta IFNs have also been designated as type I (acid-stable) and gamma IFN as type II (acid-labile). The alpha IFNs, which are produced by a variety of different cells including macrophages, null cells (non-B- and non-T-lymphocytes) and transformed B-cell lines, are a family of at least 14 species that share a 75% or greater amino acid sequence homology. Beta IFN, which shares *ca* 30% amino acid sequence homology with alpha IFNs, is produced mainly by fibroblasts and epithelial cells, but may also be produced by human tumor cells derived from leukocytes (e.g., the Namalwa cell line). Gamma IFN is predominantly a product of T-cells and natural killer (NK) cells and shares little homology with type I IFNs.

Kurt Paucker and colleagues [2] were the first to describe a nonantiviral activity for murine IFN; namely, the ability to inhibit cell growth in vitro, a phenomenon later observed with homogeneous human IFN [3, 4]. Subsequently, it was reported that IFN had potent immunoregulatory effects, as measured by its enhancement of the activity of effector cells of the immune system [5]. The list of biological effects exhibited by IFN has increased significantly (reviewed by Stewart [6]). However, despite these promising preclinical indications that IFNs could be effective pharmacological agents, the initiation of clinical studies in malignancies and viral diseases was delayed for many years due to extremely limited supplies of purified material. A small number of clinical trials were conducted in the mid to late 1970s utilizing alpha IFN that was partially purified from normal human leukocytes through a series of acid-alcohol extractions and pH precipitations [7]. It was

the cloning of the gene for a single subtype of human leukocyte IFN in *E. coli* [8] that made feasible the production of virtually unlimited quantities of purified material for phase I and phase II clinical trials [9]. Extensive clinical trials have now been conducted in all categories of malignancy, and a significant expansion of preclinical investigations of IFN's biological activity has occurred. This experience, as well as a variety of new developments in our understanding of oncogenesis and the biological properties of cancer cells, make it appropriate to review the potential biochemical and biological mechanisms for the antitumor activity of IFN. The major developments that make our understanding today different than that of a decade ago include the following:

1. With the use of highly purified recombinant DNA-derived IFNs the human pharmacokinetics have been characterized. Clinically achievable levels of IFN and its associated toxicities have been established. In addition, the availability of purified recombinant IFN has permitted the demonstration that the previously described diverse biological effects are an inherent property of IFN and are not produced by contaminants.
2. Specific cell surface receptors for IFN have been identified. It has been established that there are distinct receptors for type I and type II IFNs and that a variety of normal and malignant cells express both receptor types. Physicochemical characterization of the IFN-receptor interaction has been initiated, and it appears that the specificity of IFN binding will have major implications for understanding IFN's selective activity against certain tumor types as well as the biochemical basis for its biological activity.
3. Knowledge of the immune system and the nature of the immune response has advanced dramatically in recent years. Further dissection of the numerous arms of the lymphocyte/macrophage system as well as highly sophisticated identification of specific immune effector cells continues to be accomplished. Much new information is now available on the complex interaction of cytokines like IFN and the interleukins with the various effector cells of the immune system.
4. A new understanding of differentiation mechanisms in both malignant and normal cells has evolved. Both types I and II IFN have demonstrated the ability to act as either inhibitors or promoters of differentiation in a variety of model systems, which may be an important aspect of their antitumor activity.
5. Cancer researchers have identified new targets in the cancer cell to which drugs might be directed: specifically, the cell membrane and certain intracellular enzymes that had not previously been fully characterized. IFN may exert cytostatic or cytotoxic actions through such mechanisms, since it has been shown to alter the infrastructure and dynamics of the cell surface membrane. Additionally, IFN can affect the levels of various intracellular enzymes critical for the growth and differentiation of cells.

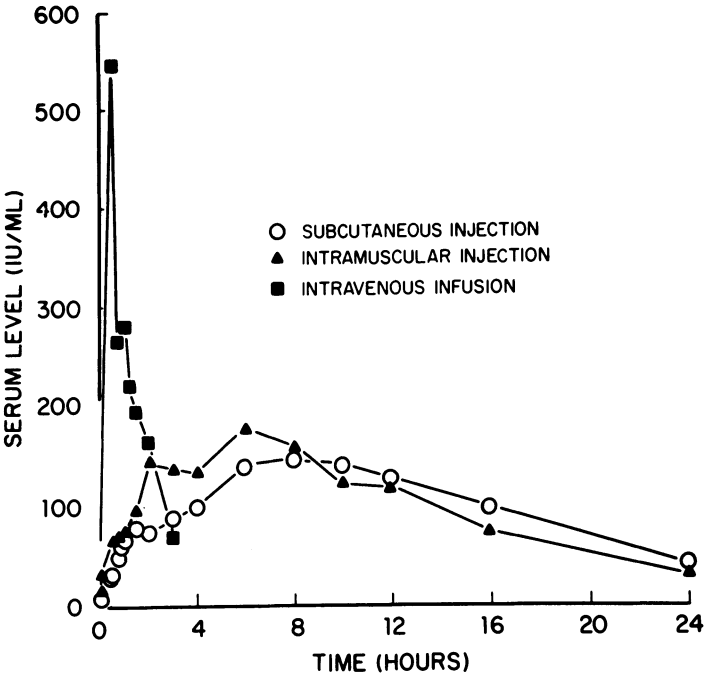

Figure 1. Mean serum level-time curves of IFN following single 10×10^6 IU doses of IFN alpha-2 administered by three different parenteral routes.

6. In vitro and animal studies have indicated that IFN can act synergistically with numerous conventional antineoplastic agents. These interactions are probably based on several types of mechanisms. The fact that IFN may potentiate the effects of cytotoxic chemotherapy is of great interest and may significantly increase its clinical utility.

These developments in IFN research have been extensively reviewed in a number of excellent volumes to which the interested reader is referred [10, 11–24]. It is our purpose to summarize current concepts related to the mechanism of IFN's antitumor activity.

Pharmacokinetics

The availability of purified recombinant IFN has permitted the characterization of IFN pharmacokinetics following administration by a variety of routes and schedules. Figure 1 depicts the results obtained using recombinant alpha-2b IFN administered via intravenous, subcutaneous, and intramuscular routes. Intravenous infusion promptly produces high blood levels. However, following bolus administration there is a half-life of only 30 minutes and levels return to baseline within two hours. Peak levels in the range of 600 to 1,000 I.U. can be attained following bolus administration of

10 million I.U. Studies employing continuous intravenous infusions have demonstrated the feasibility of achieving sustained levels. In contrast, subcutaneous and intramuscular dosing of 10 million I.U. produce slowly rising blood levels with peak levels reached in *ca* 4 hours and sustained plateaus for 8–12 hours. It is noteworthy that the peak levels obtained following intramuscular or subcutaneous doses are in the 100–200 I.U. range, which can produce immunomodulation in vitro [4] as well as direct antiproliferative activity in the human tumor clonogenic assay [25,26].

In clinical phase I and phase II studies of alpha IFNs, doses of 3–10 million I.U./day (3 times/week) have been well tolerated subcutaneously or intramuscularly. Intravenous doses up to 50 million I.U./day can be relatively well tolerated. The major toxicity observed clinically following IFN administration has been 'flu-like' symptoms (fever, chills, malaise, and myalgia), which can be expected to occur in almost all patients, and certain CNS toxicities (somnolence, confusion, and depression). The latter appear to be dose-related, occur in up to 10% of patients and may be more pronounced in elderly patients. Myelotoxicity is usually mild and rapidly reversible. Other side effects include mild elevations of liver enzymes in *ca* 10% of patients. All of these side effects are rapidly reversible upon discontinuation of IFN administration.

IFN receptors

It is well-established that the binding of both human and murine IFN to a specific cell surface receptor is the first step leading to the expression of a variety of biological activities [27,28]. Direct ligand binding studies employing radiolabelled IFN have demonstrated that binding can be correlated with the biological response [29–31]. The number of receptors per cell for both human alpha and gamma IFNs has been reported in the range of 650–13,000 with apparent dissociation constants of $10^{-10} - 10^{-11}$ M [30,32]. The latter values support the presence of a high affinity complex between receptor and ligand. Chemical crosslinking of radiolabelled human alpha IFN to the cell surface receptor has indicated a molecular weight for the interferon-receptor complex of 140,000–150,000 [31,33]. On the assumption that each receptor interacts with one IFN molecule, the apparent molecular weight of the receptor for human alpha IFN is 120,000–130,000. Physical characterization of the alpha IFN receptor solubilized from the plasma membrane has indicated that it is an asymmetric, hydrophobic membrane protein [33]. Joshi and coworkers [31] also observed that U-2 OS, a human osteosarcoma cell line relatively insensitive to the antiviral activity of human alpha IFN, did not demonstrate specific, saturable binding of radiolabelled alpha IFN and hence did not yield the 150,000 molecular weight complex upon chemical crosslinking of cells incubated with IFN. These data suggest that the failure of some cells and tumors to respond to IFN may reside, at

least in part, in the absence of the IFN receptor or in an unusually low affinity of IFN for the receptor [34]. It is consistent with this conclusion that the level of expression of the human IFN receptor on certain cells has been correlated with their sensitivity to the biological effects of IFN [35]. However, although binding to the cell surface receptor is a necessary prerequisite for IFN's activity, other events that follow binding clearly must have an equally important role. Thus, two variants of the Friend leukemia cell line have demonstrated IFN binding equivalent to that observed with the parental IFN-sensitive wild type strain but do not respond to IFN treatment [36].

Similar to the behavior of a number of polypeptide hormones and protein ligands, at least a portion of the IFN molecules are internalized by receptor-mediated endocytosis [30, 37] and appear within the cell as the IFN-receptor complex [38]. Ultrastructural studies support the concept that IFN enters the cell by clustering of the receptor-ligand complex into clathrin-coated pits followed by their transfer to receptosomes [30] — a feature also shared with polypeptide hormones. The role of this internalization in the biochemical mechanism of IFN action remains unknown. It is possible that IFN, its degradation products, or the IFN-receptor complex represents the signal that triggers the induction of cellular genes required for expression of biological activity. Alternatively, internalization may function simply to terminate IFN's action or as a self-regulating system for cell surface receptor number. Other data have suggested that binding to the cell surface per se without internalization may be sufficient for induction of the antiviral state [39].

When Daudi cells are incubated with radiolabelled alpha IFN there is a time-dependent decrease in the amount of cell-bound radioactivity. Similarly, if these cells are incubated with unlabelled alpha IFN, a progressive loss in binding of radiolabelled IFN occurs over a period of several hours [40]. These data support a steady state in which IFN-receptor complexes are internalized at a faster rate than new, unoccupied receptors are reinserted in the cell surface membrane. This phenomenon is generally referred to as 'down-regulation' of receptors. Down-regulation is apparently reversible since receptor binding activity can be regenerated upon incubation of down-regulated cells in IFN-free medium [40]. Down-regulation has also been reported for binding of IFN gamma to its receptor [32]. It will be important to ascertain whether patients receiving IFN therapy also demonstrate a similar down-regulation of the IFN receptor, and whether this behavior can be correlated with clinical response.

Specific binding of radiolabelled alpha IFN to human cells can be competitively displaced by human alpha or beta IFNs, but not by gamma IFN [31, 40]. Conversely, binding of radiolabelled gamma IFN to human cells is competitively inhibited by unlabelled gamma IFN, but not by alpha IFN [32, 40]. These data suggest that there are distinct, nonoverlapping receptors for alpha and gamma IFNs. Although the majority of reports have indicated that beta IFN binds to the same site as alpha IFN, competition between beta

IFN and gamma IFN has also been reported [41]. Chemical crosslinking studies indicate that the molecular weight of the IFN gamma-receptor complex is 105,000–125,000 [32,38], somewhat lower than the value for the alpha IFN-receptor complex. Despite the fact that the receptors are distinct, both IFNs induce the synthesis of several polypeptides in common, although gamma IFN stimulates de novo synthesis of an additional 12 distinct polypeptides [42,43]. These data suggest that the biochemical pathways induced by the two IFNs in response to binding to their receptors are different but do share common elements.

Of special importance is the fact that type I (alpha/beta) and type II (gamma) IFNs are synergistic in their biological activity, including in vitro antiviral [44], immunomodulatory [45], and antitumor effects [46,47]. This synergistic interaction is consistent with the fact that the molecular mechanisms of action for the two IFNs are unique. Consequently, a combination of alpha and gamma IFNs may be a more effective therapeutic regimen than either agent alone, provided that the toxicities are not also enhanced. The schedule for combining the two IFNs must be carefully designed, however. It has been demonstrated with human neuroblastoma cells that pretreatment with human gamma IFN decreases specific binding of alpha-2 IFN, with consequent inhibition of both the antiviral and antiproliferative activities [34].

Immunomodulatory activity

Several years after the discovery of IFN as an antiviral agent IFNs of all classes were shown to influence a variety of immune responses (reviewed by Vilcek and De Maeyer [22]). Several types of immune effector cells can be activated by IFN, including NK cells, cytotoxic T-lymphocytes, macrophages, cells involved in antibody-dependent cytotoxicity, and mast cells. In addition, the regulation of antibody formation by IFN has been described in both in vivo and in vitro models [48,49]. It is notable that IFN can both stimulate and inhibit various aspects of cellular and humoral immune function. Whether immunostimulation or immunosupression occurs depends, in general, on the IFN concentration, with higher concentrations favoring inhibition. The stimulatory effects of IFN on cytotoxic effector cell function and antibody formation may well represent major mechanisms of IFN's therapeutic activity. We will briefly review below selected aspects of IFN's immunomodulatory activity.

Although relatively impure preparations of alpha and beta IFN were first employed to demonstrate activation of NK activity in vitro [50], subsequent studies with purified recombinant IFNs have confirmed that this activity is an inherent property [51–53]. In distinction to a reproducible in vitro demonstration of NK activation by IFN, measurements of NK cell activity following administration of human alpha IFN in the clinic have demonstrated cases of both enhancement and depression as well as no response [54–56]. The mechanism for the enhancement of NK activity by IFN is

complex, involving increased growth and differentiation from NK precursors, conversion of inactive large granular lymphocytes into cytolytic effector cells, enhanced ability to interact with multiple target cells, and direct effects on the rate of cytolysis. The relative importance of these various mechanisms may be dependent on the nature of the target cell as well as the source of NK cells. IFN has also been shown to protect certain types of target cells from NK lysis [57], a phenomenon that may function in the selection of certain targets for lysis in vivo.

Another important feature of IFN's immunomodulatory activity that has been demonstrated both in vivo and in vitro is an induction of macrophage tumoricidal and tumoristatic activities [52, 58, 59]. Alpha, beta, and gamma IFNs are capable of activating the macrophage-mediated killing of a number of different tumor cells in addition to modifying a series of macrophage functions (e.g., phagocytosis, enzyme secretion, and bacteriocidal activity) [58]. Based on antiviral activity, gamma IFN has been reported to be several hundred times more active than alpha or beta IFNs in activating tumor killing by macrophages [60]. These data are difficult to interpret, however, since it is not clear to what extent differences in specific antiviral activity account for the apparent differences in macrophage activation.

All three classes of IFN appear to induce the expression of class I major histocompatibility antigens on various types of cells (reviewed by Hokland [61]). The latter antigens appear to be important in the lysis of virus-infected and tumor cells by cytotoxic T lymphocytes. This effect is dependent on an intact system for protein synthesis [62]. In addition, IFN's enhancement of the expression of the $F_c$gamma-IgG receptor on lymphocytes and macrophages may also produce enhanced immune function by increasing antibody-dependent cytotoxicity or macrophage-mediated phagocytosis [63]. Gamma IFN is more effective than alpha/beta IFNs in increasing class II (Ia-type) major histocompatibility antigen expression, predominantly on cells of the monocyte/macrophage lineage [64, 65]. Class II antigens play a fundamental role in accessory cell function, since cells on which Ia is induced acquire the ability to induce antigen-specific T-cell proliferation. Thus, these antigens are essential for the ability of macrophages to function as antigen-presenting cells. By virtue of its stimulatory effect on class II antigens, gamma IFN must have an important effect on immune responsiveness.

All three classes of IFN appear to inhibit antibody production, possibly by a direct effect on B-cells. However, under certain conditions of dosing and time of addition of IFN to the system, activation has been observed [66]. In vivo experiments in mice also indicate that both stimulation and inhibition of antibody formation can be obtained [48, 67].

Anticellular and differentiation effects

IFN can inhibit the growth of both normal and malignant cells in culture (reviewed by Taylor et al. [68]). The use of highly purified recombinant

human alpha-2 IFN has definitively established that these antiproliferative effects are an inherent property of the molecule and not due to contaminants in the preparations [4]. A wide range of in vitro sensitivities has been reported, including variable responses in cell lines derived from tumors of the same histological type [69]. The observation of variable sensitivity has recently been extended to the effect of purified recombinant alpha IFNs on fresh tumor biopsies in the human tumor clonogenic assay [25]. It is probable that a number of reasons exist for the variability in response, including differences in the affinity of IFN for the receptor or the number of receptors per cell. The biochemical pathway that mediates the antiproliferative effects may also vary either quantitatively or qualitatively among the different cell types and hence account for the observed differences in sensitivity.

Most in vitro data have shown the anticellular activity of IFN to be cytostatic rather than cytotoxic. The fact that the growth inhibitory effect can be reversed by removing the IFN provides support for the lack of cell killing [70]. However, cytotoxicity has been demonstrated for high concentrations of human gamma IFN in vitro [71]. It is significant that after removal of human beta IFN, cells can be maintained in the antiproliferative state by repeated application of a relatively low dose of IFN [72]. These data suggest that clinical protocols might incorporate long-term low dose maintenance therapy.

A number of studies have been performed to analyze the antiproliferative effect of IFN with respect to changes in the cell cycle. The distribution of cells within the cell cycle is generally measured by flow cytometry. IFN has been observed to elongate all phases of the cell cycle for asynchronously growing cells [73]. IFN treatment of various cell types can also result in a selective block in different cell compartments. For example, it has been reported that IFN causes selective extension of G_1 and G_2 in murine and human cell lines and delays passage through the cell cycle for cells treated with IFN while in G_1/G_0 [74]. Although these data suggest that IFN might be most effective as an antiproliferative agent when administered during gap periods (G_0, G_1, or G_2), it is not established that the majority of tumor types will demonstrate a similar response.

The role of IFN's direct anticellular effects in the mediation of antitumor activity in vivo is yet to be established. It is likely that the relative importance of host immune modulation compared to direct effects on the tumor will depend on the biochemical characteristics of the tumor (e.g., physical properties of the IFN receptor or the local release of proteases that may degrade the IFN) as well as the responsiveness of the host immune system. Preclinical models suggest that the direct antiproliferative effect can result in significant antitumor activity. Thus, antitumor activity has been demonstrated in the athymic nude mouse model employing a human osteosarcoma [4], a renal cell adenocarcinoma [75], and a breast carcinoma [76]. Since the recombinant human alpha subtype employed in these studies demonstrates poor activity on murine cells, it is reasonable to assume that the antitumor

activity does not involve a significant participation of the host immune system. This conclusion is consistent with previous studies documenting a failure of inhibition of the host immune response to eliminate IFN's activity against murine tumors [77]. These results suggest that the human tumor clonogenic assay on fresh tumor biopsies may be useful in selected cases in the prediction of tumor responsiveness to IFN. A number of tumor types that have responded to alpha IFN in clinical trials (e.g., melanoma) have also been responsive in the clonogenic assay [26].

Another direct cellular effect of IFN is the ability to modulate the state of differentiation of a variety of cell types. Both inhibition and acceleration of differentiation have been reported [68]. For example, both crude and recombinant alpha IFN accelerate normal myogenesis in human skeletal myoblasts, an effect antagonized by tumor promoters [78], while inhibition of differentiation by IFN is observed with chicken myoblasts [79]. Other examples of differentiation effects of type I IFN include both an enhancement and inhibition at low and high IFN concentrations, respectively, of erythroid differentiation [80, 81]; inhibition of spontaneous and insulin-stimulated adipogenesis in murine 3T3 cell lines [82, 83]; and a concentration-dependent inhibition of spontaneous and hormone-stimulated melanogenesis in murine B-16 cells [84]. Gamma IFN has also been demonstrated to affect the state of cellular differentiation. Thus, highly purified natural and recombinant gamma IFN can induce the differentiation of HL-60, a human promyelocytic leukemic cell line, into macrophage-like cells [85]. Neither human alpha nor beta IFN alone can induce differentiation of these cells [86]. In contrast, human alpha IFN can potentiate the ability of other inducers of differentiation (e.g., dimethylsulfoxide or retinoic acid) to stimulate the differentiation of both sensitive and resistant cell lines [86]. Synergy occurs in the interaction of human beta IFN and meserein, an antileukemic agent, in enhancing terminal differentiation of human melanoma cell lines [87]. IFN can also act synergistically with phorbol ester tumor promoters in the inhibition of differentiation [82, 84].

The induction of differentiation in tumor cells mediated by IFN alone or in combination with other agents may represent at least a part of its in vivo antitumor activity. This hypothesis is based on the observation that, for a given tumor type, there appears to an inverse relationship between the degree of cellular differentiation and the rate of proliferation. As cells differentiate, their proliferative as well as metastatic potential is lost, resulting in terminally differentiated populations that are essentially nonproliferative and noninvasive. The in vitro synergistic interaction of IFN with other agents in promoting differentiation provides further support for designing clinical trials based on these combinations. An understanding of the mechanism through which this synergy occurs may provide a basis for selecting a patient population most likely to respond favorably. It is yet to be established whether the site of interaction is primarily at the cell surface (e.g., in the generation of the appropriate membrane signal) or in the

cytoplasmic and nuclear compartments where the various induced proteins and genes are located. The elucidation of the biochemical mechanism for regulation of differentiation by IFN represents an important area of future investigation.

Effects on intracellular proteins and the cell membrane

The biochemical basis for any of IFN's actions may reside at a number of different points in cellular function and, in fact, may represent a composite of several mechanisms acting in concert. A variety of diverse biochemical effects appear to be triggered following IFN's binding to its receptor. These have been extensively documented in a number of different cell types. Thus, IFN has been observed to produce changes in the levels of intracellular enzymes critical for specific functions; alterations in the properties of the cell surface membrane; direct effects on internal structural elements of the cytoskeleton; and changes in the levels of critical metabolites like cyclic nucleotides and prostaglandins. As previously noted, IFN also causes an increase in the expression of a number of class I and II histocompatibility antigens, which have an important role in immune function. Of special interest are reports that alpha IFN can induce tumor-associated antigens [88]. It is reasonable to speculate that these cell-surface alterations may function in the recognition and lysis of malignant and virus-infected cells. It is beyond the scope of this chapter to review all of these reports in detail; the interested reader is referred to a number of excellent summaries and reviews [89–93]. We will attempt to highlight certain of these mechanims that appear to have the highest likelihood for representing the in situ target for IFN's varied effects.

The most widely investigated biochemical action of IFN that has been implicated in its antiviral activity against certain viruses is its induction of the synthesis of two enzymes that inhibit protein synthesis, each of which requires double-stranded RNA for activation [94]. The first is a protein kinase that catalyzes the phosphorylation of the alpha subunit of initiation factor eIF-2, resulting in inhibition of protein synthesis. The second is the enzyme 2′,5′-oligoadenylate synthetase, which catalyzes the formation of an oligomeric series of phosphorylated adenosine derivatives linked by unusual 2′,5′-phosphodiester bonds. These low molecular weight compounds activate a latent endonuclease activity, resulting in cleavage of messenger and ribosomal RNAs and inhibition of protein synthesis. The latter mechanism apparently derives its specificity for cleavage of viral versus host RNA from the localized activation of the endonuclease by double-stranded RNA present in the viral structure.

Identification of the biochemical changes that mediate the direct antiproliferative activity of IFN has not yet been achieved. Various reports have appeared correlating increases in 2′,5′-oligoadenylate synthetase with

decreased states of growth [95, 96] or the state of cellular differentiation [97]. Other cells with high sensitivity to growth inhibition by low concentrations of alpha IFN do not demonstrate increased levels of 2′,5′-oligonucleotides [98]. It is clear that alternative biochemical mechanisms must be sought to explain the antiproliferative effects. Other candidates include an elevation in cyclic adenosine monophosphate levels [99], which may be growth-inhibitory at least to certain cell types, and interference with polyamine biosynthesis through inhibition of the enzymes ornithine decarboxylase and S-adenosylmethionine decarboxylase [100, 101]. It may be relevant that difluoromethylornithine, an inhibitor of ornithine decarboxylase, is synergistic with IFN for inhibition of murine B16 melanoma growth. However, measurements of ornithine decarboxylase or polyamine levels alone do not predict IFN's antitumor activity in an animal model [75].

Substances that act to disrupt microtubules (e.g., vinblastine) also inhibit IFN's action, suggesting a direct effect of IFN on the cytoskeleton of the cell [102]. Subsequent studies demonstrated that IFN can affect the microfilamentous organization through an increase in the number of actin cables at the plasma membrane [103]. These effects could, in principle, alter cell motility and hence influence a variety of biological functions. Changes in the cell surface induced by IFN have been reported, including increased negative charge, an increase in the buoyant density of plasma membranes, and an increase in the concentration of intramembraneous particles [89]. In addition, a number of investigators have observed that type I and type II IFNs induce various unidentified membrane proteins, a number of which are shared in common between the IFN classes [42, 43]. Recent reports have indicated that IFN can reduce the expression of cellular oncogenes [104]. The relevance of any of these effects to IFN's antiproliferative or direct antitumor activity remains speculative.

IFN/drug interactions

Both in vitro and animal experiments have suggested that synergistic or additive interactions might be achievable clinically with combinations of IFN and chemotherapeutic agents. Synergy was first reported by Gresser and coworkers [105] between daily injections of murine alpha/beta IFN and a single dose of cyclophosphamide, an observation later confirmed in other murine models [106, 107]. Positive interactions have also been observed in vivo between BCNU and murine alpha/beta IFN against murine leukemia [108] as well as between human alpha IFN and either cyclophosphamide or doxorubicin against a human breast tumor xenograft in a nude mouse model [109]. However, a number of cytotoxic drugs have failed to demonstrate synergy with alpha/beta IFN against a murine L1210 leukemia, including cyclophosphamide, cytosine arabinoside, and adriamycin [110], and antagonism has been occasionally observed as, for example, in the interaction of rat fibroblast IFN and 5-fluorouracil [111]. The demonstration of synergy is

likely to be dependent on the nature of the tumor, the mechanism of action of the cytotoxic agent, as well as the scheduling of IFN and the cytotoxic agent. For example, in studies in which IFN was administered prior to cyclophosphamide, IFN apparently antagonized the cytotoxic action of the drug [62, 112]. IFN's anticellular activity has been demonstrated to be enhanced by combination with hyperthermia in studies on human and murine cells in culture [113, 114].

Interactions between IFN and cytotoxic drugs have also been examined in vitro utilizing the soft agar human tumor clonogenic assay [25, 115]. Statistically significant synergy has been observed in this assay with either established cell lines or fresh tumor biopsies employing combinations of recombinant human alpha IFN with vinblastine, cisplatin, and doxorubicin. Interestingly, the demonstration of synergy in these studies appears to be highly schedule-dependent. When cells were treated first with alpha IFN followed by long-term exposure to doxorubicin (or vice versa), a synergistic effect was not observed, compared to a dramatic potentiation obtained in this system when both agents were present throughout the assay [25]. Other drugs tested (e.g., bleomycin, methotrexate, other vinca alkaloids, and 5-fluorouracil) generally were not synergistic with IFN, but yielded additive or subadditive effects.

The mechanism for the apparent synergy between IFN and cytotoxic drugs remains speculative. One hypothesis is that IFN produces alterations in the metabolism of the drug through, for example, inhibition of the cytochrome P-450 mono-oxygenase system [116]. Studies in the athymic nude mouse model have strongly suggested that it is the tumor itself, rather than the host's drug-metabolizing enzymes in the liver, that is the primary site for positive interactions between alpha IFN and either doxorubicin or cyclophosphamide]117]. The mechanism for the synergy observed in this model appears to be an increased proportion of tumor cells in the S phase, thus increasing their sensitivity to these phase-specific drugs.

Conclusion

Many of the agents currently utilized as antineoplastics were brought to the clinic 20 to 30 years ago with mechanisms of action that were either unknown or, in retrospect, poorly understood. Extensive research efforts are still ongoing to determine the specific molecular targets for compounds such as the alkylating agents, anthracyclines, and antimetabolites. The nature of metabolic changes that result from administration of these cytotoxic agents also remains unknown. Similarly, although IFN has been introduced in the clinic as the prototype biological response modifier based on in vitro and in vivo preclinical testing, a complete understanding of its mechanism of antitumor activity is still not available. The magnitude of this problem is emphasized by the variety of cell types with which IFN can interact and the

complexity of the intracellular changes triggered by IFN's interaction with its receptor. The fact that highly purified recombinant human IFN has resulted in clinical efficacy has definitively eliminated the early concern with crude preparations of IFN that contaminants (e.g., other lymphokines) were true mediators of the responses.

The mechanism of IFN's therapeutic activity may involve a direct anticellular action on the malignant cells, an indirect effect mediated through an alteration in the response of the host immune system, or a combination of the two effects. As described in this review, a high degree of complexity exists within each of these categories. Direct effects include both cytotoxic and cytostatic responses, which may be associated with changes in the state of cellular differentiation, as well as increases in tumor-associated antigens, which may enhance immune rejection. The most cogent preclinical evidence for the involvement of a direct anticellular effect in IFN's antitumor activity are reports indicating that human tumors implanted in athymic nude mice are responsive to human IFN despite the fact that due to IFN's high species specificity the host immune cells are poorly responsive [4]. Positive interactions between alpha IFN and cytotoxic agents also appear to result from direct antitumor action [117]. Alternatively, indirect effects include stimulation of cytotoxic effector cells (e.g., natural killer cells or macrophages), enhanced expression of histocompatibility antigens and receptors for IgG, and acceleration of antibody formation. The pioneering studies of Ion Gresser and colleagues [118] demonstrated clearly that leukemic cells nonresponsive to IFN in vitro were sensitive following implantation in mice, as measured by prolongation of survival. It is likely that the choice between the direct or indirect effects of IFN as a basis for understanding the antitumor activity will be unresolved for some time and may be variable in different clinical settings. Interestingly, the IFN-activated state of the cell may be transferred to other cells by contact [119]. The additional possibility exists, therefore, that the direct antitumor effect may be derived, in part, from contact of the tumor with host activated leukocytes. Other critical factors that must be considered include the type of IFN administered, its dose and scheduling, the immune status of the host, and the biochemical characteristics of the tumor. No uniform mechanism can be anticipated to apply to all clinical responses to IFN.

It has been demonstrated that IFN may induce other lymphokines and biological response modifiers including interleukins -1 and -2 [120]. This effect leads to a complex pathway of feedback loops and interactions in various cell types of the immune system. Although the relative significance of these various immunoregulators in the biological response to IFN is yet to be established, the interesting speculation arises that combinations of these factors may lead to effects that are not observed with any of the agents alone. For example, strong synergistic antitumor activity has recently been reported between gamma IFN and tumor necrosis factor [121]. The synergistic or additive interaction of biological response modifiers

offers promise in the development of successful treatment regimens, but much basic research is still required to clarify the optimal conditions for their combination.

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7. Acivicin: A new antimetabolite

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Acivicin is an L-glutamine antagonist which underwent phase II clinical efficacy evaluation as an anticancer drug in 1985–1986. The biochemical pharmacology of this agent is unique among antimetabolites and offers several interesting possibilities for combination therapy with agents whose activity can be potentiated by acivicin, with agents which can interfere with cellular resistance to acivicin, and with agents which can lessen the dose-limiting toxicity of acivicin. This review will cover some of the experimental background of current clinical research involving acivicin, will relate clinical findings to the preclinical pharmacology of the drug, and will suggest some new directions for both clinical and preclinical research.

Biochemical pharmacology

The biochemical pharmacology of acivicin has been reviewed in detail [1, 2]. Antimetabolite activity was found in screening the fermentation broth of *Streptomyces sviceus* var. *sviceus* using bacterial cultures grown on defined media which were specifically designed as a screen for such activity [3]. This broth was found be active against the L1210 murine ascitic leukemia model even in impure form [4], and after isolation and purification the active principle was determined to be acivicin, or (αS, 5S)-α-amino-3-chloro-4, 5-dihydro-5-isoxazoleacetic acid [5]. The inhibition of ^{3}H-TdR incorporation into DNA by acivicin is antagonized by L-glutamine and by a combination of cytidine and guanosine [1]. Acivicin inhibits several (but not all) glutamine-dependent amidotransferases [6], with most significant effects on CTP synthetase (UTP:L-glutamine amido-ligase) and XMP aminase (XMP: L-glutamine amido-ligase). Other enzymes inhibited by acivicin include carbamyl phosphate synthetase II (CPS II) and FGAR amidotransferase, which have important roles in purine and pyrimidine metabolism. These inhibitions result in increases in cellular UTP concentration, accompanied by decreases in CTP and GTP concentrations with no alteration in ATP or ITP pools [7]. The antineoplastic effect of acivicin may require the inhibition of these pathways for a significant part of the average cell cycle duration.

Acivicin irreversibly inhibits CPS II in vitro and FGAR amidotransferase in vivo at high doses [8, 9]. This implies that the observed recovery of these important biochemical pathways after brief exposure to acivicin is dependent on synthesis of new enzyme and that synergism might be expected between acivicin and agents which interfere with DNA transcription and translation. Alternatively, the delivery of sustained drug levels by continuous drug infusion regimens could be effective due to inactivation of newly synthesized enzymes.

In addition to these general biochemical actions of acivicin, recent studies have found specific differences between tumors and normal tissues in the patterns of metabolite pool sizes and enzyme inhibition by acivicin, and these factors may account for the selective cytotoxic effects of the drug against tumor cells [9]. Rat hepatoma, renal cell carcinoma, and sarcoma, and human renal cell carcinoma and colon carcinoma have increased amidophosphoribosyl transferase, GMP synthetase, CPS II, and CTP synthetase relative to the corresponding normal tissues, and glutamine content is decreased in the more rapidly-growing tumors. These key enzymes were inhibited in vivo by acivicin, and selective decreases in CTP and GTP pools occurred. There is thus biochemical evidence that a favorable therapeutic ratio can be achieved between the effects of acivicin on certain tumors and on the corresponding normal tissues.

Acivicin also inhibits gamma-glutamyltranspeptidase, an enzyme which has an important role in glutathione [GSH] metabolism and in amino acid transport by cells, including those of the renal tubule [10–12]. In vitro, an acivicin concentration of 0.3 mM is required to inhibit this enzyme [11], and this concentration exceeds the level which can be achieved in the clinic. Such inhibition might be expected to decrease intracellular GSH and cysteine levels while increasing circulating GSH [13]. Although exogenous GSH can protect against acute nephrotoxicity of cisplatin [14], and GSH is a factor in the efficacy and toxicity of a variety of antineoplastic drugs and other treatment modalities [15–17], it appears unlikely that acivicin will exert important clinical interactions through this mechanism.

Preclinical activity

Clinically achievable concentrations of acivicin exert cytotoxic effects in vitro. The ID_{90} values for L1210 and P388 murine leukemia cells and for KB human epidermoid carcinoma cells are 0.20, 0.19, and 2.2 μg/ml, respectively. At these concentrations, there is a significant inhibition of ^{3}H-TdR incorporation into DNA, with no inhibition of RNA or protein synthesis as measured by incorporation of ^{3}H-UR or ^{14}C-valine. However, these effects are highly dependent upon the duration of exposure of cells to the drug, with 24 hour exposure required to attain maximal growth inhibition. Following intraperitoneal injections of acivicin, ascitic L1210 cells rapidly recover

the ability to incorporate ^{3}H-TdR as drug levels decline in the animals [1], despite the irreversible nature of the in vitro enzyme inhibition studies noted above.

In vitro, acivicin inhibits cell cycle progression in G_1 phase in human breast carcinoma cell lines, and the magnitude and irreversibility of the effect are greater in the line with higher gamma-glutamyltranspeptidase activity [18]. The MiaPaCa-2 human pancreatic carcinoma cell line undergoes marked reduction of cell growth rate during late G_1 and early S phases when exposed to acivicin, although the effect is reversible by drug removal and there is little effect on colony-forming activity [19]. Similar observations have been reported in cultured Burkitt lymphoma cells, with loss of colony-forming activity noted with prolonged (36 hour) exposure to clinically-relevant concentrations (5-50 μM) of acivicin [20]. These results further support the concept that acivicin must be continuously present at effective levels in order to inhibit cell growth.

Acivicin is active by the intraperitoneal, subcutaneous, and oral routes of administration in tumor-bearing animal models. Activity in the L1210 murine ascitic leukemia model is schedule-dependent, with optimal results (91% ILS) obtained by doses administered every 3 hours on days 1, 5, and 9; the single injection regimen is much less effective (37% ILS). The spectrum of activity is broad; in addition to good activity in L1210 and P388 ascitic murine leukemias, acivicin causes complete regression in the subcutaneous M5076 reticulum cell sarcoma model and is active in the human lung (LX-1) and mammary (MX-1) tumor xenografts in nude mice [21, 22]. In the latter model, acivicin has schedule-dependent activity; daily administration is much more effective than is intermittent administration [23]. Acivicin shows little effect on human colon tumor xenografts or on B16 melanoma or Lewis lung carcinoma systems [21, 22].

Preclinical toxicology

Murine toxicity

Female mice are more vulnerable to the toxic effects of acivicin than are males, and younger animals are more susceptible than are older ones. Histopathologic studies reveal lesions of the intestinal epithelium, including shortened villi and degeneration of crypt cells; clinically, this results in severe diarrhea and dehydration. Testosterone alleviates the differential toxicity in young males and in young and older female mice [6]. This differential toxicity is related to decreased clearance of acivicin in younger animals and in females. Clearance can also be converted by testosterone in these mice to the pattern seen in older males [24]. Continuous infusions of acivicin at various levels have been used to demonstrate that toxicity in mice is related to the total dose administered rather than to the infusion rate [25].

Table 1. Qualitative toxicity of acivicin in large animal species.

Toxicity	
Clinical	
Diarrhea	Convulsions
Hematochezia	Alopecia
Anorexia	Rashes
Adipsia	Emesis
Hyperthermia	Gait Disorders
Oligodipsia	Pulmonary Distress
Emaciation	Somnolence
Laboratory	
Myelosuppression	Elevated Creatinine
Marrow Aplasia	Proteinuria
Transaminasemia	Glycosuria
BSP Retention	Electrolyte Disturbances
Specific Gravity Changes	
Histopathologic	
Reversible Hepatocellular Vacuolization and Peripheral Degeneration	
Renal Tubular Necrosis	
Renal Pelvic Hemorrhage	
Renal Interstitial Edema	
Reversible Lymphoid Depletion (Nodes and Thymus)	
Adrenal Cortical Necrosis and Hemorrhage	
Widespread Gastrointestinal Lesions	
Male Reproductive System Lesions	
Prostatic Inflammation and Necrosis	

Large animal toxicity

In larger species, toxicity of acivicin does not appear to be sex related, in contrast to the murine system. Toxicity is schedule-dependent in dogs, however; on the daily × 5 schedule, the lethal dose [LD] is 80 mg/m^2/course, and the toxic dose low (TDL) is 10 mg/m^2/course, in contrast to the single bolus dose schedule, where the LD is 1000 mg/m^2 and the TDL is 125 mg/m^2. Monkeys tolerate doses which are twice as high as those giving similar quantitative toxicity in the dog on the daily × 5 schedule. Qualitative toxicity is similar in both species and is predominantly gastrointestinal (table 1). There is no evidence of local tissue reactions after injections [22].

Phase I clinical trials

Five clinical phase I trials have been conducted under the auspices of the Cancer Therapeutics Evaluation Program (CTEP) of the U.S. National Cancer Institute. Schedules included 24 hour infusion [26], 72 hour infusion [27], brief infusion daily × 5[28, 29], and brief single infusion every three weeks [30]. At doses lower than the maximum tolerated dose (MTD) in each study, gastrointestinal toxicity (including nausea, vomiting, diarrhea, and stomatitis), hematological toxicity (including leukopenia and throm-

bocytopenia), minimal alopecia, dermatitis, malaise and anorexia, and mild and reversible hepatotoxicity were seen. Neurotoxicity was found to be the dose-limiting toxic effect in trials employing single brief infusion, 24 hour infusion, and 72 hour infusion, while myelosuppression was dose-limiting and neurotoxicity was relatively mild with the daily × 5 regimen. The nature of acivicin-induced neurotoxicity will be discussed in detail below.

Although these phase I tolerance studies treated a mixture of solid tumor types at a wide range of doses and were usually conducted in patients who had failed to respond to other forms of chemotherapy, beneficial antitumor responses were observed in some patients. One patient with gastric carcinoma enjoyed an objective partial response lasting two months [36], minor responses or improvements were seen in five patients with colon carcinoma [26–29], one patient with melanoma [27], one patient with epidermoid lung cancer [27], one patient with pulmonary adenocarcinoma [29], one patient with ovarian carcinoma [28], and two patients with breast carcinoma [28]. A mixed response was noted in one patient with pancreatic carcinoma [28], and stable disease was seen in one patient with small cell lung carcinoma [30]. These findings encourage trials of the clinical efficacy of acivicin in the diseases where responses occurred; they do not preclude the possibility of clinical efficacy in other types of cancer.

Pharmacokinetics

Assay methods

Studies of the pharmacokinetics of acivicin have been hampered by the lack of a conventional assay for determination of the drug in biological fluids. Most data have been collected using a microbiological assay which is based on inhibition of *Bacillus subtilis* growth [24]. Bioautographic studies suggest that this assay is specific for unchanged drug, and the assay is sensitive to levels as low as 0.06 μg/ml in plasma and 0.6 μg/ml in urine [31]. A new assay uses chemical ionization mass spectrometry as a detection method for derivatized acivicin and its bromo analog (internal standard) after separation on a capillary gas chromatographic column [32]. This method can quantitate acivicin at concentrations as low as 25 ng/ml in plasma and accurately confirms data from the microbiologic method but has not yet been employed in formal pharmacokinetic studies. Both methods detect only intact acivicin; the amount and nature of the metabolites of the drug are unknown.

Preclinical

Acivicin follows first-order elimination kinetics after intraperitoneal administration in mice, with half-lives ranging from 15.6 to 77.9 minutes. As noted above, the half-lives were longest in young males and in females.

Volume of distribution ranged from 0.57 to 1.49 l/kg; clearances ranged from 0.17 to 0.93 ml/min. The drug concentrates in kidney and, to a lesser extent, in muscle; both tissues showed higher tissue/plasma concentration ratios in male mice relative to those found in female mice [24]. In rhesus monkeys, acivicin plasma concentrations after intravenous injection followed a biphasic elimination pattern, with a mean distribution half-life of 1.01 ± 0.16 hr and a mean elimination half-life of 3.04 ± 0.33 hr. CSF drug levels obtained in rhesus monkeys by means of Ommaya reservoirs were 10% to 17% of the simultaneous plasma levels [33].

Clinical

Two of the phase I trial reports contain detailed pharmacokinetic information [26, 27], and data from other patients have been reported recently [31]. Data from the daily $\times 5$ and single dose trials in man reveal the kinetics of acivicin to be dose-independent over the range of 6.8–150 mg/m^2. The mean distribution phase half-life is 0.32 ± 0.28 hours, the mean excretion-phase half-life is 9.92 ± 3.91 hours, total body clearance is 1.69 ± 0.48 l/hr/m^2, and urinary recovery is 11.1 ± 12.2% of the administered dose [31]. Single brief infusion and daily $\times 5$ intermittent brief infusion regimens do not achieve sustained levels of the drug. When acivicin was infused over a 24 hour period, plasma concentrations increased steadily to peak levels which correlated linearly with the infused dose. Following infusion, plasma concentrations declined biphasically, with terminal half-lives ranging from 6 to 9 hours. Urinary recovery of intact acivicin during infusion ranged from 4% to 19%, and following the infusion ranged from 5% to 9% of the administered dose [26]. When a 72 hour infusion schedule was employed, plateau plasma levels of acivicin were reached after 24–36 hours and correlated with the infusion rate [27]. Following infusion, there is a brief distribution phase which is difficult to quantitate (as expected with a prolonged infusion regimen), with a half-life of approximately 0.45 hours. The distribution phase exhibits a mean half-life of 8.3 hours; overall urinary drug recovery averages 35% of the administered dose in those patients who have technically satisfactory urine collections. Nonrenal clearance accounts for 67% of the total acivicin clearance in these patients, and renal acivicin clearance is less than the creatinine clearance, implying either tubular reabsorption or significant protein binding of acivicin. The former mechanism is somewhat favored by the finding that acivicin clearance exceeds the creatinine clearance in mice. In that species, tubular secretion apparently dominates over reabsorption, and urinary drug recovery ranges from 53% to 82% of the administered dose [24]. Comparison of these data suggests that there are significant differences between humans and mice in acivicin clearance mechanisms. Metabolism seems to dominate acivicin clearance in humans, while mice excrete most of the drug. Since CNS toxicity predominates in the former species and is not detectable in the latter, experiments which investigate the

role of metabolites of acivicin in the CNS toxicity of the drug may be important.

Phase II clinical trials

Efficacy

Published phase II trial results include studies of acivicin in advanced non-small cell lung cancer [34], colon cancer [35,36], breast cancer [37], and ovarian cancer [38]. All five trials employed five daily brief infusions of the drug, administered every 21–28 days, at doses of 60 or 75 mg/m^2/course. In 23 evaluable patients with previously untreated non-small cell lung cancer, acivicin produced three objective partial responses (PR), five minor responses (MR), and three stable diseases (SD), for an objective response rate of 13.4% and possible clinical benefit in 47.9% of the patients [34]. Among 17 evaluable patients with previously untreated colon carcinoma, there were no objective responses; nine patients (52.9%) had stable disease [35]. In a similar trial, seven patients with evaluable, previously treated (5-FU or combination regimens containing 5-FU) colon cancer had no objective responses, but 28.6% had stable disease [36]. Of 22 evaluable patients with previously treated breast carcinoma, there were no objective responses; 22.7% had stable disease [37]. Of 24 evaluable women with previously treated epithelial ovarian cancer, one patient achieved a partial response lasting five months [38]. These results suggest that acivicin may have a role in the treatment of non-small cell lung cancer, and that trials employing improved regimens in colon and ovarian cancer may have promise.

Toxicity

Despite the relative lack of CNS toxicity in phase I trials of this regimen, all of these studies report significant CNS effects of acivicin. The incidence of such toxicity ranged from 33% to 46% of patients treated, where reported. In most studies only a subset of patients who were treated with the daily × 5 regimen experienced dose-limiting CNS toxicity, and myelosuppression was more severe in most cases. An exception was seen in the ovarian carcinoma trial, in which neurotoxicity was more severe [38]. There was no correlation between the presence of ascites and the incidence of neurotoxicity in this study. Acute gastrointestinal toxicity was also seen in all studies. These phase II trials did not identify toxicities which were qualitatively different from those seen in phase I studies.

Future trials

Ongoing or proposed phase II trials of acivicin are listed in table 2. There is a continuing interest in acivicin by clinical researchers, although the progress

Table 2. Ongoing and proposed phase II clinical trials of acivicin as single agent chemotherapy.

Disease	Schedule	Dose [mg/m^2/course]	Institution or Group	Preliminary Results
Colon	72 hour infusion	60	ECOG	+
Lymphoma	QD × 5	60	NCIC	+
Breast	72 hour infusion	60	ECOG	—
Melanoma	72 hour infusion	60	ECOG	—
Ovary	72 hour infusion	60	ECOG	early
Mesothelioma	72 hour infusion	60	ECOG	early
Glioma	QD × 5	60	SWOG	

of several studies has been hampered by technical problems with supplies of formulated drug. These have now been solved, and a broad program of phase II trials is presently being conducted under the auspices of the CTEP.

The best dose and schedule for treatment of human cancer with acivicin have not yet been established. Preclinical efficacy and clinical toxicity of acivicin are highly schedule-dependent. Results obtained with any regimen in a specific tumor cannot be expected to predict the results or trials which employ a different schedule in treatment of the same cancer. Since prolonged exposure of cells to acivicin seems to improve the cytotoxic effect, prolonged infusion at relatively low doses may achieve improved antitumor activity with reduction of toxic effects which are dependent upon other mechanisms. In addition, acivicin has been found to display a steep cytotoxic dose-response curve against clonogenic cells from human breast carcinomas [39]. These results suggest that regional infusion methods which achieve high local drug concentrations might enhance the moderate efficacy of acivicin in disease such as colon carcinoma metastatic to the liver. A clinical trial to test this possibility is in progress. The steep dose-response curve also implies that any method which alleviates the dose-limiting neurotoxicity of acivicin and permits the administration of higher doses of the drug, has significant potential for improvement in the therapeutic results.

Neurotoxicity of acivicin

Neurotoxicity was dose-limiting in both phase I and phase II clinical trials employing single brief infusion, 24 hour infusion and 72 hour infusion schedules, while myelosuppression was dose-limiting in most trials using the daily × 5 infusion schedule. In the last, the MTD was 75–100 mg/m^2/course. No myelosuppression was seen in the single brief infusion trial, and the MTD was 120 mg/m^2/course. In contrast, the 24 hour infusion schedule found an MTD of 160 mg/m^2/course, while the 72 hour schedule found an MTD of 75 mg/m^2/course; both of the latter groups observed myelosuppression in some patients. There seem to be schedule-related differences in the

Table 3. Calculated pharmacokinetic parameters and toxicity of four Acivcin schedules.

Schedule	Total Dose [mg/m^2/course]	Peak Level [μg/ml]	AUC [g/min/ml]	Duration >0.9 μg/ml [hr]	Toxicity	Ref.
Bolus	120	13.85	5.86	26.6	CNS	[30]
24 hour infusion	160	4.66	7.69	41.3	CNS	[26]
72 hour	90	0.99	4.26	46.1	CNS	[27]
infusion	75	0.82	3.55	0	Rare CNS	[27]
QD × 5	75	2.05	3.56	16.9	Hematologic + mild CNS	[28]
	60	1.64	2.85	9.7	Hematologic	[28]

Table 4. Clinical symptoms and signs of acivcin intoxication.

Mild neurotoxicity (lower doses):	
Somnolence	Confusion
Lethargy	Depression
Fatigue	Mood Changes
Asthenia	Vivid Dreams
Disorientation	Nightmares
Headaches	'Large thoughts'
Severe neurotoxicity (higher doses):	
Dysarthria	Hallucinations
Ataxia	Paranoia
Amnesia	Hostility
Transient unconsciousness	Anxiety
Tinnitus	Dizziness

toxicity of acivicin in man, both in terms of the type of toxicity which is observed and the dose of drug required to induce toxicity. Based on simulation of the pharmacokinetics of acivicin in man, it was suggested that neurotoxicity did not correlate with peak serum drug level, but was related either to the integral of the concentration-time curve (AUC) or to the maintenance of a serum acivicin level in excess of 0.9 μg/ml for greater than 16 hours [27]. Myelosuppression, in contrast, appeared to be related to the duration of treatment rather than to peak level or to the AUC. The data which support these hypotheses are shown in table 3 (after [27]).

The neurotoxic effects of acivicin are variously described but uniformly unpleasant (table 4). Unequivocal peripheral nerve toxicity has not been reported. Diazepam proved ineffective in prevention of CNS toxicity [30]; there may have been more CNS toxicity in patients receiving opiate analgesia or with increased third-space fluid [28]. Diffuse slowing of EEG suggestive of metabolic encephalopathy was seen in two patients [28]. In all cases, neurotoxicity was reversible.

The CNS toxicity of acivicin at doses which do not cause major myelosuppression or acute gastrointestinal toxicity was not predicted by preclinical

toxicology studies, and careful search for an animal model of this toxicity has been unrewarding. The adult cat exhibits dose-related ataxia and sedation, but sensitive rodent methods have not revealed CNS changes attributable to acivicin [40]. The major CNS effects of acivicin in man appear to be relatively specific for our species.

There have been several theories proposed to explain the CNS toxicity of acivicin, but relatively little experimental data supporting them has been published. The simplest is that the drug may have intrinsic activity as a stimulatory or inhibitory neurotransmitter, or inhibits the action of one or more naturally-occurring neurotransmitters. Acivicin has structural features in common with certain known agents which have CNS activity, including ibotenic acid [41] and muscimol [42]. The former is an agonist for aspartate-mediated neurotransmission, and the latter for γ-aminobutyric acid. One defect in this hypothesis is that it does not explain the apparent species-specificity of the effect; one must hypothesize an interaction with a neurotransmission system which is physiologically important in humans and in cats, but not in rodents, dogs, or monkeys.

Alternatively, acivicin may be converted by metabolism into a neuroactive metabolite. Since there are demonstrable differences between species in the total body economy of acivicin (noted above), this hypothesis is attractive. Present measurement methods detect only intact or biological active acivicin; there is no information regarding metabolites of the drug in any species. The advent of ^{14}C-labelled acivicin will provide a powerful tool for metabolic investigations in the near future. Such studies must address the question of schedule specificity. Pharmacodynamic analysis of clinical data predicts that exposure to the CNS-active metabolite must be dependent upon AUC of the drug or duration of exposure to the drug, rather than upon the peak level of acivicin which is achieved in plasma.

The antimetabolite actions of acivicin may affect brain nucleotide or amino acid metabolism. Both de novo synthetic and salvage pathways for purines and pyrimidines are active in brain; alterations in nucleotide metabolism may produce physiological effects through alterations in macromolecular synthesis and in reactions which employ nucleotide cofactors [43]. L-glutamic acid may act as a neurotransmitter in the CNS [44] and plays a central role in the synthesis of γ-aminobutyric acid, aspartic acid, glutamine, and glycine in the brain. Each of these compounds has been linked to neurotransmission [45], and the interrelationships between the handling of these amino acids in different compartments of the CNS provide the basis for important concepts of brain biochemistry and function [46–48]. The effects of a glutamine antagonist on pool sizes, synthesis, storage, and release of these amino acids might be expected to produce CNS effects. In cats, which exhibit behavioral effects after acivicin administration, twofold elevations of glutamate, aspartate, and γ-aminobutyrate have been found in the cerebrum but not in cerebellum or midbrain after acivicin treatment [40]. Glutamine content was unchanged by acivicin treatment. Other actions

of acivicin may influence brain amino acid pools. Acivicin increases circulating GSH and decreases circulating cysteine in rats, probably through inhibition of γ-glutamyltranspeptidase, with concomitant decrease in brain cysteine and GSH [49]. This experimental observation is difficult to relate to CNS toxicity in a straightforward manner, since rats do not show detectable CNS effects when treated with acivicin. The physiological and behavioral sequelae of such effects must be defined by further study in sensitive species.

Alterations in circulating asparagine or glutamine concentrations may be associated with clinical neurotoxicity similar to that of acivicin. In 33% of 39 patients evaluable for neurotoxicity, L-asparaginase produced depression, drowsiness, confusion, and disorientation. CNS leukemia, bleeding, meningitis, and electrolyte imbalance were excluded as possible causes. In the seven patients with the most severe effects, two exhibited symptoms within 24 hours and five had delays of greater than one week before symptoms developed; symptoms lasted nearly one month [50]. This timing differs from that of acivicin-induced CNS toxicity, which usually begins within one day of treatment and clears within a week. Similar observations resulted from a larger trial, in which 33% of 147 children and 46% of 156 adults experienced CNS toxicity after asparaginase treatment [51]. Succinylated *Acinetobacter* glutaminase-asparaginase also induced coma in five leukemic patients in another trial, but all had either CNS leukemia or a history of cranial irradiation and intrathecal methotrexate [52]. Infusions of asparagine improved the CNS status in three of the severely-afflicted patients in one trial [50]; it remains to be seen if asparagine or glutamine infusions affect the CNS effects of acivicin.

Some of the hypothetical mechanisms for the CNS toxicity of acivicin involve the action or metabolism of the drug in brain. Acivicin crosses the blood-brain barrier in mice [24], dogs [J.P. McGovren, unpublished], and monkeys [33]. In tumor cells in vitro, one study suggests that acivicin transport is mediated by the L-system common to large neutral amino acids [53]. In a study which employed a different tumor line, transport was inhibited by L-glutamine [54]. Brain transport of large neutral amino acids is thought to be mediated by a carrier which is specific and saturable and which shows competitive effects between different substrates [55]. It has been hypothesized by Dr. J.P. McGovren that the CNS effects of acivicin can be lessened or prevented if transport of the drug across the blood-brain barrier can be inhibited, and that large neutral amino acids might be effective competitors for such transport [J.P. McGovren, unpublished]. This possibility is currently the object of both preclinical and clinical research.

A solution to the general problem of acivicin CNS toxicity will probably await either identification of a toxic metabolite from radiolabelled drug studies or the refinement of a suitable animal model in which neuropharmacological methods can be employed to identify the key biochemical events. This area is of high priority, since methods which can reduce this dose-limiting side effect of the drug have great potential to enhance its therapeu-

Table 5. Rationale for use of acivcin in combination chemotherapy.

(1) Active in human cancer (colon, lymphoma, and lung).
(2) Mechanism of antineoplastic action not shared by other anticancer agents.
(3) Dose-limiting toxicity not shared by most other anticancer agents.
(4) Synergistic activity with other anticancer drugs in preclinical tumor models.
(5) Inhibits biochemical reactions whose products compete with certain other anticancer agents.
(6) Resistance mechanism subject to inhibition by a modulatory agent.
(7) Collateral sensitivity with other agents in resistant cell lines.

tic use in man. In any case, such studies may result in important new insights into comparative brain biochemistry and its behavioral correlates.

Combination chemotherapy

The most impressive results in clinical cancer chemotherapy have come from the use of empirical or rational combinations of antineoplastic drugs. Acivicin has potential in this arena (table 5), although no experimental proof of its contribution to a clinically useful regimen has yet been generated. In the near future, careful study of acivicin in combination with other drugs may increase knowledge of the factors which are involved in successful combination chemotherapy.

Inhibitors of DNA function

Acivicin and actinomycin D synergise in vitro, as measured by growth inhibition and loss of clonogenic units. CTP and UTP concentrations increase, and there is a minor decrease in ATP in cells treated with actinomycin D alone, consistent with inhibition of RNA polymerase by this drug. Acivicin has the opposite effect on CTP, and also decreases GTP and, to a lesser extent, ATP. The combination produces normal GTP levels and increased CTP and UTP, with decreased ATP levels. Overall, there appears to be decreased de novo pyrimidine synthesis induced by acivicin, in combination with decreased pyrimidine utilization induced by actinomycin D [56]. The initial clinical trials based on this combination should be conducted in tumors which are sensitive to both agents and hence must await more complete definition of the spectrum of acivicin's clinical activity and optimal schedule.

Amino acid depletion

Acivicin does not inhibit *Acinetobacter* L-glutaminase-L-asparaginase [57]. The enzyme depletes plasma glutamine and asparagine and thus might be expected to potentiate glutamine or asparagine antagonists. Treatment of nontumored BDF_1 female mice with the enzyme decreased the LD_{50} of

concomitantly-administered acivicin by a factor of 25, using a daily $\times 6$ schedule. The potency of acivicin toward L1210 leukemia in these mice was also increased by the enzyme, but with no gain in absolute efficacy when compared with higher doses of acivicin. In contrast, improved efficacy was found for the combination against the sc Ehrlich ascites tumor in ICRF mice [58]. Acivicin and succinylated glutaminase-asparaginase achieve synergistic inhibition of nucleic acid synthesis in P388 tumor cells in vitro [59]. As noted above, L-asparaginase has been associated with CNS toxicity which resembles that of acivicin in man; trials of the combination may find unacceptable synergistic toxicity and should be initiated with careful dose-ranging studies. Since glutaminase-asparaginase finds its major application in the therapy of acute leukemia, a positive clinical trial of acivicin in that disease must occur before the combination is tested.

Purine antimetabolites

Acivicin augments the accumulation of 6-thioguanine (6-TG) in L1210 cells in vitro, probably through increased formation of the mono- and triphosphates of the drug. This biochemical modulation of the purine antagonist may be mediated by increased PRPP levels. Incorporation of 6-TG into DNA and RNA is reduced, however. Sequential administration of acivicin followed by 6-TG potentiates the cytotoxic effects of 6-TG. While this may be due, in part, to biochemical modulation, acivicin also increased the population of L1210 cells in S phase, which is the phase of the cell cycle which is most sensitive to 6-MP effects [60]. There is thus early experimental evidence that the combination of acivicin with purine antimetabolites may be of clinical interest as well as of biochemical interest.

Pyrimidine antimetabolites

The effects of acivicin on de novo pyrimidine biosynthesis and on conversion of UTP to CTP have stimulated preclinical studies of potential synergistic combinations with agents whose mechanisms of action relate to pyrimidine metabolism, but not all possibilities have been explored.

Inhibition of CPS II by acivicin has suggested possible combinations to several investigators, since this enzyme catalyzes the initial and often rate-limiting reaction in de novo pyrimidine biosynthesis. CPS II is part of a threefold enzyme, the CAD protein, which also carries aspartate transcarbamylase (ATCase) and dihydroorotase activity, and all three activities are coded by a single gene [61]. Functionally, this arrangement channels intermediates through the initial three steps of pyrimidine biosynthesis with conservation of metabolic intermediates [62]. Not surprisingly, Syrian hamster cells which are selected for resistance to N-[phosphonacetyl]-L-aspartic acid (PALA), an inhibitor of ATCase, have an amplified CAD gene complement and elevated levels of all three enzymatic activities associated with

the CAD protein [63]. Similar concomitant elevation of CPS II and ATCase was found in one line of Lewis lung carcinoma cells which was selected for resistance to PALA, although another resistant line had normal levels of ATCase but elevated levels of CPS II [64]. This indicates that the concordance between the two enzymes is not strict; the mechanism by which a portion of the CAD protein can show increased activity relative to other portions remains unclear. If increased CPS II is a frequent concomitant of elevated ACTase, resistance to PALA is due to two mechanisms; the specific target enzyme is increased in activity, and the competing substrate, carbamyl phosphate, is provided in increased amounts by CPS II. Inhibition of CPS II by acivicin would be expected to have a synergistic effect with PALA, and this has been experimentally demonstrated both in wild-type L1210 cells [65] and in PALA-resistant Lewis lung carcinoma cells in vivo [66].

P388 murine ascitic leukemia cells can develop acivicin resistance by repeated treatment with acivicin in vivo. Such cells acquire collateral sensitivity to PALA, to which the parent cell line is resistant [67]. When treated with PALA, the acivicin-resistant cells undergo greater inhibition of CPS II, pyrimidine nucleoside kinases, adenine phosphoribosyl transferase, and hypoxanthine phosphoribosyl transferase than did the parent line. PALA decrease UTP and CTP pools and increases ATP and GTP pools in the acivicin-resistant line but not in the parent line. This deficiency of pyrimidine relative to purine nucleotides was thought to be the basis for the observed collateral sensitivity. Further studies indicate that the resistance to acivicin may also be related to reduced uptake of the drug by a transport mechanism which is shared by L-glutamine [54]. In any case, the observation of collateral sensitivity suggests a rationale for clinical regimens which combine acivicin with PALA.

Other antineoplastic agents whose activity may be modulated by the effects of acivicin on pyrimidine metabolism include 5-fluorouracil (5-FU) and cytosine arabinoside (ara-C). The classical mechanism of action of 5-FU involves inhibition of thymidylate synthetase by 5-fluorodeoxy-uridine,5′-monophosphate (FdUMP), a phosphorylated metabolite of the drug. Alternatively, the ribonucleotide of 5-FU, 5-fluorouridine, 5′-triphosphate (5-FUTP), may interfere with RNA metabolism [68]. It is not well established that either mechanism is preferentially associated with either toxicity or efficacy of 5-FU, or that such a preferential relationship may be generalized to all tumors and tissues, but it is possible that an improvement in the therapeutic index of 5-FU might be achieved by interventions which suppress or augment one mechanism versus the other.

Since synthesis of the ribonucleotide of 5-FU requires PRPP, interventions which elevate PRPP pools should preferentially shunt 5-FU into RNA. Inhibition of amidophosphoribosyl transferase by acivicin increases PRPP levels, but inhibition of CTP synthetase by acivicin increases the level of UTP, a substrate which competes with 5-FUTP for incorporation into RNA.

In addition, increases in UTP may decrease the DNA-directed action of 5-FU, by increasing the dUMP available for phosphorylation by thymidylate synthetase. The effect which predominates is likely to depend upon the individual metabolic constraints of the specific cell type in question. In addition, although the administration of acivicin 6 hours prior to 5-FU treatment is markedly more toxic to nontumored BDF_1 mice than is the simultaneous administration of these agents, an improvement in efficacy versus P388 leukemia also is seen with the sequential regimen, relative to the simultaneous regimen, and the level of ^{14}C-5-FU incorporation into RNA was also increased by sequential administration [69]. As frequently occurs in regimens combining two antimetabolites, there are thus differences between regimens which employ different timing of the agents. An improvement in absolute efficacy relative to the single agents could not be estimated from this study, however, and there was no attempt to determine the optimal ratio of the two drugs, although biochemical effects of the regimens were well investigated. Effective biochemical and therapeutic synergism with 5-FU can be achieved by subtoxic doses of PALA, which also inhibits an early reaction in de novo pyrimidine biosynthesis [70], and more effective doses of 5-FU can be used in the absence of toxicity from the modulating agent. Consequently, it is probably important to determine the level of acivicin which is necessary to produce the desired biological effect and the duration of that effect; differences due to timing of drug administration may be related to the pharmacokinetics of the initial agent as well as to its pharmacodynamics, and in man both may be different from the preclinical model. The optimal combination regimen for acivicin and 5-FU presents challenges, but has promise for improved efficacy in cancer treatment.

The reduction in CTP levels which is induced by acivicin suggests another possible synergistic interaction with a clinically-useful pyrimidine antimetabolite. Ara-C undergoes intracellular phosphorylation to the triphosphate, which inhibits DNA polymerase. Deoxycitidine competes with ara-C for kinases, and is largely derived from UTP in vivo [71]. Acivicin should thus potentiate ara-C by lowering the pools of competing substrate for anabolic reactions. In vitro synergism has been seen between ara-C and 3-deazauridine, which also is an inhibitor of CTP synthetase [72]. In preliminary experiments, marked augmentation of the toxicity and efficacy of ara-C by acivicin has been seen in ascitic murine leukemia [unpublished observations]. Many of the caveats noted above also apply to this combination; careful investigation of combination regimens incorporating these agents is indicated.

Salvage inhibitors

Resistance to antimetabolites can be mediated through the salvage of metabolites from the extracellular milieu. Acivicin is accumulated by cells by a carrier-mediated amino acid transport mechanism which is not shared

by nucleosides [54]. Exogenous nucleosides reverse the cytotoxic and biochemical effects of acivicin in vitro [1,73–75]. Since the uptake of nucleosides from the external cellular environment can be blocked by dipyridamole [76, 77], the latter agent can prevent the reversal of acivicin's cytoxic and biochemical effects in vitro by such salvage [73, 74]. This finding has been repeated in cell lines of human origin, and measurement of nucleotide pool sizes confirmed expectations [75]. This interaction occurs at levels of each agent which can be achieved in vivo and presents a potential method of enhancing the antitumor efficacy of acivicin in clinical regimens. Human colon carcinomas have elevated levels of glutamine-utilizing enzymes involved in de novo and salvage nucleotide metabolism and also have decreased glutamine levels relative to those of normal human colonic mucosa, suggesting that the combination of acivicin and dipyridamole may be efficacious in that tumor [78, 79]. In an ongoing phase I clinical trial, acivicin has been given to patients at therapeutic doses, accompanied by escalating doses of intravenous dipyridamole. Plasma dipyridamole concentrations sufficient to inhibit salvage in vitro have been achieved in these patients, with no apparent increase in toxicity of acivicin to normal tissues [80]. Biochemical pathways associated with salvage are elevated in other tumors as well [81, 82], and specific inhibition of these reactions by drugs such as tiazofurin may also show synergy with the effects of acivicin on de novo synthesis [83].

Combination chemotherapy incorporating acivicin has much promise for the future. However, it is of critical importance that clinical trials of such combinations incorporate appropriate design features. The optimal schedules, doses, and sequences of agents must be established using techniques which measure the biochemical concomitants of the regimen in man, since failure to achieve the desired biochemical result will defeat the intent of the clinical study. Appropriate consideration must be given to species-specific differences in pharmacokinetics, metabolism, and toxicity of each agent. Establishment of the clinical spectrum of acivicin as a single agent through expanded phase II clinical trials, as outlined above, is a necessary predicate to the selection of disease categories for combination programs. Finally, there are many active antineoplastic agents which have not been studied in combination with acivicin, even in in vitro systems. Further preclinical work will be necessary to discover the full potential of acivicin for combination chemotherapy in human malignancies.

Conclusion

Acivicin is an interesting new antimetabolite whose clinical development is now in progress. Early results suggest that the drug can be efficacious in some human malignancies, but the level of clinical activity which has been seen to date is less than optimal. Clinical toxicity is well defined and reversible. The major biochemical effects of acivicin are known and provide

potential for combination regimens which may be of clinical value and which may advance our understanding of normal and neoplastic cellular biochemistry. Neither the spectrum nor the degree of efficacy of acivicin in human tumors can be determined until further investigations yield a regimen which is optimal; reduction of the drug's neurotoxic effects and development of regimens which achieve desired biochemical effects in man would seem to offer the best means to that end.

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III

Specific Therapeutic Landmarks

8. Chemotherapy for malignant intracranial tumors

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Advances in neurosurgical techniques have had a dramatic impact on the prognosis of patients suffering from benign intracranial neoplasms. Acoustic Schwannomas which carried a high surgical mortality rate in Cushing's era are now removed safely and successfully as are pituitary tumors and most meningiomas. The impact of these technical advances on the management of the malignant intracranial tumors, in particular on the glioblastoma multiforme, the most common primary malignant intracranial neoplasm, have been slight. The use of radiotherapy clearly adds to patient survival but is limited in its effectiveness. Thus, there has been a search for adjunctive approaches to the therapy of malignant gliomas which could hopefully begin to improve the management of these patients. The effective use of chemotherapy has been severely limited by the lack of therapeutic agents designed for use in brain tumor patients and by the heterogeneity of the tumor cells and the drug delivery problems which often prevent the agents from reaching the tumor site. Thus, results of chemotherapy to date have been modest at best.

In this chapter some of the factors which modulate the effectiveness of chemotherapy will be described as well as the current status of chemotherapy for the different types of brain neoplasia. Inasmuch as there are now very significant advances occurring in laboratory and experimental trials, the potential value of these new approaches will also be discussed.

Factors modulating effectiveness of chemotherapy

There are a number of biologic variables related to chemotherapy of brain tumor that are either different or unique compared to other cancers. The histopathology, as commonly reported, is not necessarily related to the biologic events of the tumor and is therefore only one part of the equation. There are clear-cut differences between astrocytomas (grade I) and astrocytomas (grade II). Malignant astrocytomas (grade III) and glioblastomas (grade IV) share essentially the same survival curve through the median point and are not as distinct from each other as are grades I and II. A

review of 417 cases of intracranial astrocytoma from the Mayo Clinic (where the Kernohan grading system originated) noted that the survival patterns for grade I and II tumors were extremely similar and, in fact, combined them into what they now call 'low grade' astrocytoma. Similarly, they noted that grade III and IV astrocytomas had no significant difference and, therefore, reunited them into 'high-grade' astrocytomas [1]. Thus, they now propose two grades on the basis of histology. The studies of Winston and colleagues have demonstrated that very few of the histopathologic variables that are graded and described bear any significant relationship to survival [2, 3]. It is evident from the above information that histopathologic classification of brain tumors is far from clear. Precisely which factors observed under the light microscope are truly significant and relate to biologic events and which factors are merely observations without known biologic significance remain to be determined.

Tumor microenvironment

Recently, the concept of the 'microenvironment' of brain tumor has evolved and includes a number of factors [4]. The brain is devoid of lymphatic drainage, and, therefore, one of the major paths of egress of drugs (and metabolites) from extracellular fluid is not available. Such lack of drainage also has implications for formation of edema as well as drainage from the extracellular space into the CSF. Second, there is frequently seen, in the histology of most brain tumors, areas of necrosis in the center of the tumor, an actively proliferating edge of tumor which is well vascularized that intermingles with an outer zone, and the so-called 'brain adjacent to tumor' (BAT) [5]. Each of these areas is believed to have a different pharmacologic environment and contains viable tumor cells, but in very different proportions, and has different kinetic considerations for the cells in each of these zones.

Heterogeneity of tumor cells

There is increasing evidence of diverse biologic heterogeneity found in brain tumors [6]. Studies evaluating the chromosomal content, number, and karyotyping of freshly explanted serially transplanted brain tumors has provided evidence of marked changes in tumor characteristics within a matter of a few generations. Thus, what appears histopathologically as a single type of brain tumor cell may, in fact, have extremely different kinetic, immunologic, and metabolic activity and response to therapy.

Tumor kinetics

The kinetics of brain tumors have been elucidated only partially [7]. Of particular note is the fact that normal glia essentially does not replicate (in adults or older children). Cerebral vasculature and some of the other supporting elements do replicate, but at comparatively slow turnover times. Brain tumors, on the other hand, are in part, by their very nature, actively going through the cell cycle. Low-grade astrocytomas have been demonstrated to have extremely few cells undergoing active proliferations, whereas high-grade gliomas appear to have a greater, but nevertheless still small, percentage of cells actively replicating [8]. A very wide distribution of kinetic parameters was seen in the patients who were studied. Thus, the labeling index is on the order of 0% to 10%, the S phase is approximately seven hours to ten hours and the growth fraction is 0.30 and extremely variable. Birthrate of tumor cells is between 0.5% per hour and 1.7% per hour. The estimated turnover time is therefore between three days and seven days, which is obviously not consistent with the clinical entity. If a computed cell loss factor of 85% is included, the more common clinically accepted doubling time of six to eight weeks can be obtained. However, this has marked implications for both previous kinetic observations as well as chemotherapeutic considerations [8].

Blood-brain barrier

The brain traditionally is thought to be protected by the blood-brain barrier [9]. This pharmacologic-physiologic entity has been located in the endothelium of the majority of cerebral capillaries [10]. Pentalaminar fusions of endothelial cell membranes from relatively continuous zones of occlusions which obstruct the passage of substances having a molecular weight greater than 200 daltons. Substances that have high lipid solubility and thus are capable of passing cell membranes generally are considered as not being excluded by the blood-brain barrier. Finally, drugs must either not be ionized or have readily reversible ionization equations in order to pass through the blood-brain barrier. Although the blood-brain barrier traditionally has been cited as one of the most important factors in the choice of chemotherapeutic agents for the treatment of malignant brain tumor, it is both pharmacologically, as well as histopathologically, not intact in the midst of the tumor [11]. Capillary endothelial cells within tumor have been shown to have abnormal or discontinuous tight junctions [11]. CT and radionuclide scanning, both of which are dependent upon the entrance into the area of tumor of large protein molecules which are isotope labelled, are able to attain contrast differential between tumor and normal brain by virtue of leaks in the blood-brain barrier [12]. Studies using an extracellular peroxidase marker and horseradish peroxidase (44,000 daltons) in ex-

perimental tumor systems have demonstrated the discontinuous nature of the endothelium of brain tumor vasculature [13]. Thus, the role of the blood-brain barrier remains obscure in human brain tumor therapy.

Tumor cell resistance

With the wide variety of kinetics seen and the marked heterogeneity of cell populations, the therapist is faced with an extraordinarily difficult problem in that a radio- or chemotherapeutically sensitive tumor today may be replaced by its resistant variant within a short time. The development of resistant strains of bacteria to antibiotics is well known. However, it takes many generations before resistance becomes apparent, and it may well not be an analogous phenomenon.

General concepts of brain tumor chemotherapy studies

Chemotherapy cannot be considered independent of surgery and irradiation but must be integrated into therapeutic planning, which takes into account the biology of brain tumor. The majority of other cancers that have responded to therapeutic approaches usually have done so in relation to multimodal treatment (surgery, radiation, and chemotherapy), which has been used in either sequential or concomitant treatment schedules. The interrelationship of radiotherapy and chemotherapy has not been elucidated clearly.

The classic concept of phased studies in the application of chemotherapy is no place more clearly found than in the treatment of brain tumor. Phase I studies of new drugs may be carried out in patients with brain tumors, provided they have sufficient marrow reserve. In one sense, they are more ideal patients for such investigations, because they rarely have systemic disease and therefore do not have hepatic, renal, or pulmonary compromise. However, it is inappropriate use of patient material if patients with brain tumors are used for phase I studies if there are any other treatment modalities that should be investigated that will specifically aid patients with brain tumors. From a therapeutic point of view, the phase II study of chemotherapeutic agents in patients with brain tumor is extremely important.

Drugs entering into phase II studies are selected because they have (1) the appropriate pharmacologic characteristics that allow them to enter into the brain; (2) evidence of efficacy in one of the various experimental brain tumor models; (3) an indication from the treatment of other malignant disease that the drug in question is one which has a potential by wide application; (4) a specific biochemical mode of action that directly relates to brain tumor metabolism; and (5) suggestion or proof of efficacy in phase I trials in brain tumor patients. The conduct of the phase II study relies

primarily on the judgment of the investigator, which is supported by some objective and statistically manipulatable measurement factors. Because of the complexity of measuring outcome in patients with malignant glioma, phase II studies are extraordinarily difficult to analyze in a meaningful way. Therefore, any drug that shows a reasonable indication of value in phase II should be subjected to more carefully controlled studies.

The phase III study is clearly the most important and the most difficult to carry out in the treatment of malignant brain tumors. Strict selection criteria, careful documentation of pathology, randomization procedure, and the requirement for close and continuous follow-up severely limits the study of this disease, and the number of patients required make multicentered studies with close coordination imperative. The implication of the null hypothesis expressed by the randomization procedure places the surgeon in an antithetical position, that is, his training and inherent disposition is that of decision making on behalf of and for the benefit of this patient. When he must state that he does not know which treatment is better and therefore must revert to the randomization procedure, a discordant note is rung. Nevertheless, the phase III control study for evaluating chemotherapeutic efficacy remains the most effective way of *proving* efficacy.

Chemotherapy of specific central nervous system (CNS) neoplasms

Inasmuch as one is often faced with patients other than those with 'glioblastoma multiforme,' the most frequent target of our chemotherapeutic armamentarium, the role or lack of role of chemotherapy in many of the common intracranial neoplasms will be described.

Chemotherapy of high-grade astrocytomas

The greatest amount of work and some of the most significant effects of both radiotherapy and chemotherapy have been seen in patients with high-grade malignant gliomas. Included within this category are glioblastoma multiforme, malignant astrocytoma, and Kernohan grades III and IV. The vast majority of these patients also have undergone surgical resection, and a smaller portion have undergone radiotherapy. These factors must be taken into consideration when examining adjuvant chemotherapy.

Almost every drug which has been used for the treatment of malignancies has been applied to the treatment of brain cancer [14]. The vast majority of these studies are uncontrolled, using patients with different histologies who appear at different times during the course of their disease, and who, by and large, and both recurrent and debilitated. Intercomparison between these studies is extremely difficult because the advertent and inadvertent selection factors that modulate the choice of patient populations often may have a

greater effect than some of the therapies available. Thus, controlled perspective randomized trials become extremely important, because a majority of these variables will have less impact on the results. The first multi-institutional control perspective randomized trial in the treatment of malignant glioma was carried out by the Brain Tumor Study Group (BTSG) (a clinical cooperative group of neurosurgeons, neurologists, radiotherapists, and neuropathologists under the aegis of the National Cancer Institute) [15]. In this study, the use of mithramycin was compared to no mithramycin in patients who had a surgical resection and some radiotherapy. The importance of this study is not so much that it definitively demonstrated that mithramycin is an ineffective agent for the treatment of malignant glioma, but in that it established that controlled perspective randomized trials can be carried out in the treatment of this disease and develop meaningful results.

The nitrosoureas were among the first rationally designed chemotherapeutic agents that have the specific properties necessary to cross the blood-brain barrier. They are highly lipid soluble, of small molecular weight, and are not ionized, and, therefore, they will not only penetrate normal brain tissue but they will equidistribute throughout the body. 1-3-bis-2-chloroethyl-1-nitrosourea (BCNU) was the first of these to be used clinically. Early phase II studies demonstrated improvement in approximately 50% of patients treated with doses of 80 mg/m^2/day to 100 mg/m^2/day on three successive days, delivered intravenously every six to eight weeks [16, 17]. However, the median time to progression was brief and in the order of 12 weeks to 20 weeks.

Following these initial observations, a wide variety of trials of both controlled and uncontrolled design have been undertaken and are summarized in table 1. Contained within this table are important factors, such as tumor type and the nubmer of patients, that were both entered into the study and became part of the evaluated group. The results are reported as either median time to progression (MTP) or median survival time (MST) in weeks. One of the major deficits in most of these trials was the lack of an adequate number of patients in order to demonstrate true efficacy or to satisfy the null hypothesis. Only three of the randomized trials had over 100 patients in all groups for purposes of analysis.

The subsequent study of the Brain Tumor Study Group attempted to define the quantitative (in contradistinction to the qualitative) value of radiotherapy. It clearly demonstrated that the use of 5,000 to 6,000 rad whole-head through bilateral opposing ports increased median survival by 150%, whereas the use of BCNU alone increased median survival by an insignificant 30% [18]. However, at 18 months, approximately 20% of the patients who received both BCNU and radiotherapy were still alive, while less than half as many patients who received monotherapy were still alive.

In a study using radiotherapy as a control and examining the use of methyl-CCNU alone and in a combination with radiotherapy versus the best arm from the prior study of radiotherapy and BCNU [19], it clearly was

demonstrated again that nitrosourea alone is inadequate treatment, that radiotherapy is an effective mode of treatment, and that the combination of BCNU and radiotherapy is modestly better than BCNU alone.

A number of other studies have examined CCNU, as it is an oral nitrosourea and therefore relatively easy to deliver. The EORTC in a carefully controlled trial, gave CCNU immediately after surgery and radiotherapy and compared this to giving it after the patient demonstrated progression [20]. The median time to progression for both groups was exactly the same; however, a significant improvement was seen in patients following progression who then received CCNU. In a smaller study of patients with poor performance status and requiring steroids, they demonstrated that patients who received CCNU had a better median survival time than those who did not. Several other studies have been unable to demonstrate the specific value of CCNU [21–23]. Some studies in table 1 of nonrandomized nature have examined various forms of combination chemotherapy [24–27]. The majority of these have failed to substantiate the significance of their combination.

Eagan and coworkers (table 1) treated a randomized group of 42 patients with dianhydrogalactitol and found a remarkable improvement in median survival (67 weeks) as compared to those who received radiotherapy alone.

Procarbazine has been reported as an effective treatment for malignant glioma and, therefore, has been brought forward into the current BTSG study. Following definitive surgical resection, all patients will receive 6,000 rad of radiotherapy and will be randomized to procarbazine, high-dose corticosteriod, and high-dose corticosteroid plus BCNU versus BCNU alone. The purpose of this study is to evaluate the oncolytic effect of corticosteroids in comparison with their well-known ability to control cerebral edema, the effect which they might have in combination with BCNU, and the efficacy of procarbazine. The current trend indicates that corticosteroids have no independent oncolytic effect in the doses used and do not add to the efficacy of BCNU. Further, procarbazine appears to be an effective agent with the same approximate efficacy as BCNU. The absolute results must await the final analysis.

Several significant factors have been derived from these controlled studies. A group of pretreatment prognostic factors which play an important role in survival rates of patients with malignant glioma have been identified [28]. Such determinants as age, performance status, the duration of symptoms, and histopathologic classification all are of considerable significance and must be accounted for in intercomparisons between studies. As the treatment of high-grade glioma becomes more prevalent and patients live longer, the complications of both chemotherapy and radiotherapy can be expected to become more apparent. Late delayed radiation necrosis was identified in 4 of 25 brains examined that had received between 5,000 and 6,000 rad of radiotherapy [29]. Two of the four had received BCNU, one-dibromodulcitol, and one had received other chemotherapy. The com-

Table 1. Summary of chemotherapy trials.

Author	Study Design	Tumor type and Percent		Number (Evaluable / Entered)	Pretreatment Conditions	Treatment Groups	Results† MTP	Results† MST	Comments
Levin	Randomized	GBM Non-GBM	62 37	99/130	Surgery	RadTh and BCNU RadTh, BCNU & Hydroxyureu	31 wk 42		Significant at p = 0.04
Sweet	Randomized	Astrocytoma III and IV		21	Surgery and radiation Theory	BCNU BCNU and VM26	61 wk 94		Difference not statistically significant
Sobero	Randomized	GBM	100	102/105	Surgery randomize in 2 wk	RadTh RadTh and BCNU RadTh and CCNU	38 wk 45 wk 52 wk	45 wk 52 69	RadTh and CCNU only significantly better than RadTh alone P = 0.05
Jellinger	Consecutive, historic, selective	CBM MA	66 34	116	Surgery	Supportive Care RadTh COMP RadTh and COMP	16 wk 29 29 30	23 wk 46 46 58	Uncontrolled, consecutive and selected patients. Any therapy better than supportive care alone
Garrett	Randomized	GMB MA Other	68 29 3	69/74	Biopsy	RadTh RadTh and CCNU		35 wk 56	Not statistically significant
Heiss	Consecutive and historic	GMB	100	77	Surgery Some radiation therapy	Control CCNU Polychemotherapy COMP	11 wk 15 20 39	27 wk 28 35 35	Combined retrospective and previous series. Not stratified by RadTh. COMP appeared better
EORTC-BTG (Hildebrand)	1. Randomized	GBM Astro III-IV	40 31	81/111	Surgery and radiation therapy *Good* performance status, no steroids	CCNU (after surg) CCNU (after prog)	34.5 wk 31	43 wk 62	 Significant $p = 0.05$ Population stratified unto two groups by performance status
	2. Randomized	Other	29	22/111	Surgery and radiation therapy *Poor* performance status, steroids required	Control CCNU		21.5 wk 31	 Significant $p = 0.01$
Walker	Randomized	GBM MA Other	90 9 1	222/303	Surgery and randomize in 2 wk	Control BCNU RadTh BCNU and RadTh		14 wk 18.5 35.0 34.5	RadTh and RadTh & BCNU statistically significant from control $P = 0.001$

Shapiro	Randomized	Malignant glioma	33		Surgery	BCNU and VCR BCNU, VCR and RadTh			30.0 wk 44.5		No significant difference demonstrated
Reagan	Randomized	Astrocytoma grade III and IV		63 72	Surgery Randomized in 2 wk	RadTh CCNU RadTh and CCNU	30 17 30	wk	49 28 52	wk	Subopumal RadTh (5,000 rad) Stopped treating upon recurrence CCNU inferior to other treatments $p = 0.02$
Walker	Randomized	GBM MA Other	84 11 5	358 467	Surgery randomized in 2 wk	MeCCNU RadTh RadTh and MeCCNU RadTh and BCNU			24 36 42 51	wk	RadTh and BCNU vs RadTh, $p = 0.072$ MeCCNU vs RadTh $p = 0.048$
Wear	Randomized and crossover	Astrocytoma III and IV Other	93 7	40	Surgery randomize in 2 wk	RadTh CCNU RadTh and CCNU	23 14 31	wk	27 37 36	wk	7 RadTh crossed to CCNU 10 CCNU crossed to RadTh Those who had combination treatment survived longer
Sewer	Consecutive vs historic	GBM MA	63 37	52	Surgery Radiation therapy	CCNU Procarbazine Bleomycin Control			56 51 	wk wk 	No significant difference
Eagan	Randomized	GBM MA	71 29	42 43	Surgery Radiation therapy Randomized in 2 wk	Dianhydro-galactitol Control			67 35 2	wk	Split course RadTh given to half the patients DAG vs Control $p:002$

* GMB = Glioblastoma multiforme, MA = malignant astrocytoma.

† MTP = Median time to progression; MST = median survival time.

‡ 'COMP' = CCNU, vincristine, methotrexate, and procarbazine

(Levin VA, Wilson CB, Davis R et al; A Phase III comparison of BCNU, hydroxyurea, and radiation therapy to BCNU and radiation therapy for treatment of primary malignant gliomas. J. Neurosurg 51:526–532, 1979 Sweet DL, Hendler FJ, Hanlon K et al: Treatment of Grade III and IV astrocytomas with BCNU alone and in combination with VM-26 following surgery and radiation therapy. Cancer Treat Rep 63:1707–1711, 1979; Solero CL, Monfardini S. Brambilla C et al; Controlled study with BCNU vs CCNU as adjuvant chemotherapy following surgery plus radiotherapy for glioblastoma multiforme. Cancer Clin Trials 2:43–48, 1979; Jellinger K. Kothbauer P, Volc D et al: Combination chemotherapy [COMP protocol] and radiotherapy of anaplastic supratentorial gliomas. Acta Neurochrurgica 51:1–13, Springer-Verlag, 1979; Garrett MJ, Hughes HJ, Freedman LS: A comparison of radiotherapy alone with radiotherapy and CCNU in cerebral glioma. Clin Oncol 4:71–76, 1978; Heiss, W-D: Chemotherapy of malignant gliomas: Comparison of the effect of polychemo- and CCNU-therapy. Acta Neurochurgica [Wein] 42:109–115, 1978; EORTC Brain Tumor Group: Effect of CCNU on survival rate of objective remission and duration of free interval in patients with malignant brain glioma—Final evaluation Eur J Cancer 14:851–856, 1978; Walker MD, Alexander E Jr, Hunt WE et al: An evaluation of BCNU and/or radiotherapy in the treatment of anaplastic gliomas. [A cooperative clinical trial] for the Brain Tumor Study Group. J Neurosurg 49:333–343, 1978. Shapiro WR, Young DF: Chemotherapy of malignant glioma with BCNU and vincristine. Neurology Minn 24:380, 1974; Reagan TJ, Bisel 11J, Childs DS et al: Controlled study of CCNU and radiation therapy in malignant astrocytoma. J Neurosurg 44:186–190, 1976; Walker MD, Green SB, Byar DP et al: Randomized comparisons of radiotherapy and nitrosoureas for malignant glioma after surgery. N Engl J Med, 303:1323–1329, 1980; Weur B. Band P. Urtasun R et al: Radiotheraphy and CCNU in the treatment of high-grade supratentorial astrocytomas. J Neurosurg 45:129–134, 1976; Seiler RW, Greiner RH, Zimmerman A et al: Radiotherapy combined with procarbazine, bleomycin, and CCNU in the treatment of high-grade supratentorial astrocytomas. J. Neurosurg 48:861–865, 1978; and Eagan RT. Childs DS Jr. Layton DD Jr et al: Dianhydrogalactitol and radiation therapy: Treatment of supratentorial glioma. JAMA 241:2046–2050, 1979)

plications of prolonged nitrosourea therapy are also becoming apparent, as scattered case reports identify patients with pulmonary fibrosis, hepatic toxicity, or renal failure. Cases of second tumors in association with nitrosourea treatment also are being reported [30].

A detailed review of the results of chemotherapy trials is given in table 1.

Chemotherapy of low-grade astrocytomas

Low-grade, 'benign' astrocytomas (Kernohan grade I) have a median life span of four to five years [23, 31, 32]. Immediate chemotherapy after obtaining a biopsy and establishing the diagnosis does not appear to be logical either for kinetic considerations (the long doubling times and few cells actively proliferating) or for chemotherapeutic toxicity considerations (long-term exposure and depletion of bone marrow reserve). The usual course of events of low-grade astrocytomas is to continue in a somewhat indolent state for months to years, at which time, for unknown reasons, they appear to become more active, start to become larger in size, and frequently differentiate histopathologically. At this time, they often are reoperated upon and demonstrate a more aggressive, actively growing tumor. If the maximum cumulative dose of radiotherapy already has been used and the bone marrow has been compromised severely by several years of chemotherapy, maximum doses of both of these forms of therapy, at at time when they are most needed, cannot be used. Therefore, chemotherapy should not be considered in low-grade astrocytomas until after recurrence has been demonstrated. This is particularly true of the juvenile cerebellar astrocytomas, optic nerve gliomas, and microcystic astrocytomas, all of which may be considered as cured or at least stabilized in a reasonable proportion of patients following surgery. There is a pressing need for a detailed analysis of the long-term events of patients with low-grade astrocytomas with the hope that the natural history will make more apparent the appropriate timing for both radiotherapeutic and chemotherapeutic intervention.

Low-grade gliomas (astrocytomas, grade II) are located on the histopathology continuum of gliomas somewhere between benign astrocytomas and those that truly show malignant tendencies. To whatever extent possible, a rereview of pathologic material, the clinical course of the patient, the surgeon's notes, and a neuroradiologic presentation of the tumor should be undertaken to attempt to either declare it of the low-grade astrocytic series or recognize its malignant potential. There is some question as to the indications of chemotherapy in the low-grade gliomas of either the cerebral or cerebellar hemisphere. As noted above, the value of radiotherapy is unproven but is indeed highly suggestive. However, until effective minimally toxic agents are identified, it probably would be more appropriate to wait for evidence of recurrence following radiotherapy for patients in whom the degree of anaplasia cannot be more clearly defined.

Chemotherapy of medulloblastomas

Medulloblastoma accounts for one-fourth of all pediatric age brain tumors, and, therefore, a significant number of patients may be accrued on therapeutic trials. The advent of carefully planned maximal dose radiotherapy has resulted in a median survival of patients with medulloblastoma for some four to five years. The majority of patients often recur at the original site of disease, and chemotherapeutic trials therefore are appropriate. Most reports, unfortunately, contain less than a half dozen cases who have been treated on a comparatively ad hoc basis at the time when they became symptomatic.

There is a general impression that medulloblastomas are comparatively radioresponsive as well as chemosensitive. The duration of response is frequently brief or not reported. Table 2 contains a series of currently reported studies evaluating various modalities in the treatment of medulloblastoma. Two major prospective randomized studies are currently underway and only preliminary information is available [33, 34]. Both studies require the patient to have had a surgical biopsy and then compare radiotherapy to radiotherapy plus CCNU and vincristine. In addition, the study carried out by Evans and coworkers adds prednisone [34]. A preliminary analysis of a study being carried out by the International Society of Pediatric Oncology (SIOP) indicates a slightly greater median time to progression for patients receiving radiotherapy and chemotherapy in comparison with those who receive radiotherapy alone. A study carried out by the Children's Cancer Study Group (CCSG) has not yet reached significance. It is too early to determine if the preliminary analysis will continue to show the same trend or if, in fact, selective and prognostic factors may in the long run account for differences seen. These studies will form a basis for controlled clinical trials in the treatment of children with medulloblastoma.

Although vincristine has been reported as being useful for the treatment of gliomas, it rarely has been used alone. Rosenstock reported response in three out of four patients with recurrent medulloblastomas [35]. A wide variety of other phase II therapeutic studies in patients with recurrent or progressive tumor symptoms have added vincristine to other drugs, such as the nitrosoureas, procarbazine, nitrogen mustard, and methotrexate. Cangir had an impressive 80% response rate in ten patients with the median duration of response of 11 months in a group of ten patients who received MOPP [36]. Thomas had 100% response rate in eight patients with recurrent symptoms of medulloblastoma whom he treated with vincristine, BCNU, dexamethasone, IV methotrexate, and intrathecal methotrexate. In nine patients who were treated immediately after surgery and in combination with radiotherapy, severe toxicity was seen with four early deaths [37]. The toxicity was attributed to the intrathecal methotrexate and BCNU being delivered during ratiotherapy. Considerably less toxicity was seen when they were discontinued while radiotherapy was being delivered.

Table 2. Current chemotherapy: Studies on treatment of medulloblastoma.

Author	Study Design	Patient Population	Number	Pretreatment Conditions	Treatment Groups	Results		Comments
						MTP	MST	
Bloom (SIOP)	Randomized (preliminary)	Postoperative	191	Surgury	RadTh RadTh, VCR and CCNU	2 yr 3 + yr		Slight benefit to chemo-therapy p = 0.408
Evans (CCSG)	Randomized (preliminary)	Postoperative Age 2–16 years	128 144	Surgery	RadTh versus radTh, VCR, CCNU and prednisone	69% 2 yr survival 72% 2 yr survival		No significant difference
Crafts	Phase II	Recurrent and symptomauc	16 17	Surgery and RadTh	Procarbazine + vincristine + CCNU	Response: Stable progression	63% 31% 6%	
Cangir	Phase II	Recurrent and progressive	10	Surgery and RadTh	Nitrogen mustard + vincristine + procarbazine + prednisone	Response = 80% No response = 20% Response = 75%	11 mo 2	Moderately acceptable toxicity
Rosenstock	Phase II	Recurrent	4	Surgery and RadTh	Vincristine	Response = 75%		VCR used alone
Thomas	Phase II	(1) Recurrent	8	Surgery and RadTh	Vincrisune BCNU			IT-MTX and BCNU stopped druing RadTh
		(2) Early treatment	9	Surgery and RadTh	Dexamethosone Methotrexate IV Methotrexate IL	Response = 100% MDR = 18.8 mo		as very toxic when used early (4 deaths)

* MTP = Median time to progression; MST = median survival time.

(Bloom HJG: Prospects for increasing survival in children with medulloblastoma: present and future studies. Multidisciplin Aspects Br Tum Ther 1:245–259. 1979; Evans AE, Anderson, J. Chang C et al: Adjuvant chemotherapy for medulloblastoma and ependymoma. Multidisciplin Aspects Br Tum Ther. 1:219–222, 1979, Crafts DC, Levin VA, Edwards MS et al, Chemotherapy of recurrent medulloblastoma with combined procarbazine, CCNU, and vincristine. J. Neurosurg 49:589–592, 1978; Cangir A, van Eys J, Berry DH et al: Combination chemotherapy with MOPP in children with recurrent brain tumors. Med Ped Oncol. 4:253–261, 1978; Rosenstock JG, Evans AE, Schut L: Response to vincristine of recurrent brain tumors in children. J Neurowrg 45:135–140, 1976; Thomas PR. Duffner PK, Cohen ME et al: Multimodality therapy for medulloblastoma. Cancer 45:666–669, 1980. Crafts DC, Levin VA, Edwards MS et al: Chemotherapy of recurrent medulloblastoma with combined procarbazine, CCNU, and vincristine. J Neurosurg 49:589–592, 1978.)

The design of a therapeutic study in the treatment of medulloblastoma is complicated by the long life span and, therefore, long therapeutic period which these patients can enjoy. Radiotherapy, chemotherapy, and the combination of both can have severe effects upon the endocrine and mental function of children and their general growth and development. All of these factors must be taken into consideration when designing therapeutic studies.

Chemotherapy of ependymomas

Although ependymomas are highly responsive to radiotherapy and a prolonged high quality of life can be anticipated, recurrence eventually may take place. This may be either at the original site of the tumor or more distally, owing to metastasis. Chemotherapy has been carried out only occasionally in late-stage recurrent tumors. In Cangir's study using MOPP, one recurrent patient was treated with no response [36]. Four out of six late-recurrent ependymomas treated with the nitrosoureas were noted to have a positive response [38].

The major study that will provide the basic information on the treatment of ependymoblastoma is being carried out by the CCSG in conjunction with the previously discussed medulloblastoma study [9]. As a separate stratification, patients with ependymoblastoma will receive standard prescribed courses of radiotherapy and be randomized to no additional therapy or to receive CCNU, vincristine, and prednisone. Over 40 patients have been entered into this study; however, it is too early for results to be meaningful.

The survival curve of patients who have ependymomas is generally biphasic, in which the first half succumb rather promptly to their disease, with a median survival of approximately one year, regardless of the supra- or infratentorial location of the tumor. After five years the slope of the survival curve has flattened out markedly with approximately 25% of patients alive. Clearly, these patients describe a different biologic entity than those seen in the earlier portion of the survival curve and deserve vigorous investigation as to which characteristics might account for their increased survivorship.

Chemotherapy of tumors of brain stem and thalamus

Brain stem and thalamic gliomas are seen relatively infrequently and, therefore, often are placed on adult brain tumor chemotherapy protocols with the hope of showing some results [39, 40].

The nitrosoureas (BCNU and CCNU) produced temporary responses in 3 of 12 of a heterogeneous group of patients so studied. Symptomatic improvement was reported in one patient treated with procarbazine [40]. A complex protocol involving a six drug combination (6-mercaptopurine,

procarbazine, cyclophosphamide, methotrexate, 5-fluorouracil, and vinblastine) has been tried in a small series of patients who received concurrent radiotherapy and failed to show significant results [39].

Chemotherapy of meningiomas

Although the primary treatment of meningioma is surgical, adjuvant chemotherapy should be considered in cases where the tumor has regrown after multiple procedures and has failed to respond to radiotherapy. Under such circumstances, the tumor frequently undergoes progressive neoplastic degeneration and may take on a sarcomatous appearance. In such cases, chemotherapeutic regimens for sarcomas or adriamycin may be considered.

Experimental approaches to chemotherapy of brain tumors

Advances in chemotherapy of CNS tumors are likely to include the development of new agents with greater specificity for such tumors as well as a means of designing individualized therapy. The new agents being sought would be those with a greater ability to penetrate the blood-brain barrier, a better tumoricidal effect, and a diminished toxicity for normal organs. In addition to new agents, the use of a combination of the existing agents may prove valuable. Higher dosages to tumor may be achievable using bone marrow transplantation to reconstitute marrow after higher dose chemotherapy.

Further, the relatively small growth fraction of malignant brain tumors gives less opportunity for effective chemotherapy [41, 42]. Chemotherapy has advanced in recent decades and offers tremendous hope for improvement in outlook of patients with CNS malignancies. A major current limitation is that only one agent or group of agents, the nitrosoureas, has been proven to be consistently effective, and then only to a small degree [18]. The development of further effective agents, differing in their mechanisms of action, would significantly change the treatment of human CNS malignancies. The challenge is clear to those involved in neurooncology to develop new approaches for the diagnosis and treatment of human brain and spinal cord neoplasms.

Despite the foregoing considerations, chemotherapy remains a potential avenue in which modest success has already been seen. Such drugs as the nitrosoureas and procarbazine do possess the appropriate pharmacologic characteristics for passing the blood-brain barrier and have application in a wide variety of tumors. The development and investigation of new drugs is of extreme importance and requires the concerted effort of the medicinal chemist, pharmacologist, and neurooncologist in their design. Carefully controlled clinical trails of both phase II and phase III variety should be

carried out in all appropriate drugs to develop a backlog of information concerning the tumor and its biologic variables as well as efficacy. The use of experimental models, both in vivo and in vitro, must be expanded to provide the comparative data base necessary for evaluating new drugs and determining the relative meaning of the model system themselves.

Several noteworthy attempts have been made to circumvent what might be considered the 'rate-limiting step' of brain tumor chemotherapy. Systemically delivered drugs are distributed throughout the body as well as to the brain. By the time one has entered the cerebral circulation, its plasma concentration has been diluted to a considerable extent. Intra-arterial chemotherapy therefore might possess considerable advantage. Studies using intracarotid infusion of radiolabelled modelled substances (insulin, antipyrine, and pentobarbital) in the dog produced between 1.5 and 3 times greater concentration of drug in normal brain as compared to a similar intravenous infusion [43]. Intracarotid ^{14}C-BCNU in the monkey achieved between 1.9 and 2.8 times greater brain nucleic acid-bound drug [44]. Intra-arterial BCNU has been used in a phase II study of patients with malignant glioma and, in a preliminary report, failed to reveal marked therapeutic results [45]. In addition, there were a number of serious complications.

Because the blood-brain barrier has been implicated as a major limiting factor to the egress of chemotherapeutic agents to neoplasms in the brain, attempts have been made to temporarily disrupt the blood-brain barrier for the sake of increasing drug concentration [46]. Internal carotid artery infusions of 25% mannitol were shown to produce a transient reversible osmotic disruption of the blood-brain barrier which results in several fold higher concentrations of subsequently delivered methotrexate. Such techniques are not without hazard but are of interest in modulating the microenvironment of the brain.

The small therapeutic index possessed by BCNU limits the total amount of drug that may be delivered at any one time. The dose-limiting critical organ for the nitrosoureas is the bone marrow and, thus, if one could protect the marrow from the comparatively brief pharmacologic effects of BCNU, considerably higher concentrations of drug might be achieved in tumor. In a pilot study, 10 ml/kg of bone marrow was removed from patients under general anesthesia and stored frozen. Between 280 mg/m^2 and 600 mg/m^2 BCNU were delivered intravenously over the course of two hours, divided into three daily doses. Several days later, the autologous bone marrow was retransplanted into the patient. Some 55 patients with refractory malignancies have been so treated, with tumor responses seen in approximately half. However, considerable amounts of toxicity have been encountered and, although the hematopoietic system was protected, the next most significant dose-limiting organ would appear to be the liver or lungs. Such investigations need to continue to be carefully carried out in order to document this interesting approach.

Combination of photon irradiation with lipophylic chemotherapy agents

such as the nitrosoureas (BCNU, CCNU, and methyl-CCNU) has also been tested in a randomized fashion by the Brain Tumor Study Group. Although prolonged survival was achieved, the gain was modest and median improvement was measured in weeks [47–49]. More recently, a trial of two fractions of very high-dose CCNU (600 mg/m^2) sandwiched around conventional high dose radiation therapy is being tested by Takvorian [50]. Because of the potential of prolonged and severe myelosuppression with this regimen, bone marrow removal and storage for possible autologous marrow transfusion procedes this therapeutic attempt. This trial has been initiated too recently for any meaningful data to be available.

A new approach for the therapy of human CNS tumors involves the concept of using tissue cultures of human tumors for prediction of clinical response. Human CNS neoplasms lend themselves extremely well to this technique. In a study of 2,000 human brain tumors, about 90% of these were grown successfully in tissue culture [3]. This high percentage of success allows the use of chemotherapeutic approaches in the majority of human brain tumor patients. Several different techniques have been used, including the microtiter plate technique and the clonogenic assay [51–53]. From both of these approaches the same types of information are being derived. It appears at this time that approximately 60% of patients who will respond to a nitrosourea clinically will have an in vitro positive response by either technique. Similarly, between 90% and 100% of patients who will be unresponsive to the nitrosoureas can be predicted from the in vitro data [53]. This ability to correlate clinical and tissue culture information holds great promise. The effectiveness of this technique will be multiplied immeasurably should other chemotherapeutic agents be proven to be of consistent effectiveness in the treatment of such tumors. The development of an understanding of the resistance of cell lines may also be helpful in gaining insight as to why certain tumors are more resistant to treatment than others.

There have been two developments in the area of new systems of drug delivery. A totally implantable system for maintaining constant CSF drug levels has been developed. Initially used with methotrexate, it produced drug levels in the range of 2 to 30 micromolar at the infusion rates of 0.5 to 10 mg/day. Serum levels of the drug were in the undetectable range. The use of methotrexate did produce toxic effects within the CNS, including fever, meningismus, and transverse myelitis, but no systemic toxicity was noted [54]. The intra-arterial route of delivery appears to allow high concentrations of drug to be delivered directly to the tumor and now even to be removed prior to systemic exposure [44, 45].

Conclusion

Chemotherapy of brain tumors presents a unique challenge to the therapists because of the small tumor burden present, its highly localized position, its

failure to metastasize, and the frustrating inability to get to it because it is inextricably intertwined within vital brain parenchyma. Thus, new drugs with highly specific activity for brain tumor or various methods by which the dose-limiting steps of marginally effective drugs may be enhanced must all be explored.

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9. New antiviral compounds and impact in management of neoplastic disease and AIDS

Abraham Chachoua and Michael Green

The majority of viruses are associated with acute limited diseases in healthy hosts. For this reason, the development of safe and effective antiviral agents has until recently been slow.

Over the past decade, however, herpes viruses and retroviruses have emerged as significant causes of morbidity and mortality, in both normal and immune compromised hosts. Molecular and sero epideminological data clearly link Epstein-Barr Virus (EBV) with the pathogenesis of Burkitts lymphoma. Antibodies to virus capsid antigens, to early antigens, and to membrane antigens are significantly higher in patients with Burkitts lymphoma [1]. In addition, examination of Burkitts lymphoma biopsies by nucleic hybridization has shown the presence of numerous copies of the EBV genome [2]. Several similar studies link herpes simplex type II (HSV-2) with cervical carcinoma, although the relationship may not be causal. Most of these are sero epidemiological studies, reporting increased serum HSV-2 antibody titers in women with cervical carcinoma compared with controls [3]. In addition, antibodies to HSV-2 antigens appear to be increased in a number of cervical tumor biopsies [4]. The recent discovery of a retrovirus named HTLV III, LAV, or HIV, and the consequent development of an antibody assay has clearly implicated this virus as the etiologic agent of the acquired immune deficiency syndrome (AIDS). This has renewed clinical interest in antiviral agents and has led to the rapid expansion of compounds available for testing. Because of the significant role of herpes viruses and retroviruses in the etiology of human diseases, this chapter will review some of the compounds that have gained wide clinical use as well as some newer agents that show promise in the treatment of these viruses.

Rationale for the development of antiviral drugs

An understanding of the steps involved in viral replication is important for the development of effective antiviral drugs. In the case of DNA viruses, such as herpes viruses, replication involves:

a) attachment to specific receptors on the cell surface

b) fusion of the viral envelope and the cell membrane
c) release of viral DNA from capsids into the cell
d) transcription of viral DNA
e) synthesis of viral proteins.
f) replication of viral DNA.
g) assembly of virions
h) egress of virions from the cell

Herpes virus DNA codes for three classes of proteins, alpha, beta, and gamma; the best studied of these are the beta proteins. Several beta proteins are essential for the synthesis of viral DNA. These include thymidine kinase [5], DNA polymerase [6], DNAse [7], and ribonucleotide reductase [8]. These enzymes differ from host cell enzymes in function and potential substrates. For example, thymidine kinase can phosphorylate purine nucleosides as well as a variety of nucleoside analogs not phosphorylated by the cellular kinase. These differences allow for targeting these enzymes for specific antiviral therapy, in particular purine and pyrimidine analogs. These compounds are activated by the viral enzymes and subsequently inhibit viral DNA synthesis [9].

The replication of retroviruses differ in several aspects. First, the virus must convert its RNA genome to DNA [10]. Secondly, the free viral DNA must be integrated in the host genome and be expressed [11]. This may lead to cell death or malignant degeneration. Retroviruses contain the three basic genes necessary for replication: the gag gene coding for three internal structural proteins P18, P25, and P13; the pol gene coding for reverse transcriptase, and the env gene for the envelope [12]. Reverse transcriptase is a virus specific enzyme, which is capable of synthesising DNA from viral RNA and is the target enzyme for the majority of the currently available antiretroviral drugs.

Viral infection can be quantitated by several ways. These include measurement of serum antibodies to viral antigens, utilizing assays such as ELISA or Western blot, the detection of specific viral products such as reverse transcriptase, or culturing live virus from tissue or blood. The detection of viral products and viral culture currently provide the major means for the assessment of the efficacy of antiviral agents in clinical trials.

Antiherpes agents

Purine nucleosides

9 B-D-arabinofuranosyladenine (Ara-A, vidarabine). Ara-A is a purine nucleoside analog that inhibits viral DNA synthesis (figure 1). Once administered Ara-A is rapidly deaminated to the less active metabolite hypoxanthine arabinoside and is excreted mainly by the kidney [13]. Ara-A and its metabolite, hypoxanthine arabinoside, are converted intracellularly to the

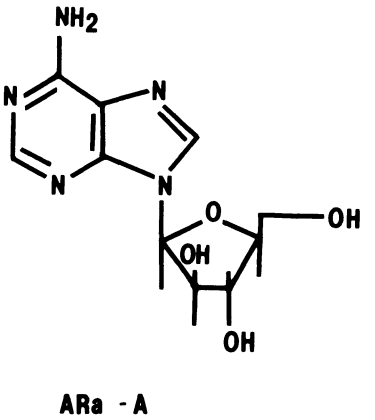


Figure 1. Structure of 9 B-D-arabinofuranosyl-adenine (Ara-A).

corresponding 5′-triphosphorylated derivative, which in turn inhibits viral DNA polymerase [14]. Because of its poor solubility, Ara-A has to be administered in large volumes of fluid. Renal impairment may lead to increased serum levels of hypoxanthine arabinoside, which may be responsible for the neurotoxicity associated with Ara-A treatment [15].

Ara-A is active against certain DNA virus infections including herpes encephalitis, neonatal herpes, and varicella zoster infection [16–18]. However, it is ineffective when used topically because of insufficient penetration into the skin [19].

Adverse effects include gastrointestinal disturbances such as anorexia, nausea, vomiting, and diarrhea. Neurological side effects include hallucinations, tremors, ataxia, dysarthria, myoclonus, confusion, and coma [19].

Ara-A monophosphate is a phosphorylated ester of Ara-A. The main advantage over the parent compound is that it is water soluble and is well absorbed after intramuscular injection. Ara-A monophosphate, however, has the same spectrum of activity and toxicity as Ara-A [20].

9-(2-hydroxyethoxymethyl) guanine (acyclovir). Acyclovir is an acyclic purine nucleoside analog which can be administered orally, topically, or intravenously (figure 2). It has potent antiviral activity against herpes simplex I (HSV-1) and II (HSV-2) as well as varicella zoster (VZV). Other herpes viruses, such as Epstein Barr and cytomegalovirus, are generally resistant. This is probably because both viruses lack viral thymidine kinase [21,22]. Acyclovir is converted by viral thymidine kinase to the triphosphate derivative, which, in turn, inhibits viral DNA polymerase and leads to early termination of the viral DNA chain synthesis [23,24]. Therapeutic drug levels can be obtained by both oral and intravenous administration. Although plasma acyclovir levels are 90% lower when given orally as compared to the equivalent intravenous dose, inhibitory concentrations to HSV

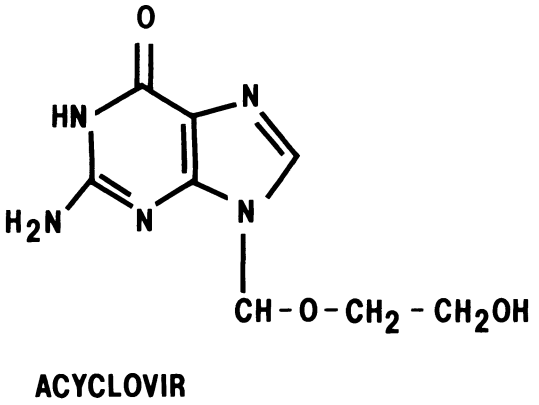


Figure 2. Structure of acyclovir.

types I and II can still be achieved [21,22]. In addition, acyclovir can penetrate the blood-brain barrier, and therapeutic levels can be achieved in the CSF [23]. Toxicity of acyclovir is mild, nausea and vomiting can occur and rises in both blood urea and creatinine have been seen, probably due to renal tubular damage [24]. Acyclovir is currently the drug of choice in the treatment of herpes simplex and zoster infections.

Resistance to acyclovir has been described. Several mechanisms may be responsible for this including deficient viral thymidine kinase production, alteration in the DNA polymerase gene which renders it resistant, and decreased efficiency of thymidine kinase in phosphorylating acyclovir [29]. It remains unclear what the clinical significance of resistance to acyclovir is and which is the predominating mechanism for this.

8,9-1,3-dihydroxy-2 propoxymethyl guanine (DHPG). DHPG is an analog of acyclovir, which has in vitro activity against HSV-1, HSV-2, and varicella zoster (figure 3). In contrast to acyclovir, however, DHPG is also active against cytomegalovirus (CMV). The mechanism of action of DHPG against HSV-1, HSV-2, and VZV is similar to acyclovir. DHPG is more efficiently phosphorylated by thymidine kinase, and the product, DHPG triphosphate, potently inhibits DNA polymerase [30]. The mechanism of action against CMV is unclear and is not mediated by thymidine kinase, since the CMV genome does not code for viral thymidine kinase [31]. DHPG penetrates into the cerebrospinal fluid (CSF), however, the CSF levels do not correlate with plasma levels [32]. DHPG is mainly excreted unchanged in the urine [33]. Several clinical trials in immunocompromised patients with CMV infection have been reported. Clinical improvement was seen in 21 of 25 evaluable patients with AIDS and CMV infection treated with 5 mg/kg intravenously twice daily for 14 days. Twelve of 17 CMV culture-positive patients became culture-negative with treatment. Toxicity included neutropenia in seven patients and mild skin rash in two patients. Clinical and

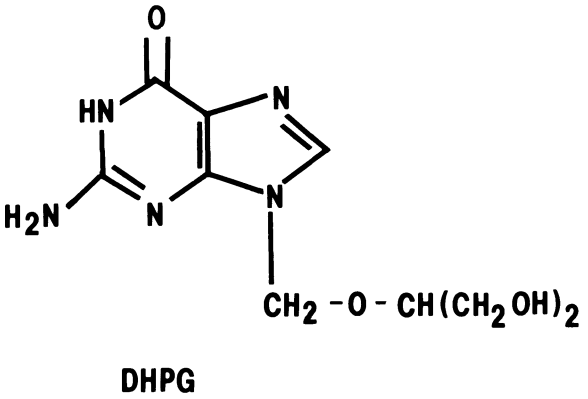
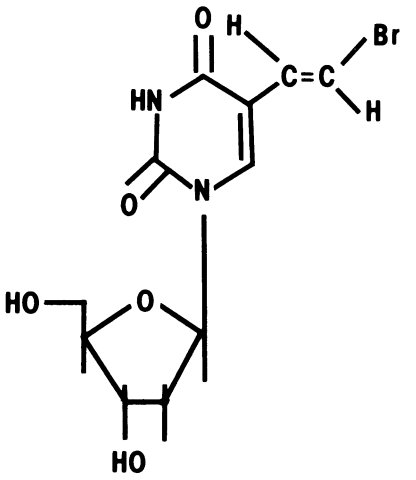


Figure 3. Structure of 8,9-1,3-dihydroxy-2-propoxymethyl guanine (DHPG).

virological relapses, however, were common after cessation of treatment [34]. In another study, 31 patients with AIDS or following bone marrow transplantation were treated with DHPG 5 mg/kg twice a day for 14 days. Response rates varied according to major site of disease. CMV retinitis improved in 66% of patients, colitis in 10%, and pneumonitis in 45%. Mental obtundation was seen in two patients and was presumably drug related. Recurrences occurred in the majority of patients on cessation of treatment, indicating that maintenance treatment with DHPG may be of value [35]. In a small nonrandomized study exploring maintenance schedules, following cessation of viral shedding with induction therapy. A total of 15 patients were treated on two maintenance dose schedules after completion of the initial therapy. Low-dose maintenance consisted of DHPG 2.5 mg/kg administered intravenously three times a week, and high-dose maintenance was 5 mg/kg intravenously daily. Recurrences occurred in patients on the low-dose maintenance therapy. In contrast, no relapses occurred in the patients on the high-dose maintenance schedule [36]. Although published reports indicate that DHPG is effective in the treatment of CMV infection, all the reported studies to date are not placebo controlled, and the majority do not involve a large number of patients. Placebo controlled trials are now being designed in patients with CMV colitis.

Pyrimidine Nucleosides

5-iodo-2'-deoxyuridine, (idoxuridine, IDU). IDU is a pyrimidine analog, which competes with thymidine for incorporation in viral and cellular DNA, leading to inhibition of DNA polymerase [37]. IDU is active against DNA viruses, primarly HSV-1, HSV-2, and VZV [38]; however, its poor solubility in polar solvent limits its use to topical application. Adverse reactions relate to the site of application. For example, when used in the treatment of



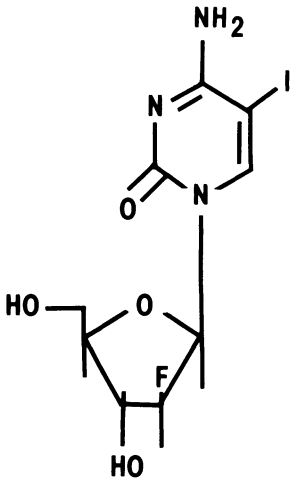
BVDU

Figure 4. Structure of E-5-2-bromovinyl-2-deoxyuridine (BVDU).

herpes keratitis, toxicities include local irritation and pain, contact dermatitis, and conjunctivitis [39]. IDU is teratogenic in laboratory animals and has largely been replaced by other antiherpetic drugs [40].

E-5-2-bromovinyl-2'-deoxyuridine (BVDU). BVDU is a selective inhibitor of HSV-1 and VZV (figure 4). The mechanism of action involves the specific phosphorylation by viral thymidine kinase and subsequent inhibition of DNA polymerase by the phosphorylated derivative [41]. BVDU can be administered topically or by the oral route. Renal excretion is the principle route of elimination. Several small studies in patients with advanced malignancies infected with herpes zoster or simplex infections have been reported. In one study, 25 patients with hematological malignancies and herpes simplex or zoster infection were treated with 7.5 mg/kg/day of BVDU. In 23 patients, treatment arrested the progression of infection within one to two days [42]. In another study, 20 patients with malignancies and severe localized herpes zoster were treated with 7.5 mg/kg/day for five days. In the majority of patients, disease progression was arrested within one day of starting treatment. No toxic adverse effects were seen [43].

Several BVDU analogs have been synthesized these include E-5-(2-bromovinyl) uracil (BVU) and E-5-(2-bromovinyl) uridine (BVRU). The antiviral spectrum of these compounds is the same as BVDU and indicates that they are probably metabolically converted to BVDU or a phosphorylated product thereof [44].



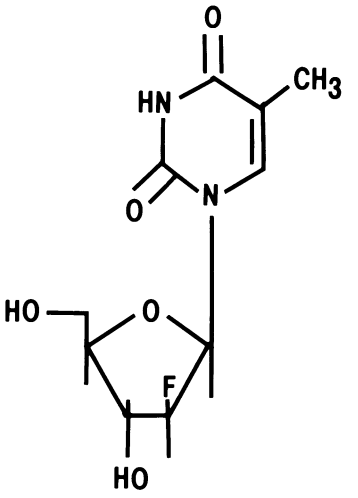
FIAC

Figure 5. Structure of 1-2 deoxy-2-fluoro-1-B-D-arabino furanosyl-5-iodocytosine (FIAC).

1-2 deoxy-2-fluoro-1-B-D-arabinofuranosyl-5-iodocytosine (FIAC), 1-2 deoxy-2-fluoro-1-B-D-arabinofuranosyl-5-methyluracil (FMAU), and 1-2-deoxy-2-fluoro-1-B-D-arabinofuranosyl-5-iodouracil (FIAU) These compounds are new pyrimidine analogs with in vitro and in vivo antiherpes activity (figures 5, 6). Their mechanism of action also involves phosphorylation by thymidine kinase. The triphosphate product competitively inhibits viral DNA polymerase while cellular DNA polymerases are less sensitive to this inhibition (45, 46).

FIAC, FMAU, acyclovir, and DHPG have been evaluated in cell cultures and animals for inhibition of herpes simplex virus. In a herpes type II infection model in mice, DHPG and FMAU were more effective in inhibiting herpetic lesions than acyclovir and FIAC. In a herpes type-1 encephalitis model, DHPG and FMAU were both effective, with FMAU being fourfold more potent than DHPG [47]. Both FIAC and FMAU are undergoing early clinical evaluation.

A phase I study of FIAC showed that 200 mg/m^2 given intravenously every 12 hours was well tolerated and did not produce significant myelosuppression [48]. Furthermore, FIAC was shown to have superior antiviral activity when compared to Ara-A in a randomized double blind study in immunosuppressed patients with herpes zoster [49]. More recent studies have focused on the oral form of this drug. Single does pharmacokinetics have demonstrated adequate serum antiviral concentrations for up to 12 hours after administration of a single oral dose of 50 mg/m^2. The recommended dose schedule is 10–30 mg/m^2 three times a day for seven days [50].



FMAU

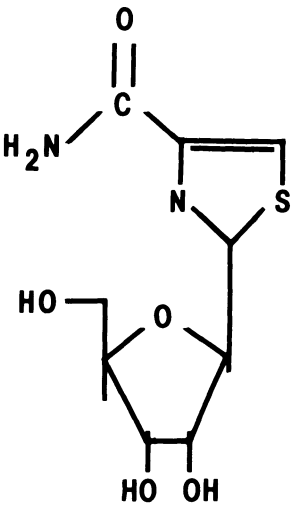
Figure 6. Structure of 1-2 deoxy-2-fluoro-1-B-D-arabinofuranosyl-5-methyluracil (FMAU).

A recent phase I study has also been reported in patients with advanced malignancies treated with FMAU. The observed toxicity was mainly neurological, with encephalopathy and extrapyramidal dysfunction seen at high doses. The MTD was 32 mg/m^2/day intravenously for five days [51].

Another analog, 2′-fluoro-5-ethyl-1-B-D-arabinofuanosyluracil (FEAU) has also been synthesized and is undergoing preclinical evaluation. FEAU is a potent inhibitor of HSV-1 and HSV-2 and is relatively nontoxic in vitro. FEAU is a weak inhibitor of thymidylate synthetase and DNA polymerase and is two-to tenfold less potent than FMAU as an antiviral agent. However, it is 150 fold less active in inhibiting precursor incorporation in rat bone marrow cells, suggesting that it would be less myelosuppressive than FMAU [52].

Triazole nucleosides

These agents are potent antiviral as well as antitumor agents. They are structurally related to guanine [53]. The antiviral and antitumor effects maybe related to the inhibition of inosinate dehydrogenase, leading to depletion of cellular guanine nucleotides. The best studied of this group of drugs is ribavirin, which will be discussed in the section on antiretroviral drugs.



TIAZOFURIN

Figure 7. Structure of tiazofurin.

2-B-D-ribofuranosylthiazole-4-carboxamide (tiazofurin). Tiazofurin resembles ribavirin in structure and in antiviral spectrum (figure 7). It inhibits DNA viruses, mainly herpes, and RNA viruses such as orthomyxo, paramyxo, and arena viruses. Phase I studies in patients with advanced malignancies, exploring a variety of dose schedules, have been conducted. Nineteen patients were treated with escalating doses on days 1, 3, 4, 5, and 6 with cycles repeated every 28 days. Dose-limiting toxicities included mental obtundation, probably related to concurrent Diamox therapy, severe headache, and myalgias. Other toxicities included myelosuppression, fever, chills, conjunctivitis, diarrhea, or constipation, drowsiness, fatigue, and cutaneous rash [54]. In addition, an analysis of 45 patients treated in a phase I trial on a five day schedule implicated tiazofurin as a cause of nephrotoxicity. Prior chemotherapy and renal impairment were associated with an increased incidence of myelosuppression with treatment [55]. In a further phase I study with a five day schedule and a variety of dose levels, the dose-limiting toxicity was pleuropericarditis. Other adverse effects seen were mainly non-hematologic and included hyperuricemia; elevations of SGOT, LDH, and CPK; conjunctivitis; malaise; myalgias; headaches; nausea; and vomiting, anorexia, and skin rash [56]. Because of the significant associated toxicity tiazofurin is not likely to be tested as an antiviral agent in humans.

Glycosylation inhibitors

2-deoxy-D-glucose

Deoxy-D-glucose is a glucose analog which inhibits glycosylation of glycoproteins and glycolipids so that longer-chain homologs are not formed and precursor molecules accumulate (figure 8). Deoxy-D-glucose can inhibit the multiplication of herpes virus by interfering with viral-mediated functions such as envelope biosynthesis and fusion [57, 58].

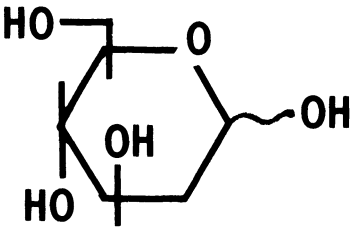
2-deoxy-d-glucose has been tested as a topical application in women with genital herpes. In a double-blind controlled study, 36 women with genital herpes were treated with topical deoxyglucose or placebo. In the case of initial mucocutaneous infection, 89% were cured. Discomfort resolved within 12 to 72 hours of therapy, and the majority of patients were asymptomatic within four days. Two recurrences were seen after 24 months. In the case of recurrent infections, 90% had a notable improvement manifested by no or less frequent recurrences, fewer lesions, or shortened duration of symptoms. In both cases, viral shedding was notably reduced by 2 deoxy-D-glucose. Controls treated with placebo failed to respond within the same time frame. The difference in time to response and recurrence rate was statistically significant [59].

Recent interest in 2-deoxy-D-glucose focuses on its antiretroviral effects, in particular against HIV. In vitro studies with persistently infected H9 cells demonstrated lowering of infectivity, unrelated to reverse transcriptase inhibition, suggesting that glycosylation inhibitors may be useful in vivo agents in the treatment of AIDS [60].

Interferons

Interferons are a group of relatively small glycoproteins produced by virtually all vertebrate cells. Isaacs and Lindemann first described interferon as antiviral agents in 1957 [61]. Recent advances in recombinant DNA technology have facilitated commercial production and subsequent clinical trials [62]. Interferons have antiproliferative and immune modulating properties as well as antiviral effects. The current nomenclature classifies them as interferon alpha, antigenically like leukocyte interferon; beta, antigenically like fibroblast interferon; and gamma, antigenically like immune interferon. They each have different properties. However, alpha and beta share the same cellular receptor.

The mechanism of antiviral activity is unclear but probably involves reversible binding to cell surface receptors with subsequent activation of cytoplasmic enzymes affecting messenger RNA translation and protein synthesis. The antiviral state may take hours to develop but may persist for days thereafter [63]. Because of the availability of other effective agents,



2 - Deoxy - D - glucose

Figure 8. Structure of 2-deoxy-D-glucose.

such as acyclovir, with low systemic toxicity, interferons have not been widely utilized as antiviral agents. Of interest, however, is a recent study demonstrating high efficacy of intralesional natural leucocyte interferon in the treatment of condylomata acuminata. Thirty-four patients were randomized to receive interferon at 600,000 IU twice weekly intralesionally or placebo. Treatment duration was eight weeks. Eighty three percent of patients receiving interferon had a complete response, compared with 8% in the placebo arm. Treatment was well tolerated with mild systemic toxicity and mild pain at the injection site [64].

A recent large, randomized, placebo controlled study, has also demonstrated the efficacy of intranasal alpha interferon spray in the prophylaxis of rhinovirus infections. A total of 448 patients, received intranasal spray with interferon or placebo after exposure to rhinovirus infection. Post exposure use of intranasal interferon resulted in 39% reduction in the incidence of respiratory illnesses. Adverse effects were limited to nasal mucosal inflammation and bleeding.

Interferon inducers

Poly ICLC. The synthetic double-stranded RNA polynucleotide (Poly [I]-Poly [C]) has at least three activities which might relate to its antiviral and antitumor activity: (1) induction of interferon, (2) nonspecific stimulation of immune mechanisms, and (3) nonspecific and specific effects on cell membranes and on macromolecular synthesis [66–71]. In a phase I–II trial of this compound in patients with cancer, however, the agent proved to be a poor interferon inducer [72]. Rapid hydrolysis may have been responsible for this lack of clinical activity [73].

Recently, a stabilized Poly (I)-Poly (C) complex has been prepared which appears to resist hydrolysis by serum nuclease and is an effective interferon inducer in subhuman primates [74]. The stabilized product is a complex of

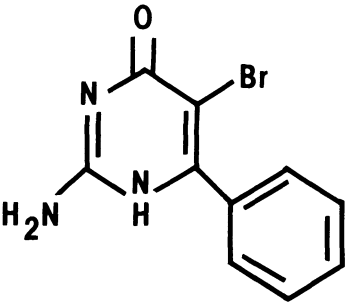
high molecular weight Poly (I)-Poly (C) and low molecular weight Poly-L-lysine, (Poly [ICLC]). Because of the low molecular weight of the Poly-L-lysine, it was anticipated that the complex would not be strongly antigenic. The preparation, which also contains carboxymethyl cellulose, is eight to ten times more resistant to hydrolysis by pancreatic ribonuclease than is the parent (Poly [I]-Poly (C)). When injected intravenously into monkeys or chimpanzees, significant serum interferon levels have been obtained [74, 75]. Rhesus monkeys have been protected against certain viral infections using Poly (ICLC) [74]. Indeed, high levels of protection against otherwise lethal simian hemorrhagic fever, rabies, yellow fever, and other viruses constitutes the first reported control of systemic virus disease in a subhuman primate by an interferon inducer [76, 77]. More recent observations suggest intermittent schedules are better tolerated and lead to more sustained interferon levels [78].

Levine and associates reported the results of a phase I clinical trial in which Poly (ICLC) was given intravenously in 15 daily doses of 0.5–27.0 mg/m^2 to 19 patients with various solid tumors, and six with acute leukemia. At least three complete trials were conducted at each of six dose levels. Toxic reactions included fever (in 100% of trials), nausea (44%), hypotension (28%), thrombocytopenia and leukopenia (68%), erythema (12%), and polyarthralgia plus myalgia (16%). Hypotension and arthralgia-myalgia appeared to be related to dose level and/or magnitude of interferon induction, but other toxic manifestations did not. Poly (ICLC) induced significant serum interferon levels in 76% of trials, and the correlation between dose and peak interferon titer was linear. The maximum tolerated dose for all patients at a given drug dose was 12 mg/m^2; at this dose, the mean peak interferon titer was 1,940 reference units per ml. At 18 mg/m^2, the mean peak interferon titer was 4,473 reference units per ml, but severe myalgia and arthralgia were intolerable in at least half of the patients, and most had significant hypotension. At 27 mg/m^2, one patient had acute renal failure. At high doses, intravenous Poly (ICLC) also induces interferon in the cerebrospinal fluid [79].

The therapeutic ratio of double stranded RNAs can be greatly enhanced by mismatching base pairs. Poly I-poly $C_{12}U$ (ampligen) is an example of this. Ampligen displays strong antitumor and immune modulating activity, including interferon induction in preclinical systems, and is currently undergoing early clinical trials [80].

Pyrimidinones

Pyrimidinones are a group of compounds which are capable of interferon induction, 2-amino-5-bromo-6 methyl-4-(1H)-pyrimidinones was the first such compound [81]. However, its toxicity was significant in preclinical systems [82]. A new derivative, bropirimine, has been shown to have anti-



ABPP

Figure 9. Structure of bropirimine (ABPP).

viral and antitumor activities in vivo and to modulate the immune system (figure 9). In 1980, Stringfellow et al. reported that a series of 6-phenyl substituted, halogenated pyrimidinones (including bropirimine) were active interferon inducers in experimental animals and in human tissue cultures [83]. In addition, these compounds were in vivo stimulators of natural killer (NK) cell and macrophage-mediated cytotoxicity, as well as B-cell responses [84–87]. Interestingly, there was no correlation between the level of induced, circulating interferon and cellular immune responses. Finally, some of these compounds were potent antiviral agents in experimental animals when given by various routes of administration, including orally, and showed no severe toxicity at active dose levels [88–90]. These data suggested that the pyrimidinones might have utility in humans as a means of treating or preventing virus infections and neoplastic diseases.

Administration of bropirimine to mice resulted in increased natural killer cell activity, increased antibody formation, and increased macrophage-mediated cytotoxicity. Bropirimine, administered orally, induced interferon in a number of species (mice, cats, dogs, cattle, rats, and monkeys) [91]. Bropirimine had antitumor activity in mice against spontaneous as well as carcinogen-induced tumors. Increases in survival rate and mean survival time, and decreases in tumor volume and metastasis rate have been documented [92–96]. Bropirimine had broad spectrum antiviral activity against both DNA and RNA viruses in various species of animals when administered orally or parenterally, or by local instillation at the portal of infection [86–90, 97].

Three phase I oral studies in man have been accomplished and have defined basic pharmacokinetic parameters. Single dose tolerance was approximately 8 grams, and weekly doses of 8 grams produced acceptable toxicity, chiefly nausea and vomiting, tachycardia, and leg cramps, (ECOG grade II

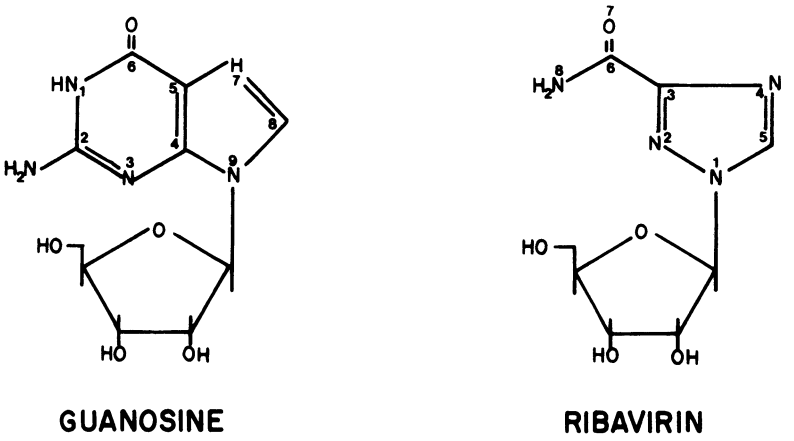


Figure 10. Structure of ribavirin.

or less). Bioavailability was improved by fractionating each dose at two hour intervals. Circulating interferon of the alpha class was detected consistently at the 6 to 8 gram level [98].

Antiretroviral agents

Several antiretroviral agents have been developed specifically to inhibit HIV, the etiologic agent of AIDS. Potential targets for anti-HIV agents include enzymes which are unique to virally infected cells or viral envelope proteins such as reverse transcriptase. With the recent recognition of likely sanctuary sites, such as the central nervous system (CNS) and semen, effective therapy must be designed to eradicate infections in these compartments.

Criteria for antiviral efficacy should include viral killing, evidenced by negative viral culture, or inhibition of viral replication, as seen by the suppression of reverse transcriptase (RT) activity. Other clinical measures of effect might include improved immunologic function, such as increases in absolute lymphocyte counts, restoration of the normal T4/T8 ratio, and positive delayed-type hypersensitivity to skin testing with recall antigens.

Ribavirin

Ribavirin is a broad-spectrum, non-interferon inducing orally active virostatic agent. Its structure is closely related to guanine (figure 10), and although its precise mechanism of action has not been fully elucidated, it is likely related to disruption of guanine nucleotide metabolism.

In vitro, ribavirin is active against both DNA and RNA viruses [99–101].

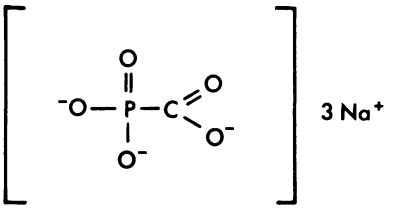
This effect is dependent on the concentration of virus used to infect cells, the characteristics of the host cell line, and the specific virus strain [102]. Recently, concentrations of 50 μg/ml of ribavirin have been shown in vitro to inhibit the replication of HIV and the release of reverse transcriptase in the supernatant [103].

Ribavirin can be administered orally or intravenously, inhaled as an aerosol, or applied topically to the skin. After oral and intravenous administration of 1,000 mg/day, serum plasma concentrations reached the in vitro mean inhibitory concentration values for herpes and influenza viruses [104]. The serum half-life of the drug is approximately nine hours (range 6.5–11.0 hours) and is about twice as long as that of other antiviral drugs such as adenosine arabinoside (Ara-A) and acyclovir [104, 105]. Small-particle aerosol administration delivered adequate drug levels to infants with influenza and respiratory syncytial virus infections [106, 107]. No significant toxicity has been seen in oral regimens of 600 mg/day for 28 successive days. Higher doses and longer periods of treatment may lead to toxicity, most notably decreases in hemoglobin content and hematocrit [108].

Ribavirin is selectively concentrated in red cells, and the monophosphate metabolite of the drug may then result in a competitive deficiency of ATP and interfere with the cell's metabolism or membrane components causing early red cell death [109]. The anemia is rarely clinically significant, is dose-related, and is reversed when treatment is discontinued [109, 110].

Extensive clinical testing in randomized double blind trials has shown efficacy of ribavirin in treating respiratory syncytial virus infections and lassa fever [111–116].

To date, experience with ribavirin in retroviral infection has been limited. In vitro concentrations of 50–100 μM interfere with the replication of HIV in cultured T-cells [103]. In phase I trials, mean plasma ribavirin concentration of 3.1 μM was achieved after an oral dose of 1,000 mg/day [104]. Following intravenous administration of 1,000 mg/day and 500 mg/day for four days, mean plasma ribavirin levels were 94 μM and 68 μM, respectively. These levels are in excess of the in vitro minimal inhibitory concentrations for the herpes virus family. In a pharmacokinetic study, ribavirin was administered orally or intravenously to 17 patients with AIDS-related complex. Single doses of 600, 1,200 or 2,400 mg were given. The mean peak and one hour postinfusion concentrations following intravenous ribavirin were 43.5 and 8.0 μM, 72.3 and 19.7 μM, and 161 and 37.1 μM, respectively. Terminal half-life was 36.2 ± 13.4 hours. Following oral administration the mean peak ribavirin concentrations were 5.1, 9.9, and 12.6 μM, respectively [117]. The long terminal half-life indicates that drug accumulation follows repetitive dosing. Oral ribavirin administered for two to four weeks at escalating dose levels from 600 to 2,400 mg, to 15 patients with adenopathy, positive HIV antibody, and low normal or reduced T4 cells, led to reversible reduction in hematocrit, transient reduction in reverse transcriptase activity, and transient increase in the T4/T8 ratios [118]. Furthermore, in a small



FOSCARNET

Figure 11. Structure of phosphonoformate.

study of five patients with AIDS receiving oral ribavirin, increased numbers and augmented responses of peripheral lymphocytes to foreign antigens were seen in the majority of patients [119].

Toxicity in these studies included moderately severe anemia and mild central nervous system toxicity consisting of headache, insomnia, irritability, and poor concentration. Ribavirin treatment of patients with AIDS has recently begun in a controlled setting.

Phosphonoformate (foscarnet)

Foscarnet, a pyrophosphate analog (figure 11), has been shown to noncompetitively inhibit several viral enzymes including the DNA and RNA polymerases [120]. Reverse transcriptases from HIV and other viruses are inhibited in vitro by foscarnet in concentrations ranging from 0.7 to 100 μM [121]. Growth of HIV-infected cells was suppressed by 98% with doses of 132 μM and was completely inhibited at 680 μM. Growth rates of uninfected cells were not affected at the lower dose and only minimally inhibited at the higher dose.

Additional experiments [122] revealed that timing of initiation and duration of treatment was important. Either postponing addition of drug to cultures or withdrawing it prematurely, incompletely inhibited RT activity and viral replication.

In cultures of T-cells from patients with ARC, foscarnet reversed the viral induced impairment of clonal lymphocyte expansion, as measured by a significant rise in T-cell colony numbers [123]. In another experiment using lymphocytes derived from patients with AIDS, 7 out of 12 T-cell cultures exposed to foscarnet showed increased colony numbers, a positive trend which did not reach statistical significance [124]. Foscarnet did not stimulate normal peripheral blood leukocytes in the presence of PHA or IL-2. The

mechanism of action of foscarnet in this regard is not well defined, but does not seem to be related to mitogenesis stimulated by PHA or to direct antiviral effect. One postulate is that foscarnet permits T-cell growth by activating colony precursors while preventing viral production, while another suggestion is that it may inhibit the production of soluble suppressive factors which impair immune response. The latter hypothesis was supported by the results of mixed lymphocyte experiments in which foscarnet reversed the suppression of colony formation of healthy control cells induced by lymphocytes from AIDS patients [123].

Foscarnet can only be administered intravenously in humans. Bioavailability after oral administration is 15%. Preliminary results from patients treated for CMV infections revealed plasma and CSF concentrations ranging from 40–75 μg/ml and 8–30 μg/ml respectively. The drug is excreted in the urine. Its plasma half-life is between one and four hours, and the drug remains in bone for up to 400 days [125].

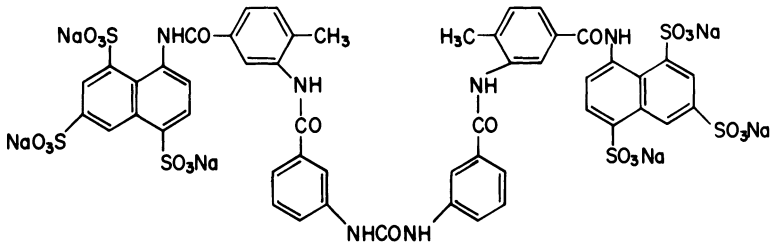
No acute toxicity was seen in the studies of foscarnet in treatment of CMV infections in man. Evidence from animal studies suggests that the drug is relatively nontoxic. Problems related to long-term intravenous infusion include local phlebitis and volume overload. There was a suggestion of nephrotoxicity in patients who had been treated for longer than two weeks.

Based on its in vitro activity against reverse transcriptase, ability to inhibit growth of HIV, and the relative lack of toxicity, foscarnet may offer some promise in the treatment of AIDS. Eleven patients with AIDS and ARC were treated in a controlled study with continuous infusion foscarnet for three weeks at 20 mg/kg/24 hours. The study is ongoing; preliminary results suggested clinical improvement and enhanced delayed hypersensitivity with viral inhibition in some patients [126].

Suramin

Suramin is a hexasodium salt derivative of naphthalenetrisulfonic acid (figure 12). Originally introduced in 1920 as a cure for African sleeping sickness, the drug has been extensively used in the treatment of trypanosomiasis and onchocerciasis. Its specific mechanism of action against these parasitic diseases is not known, but may be related to inhibition of various enzymes including hyaluronidase, ATPase, hexokinase, and DNA polymerase [127]. Suramin has also recently been shown to competitively inhibit viral reverse transcriptase in vitro. At concentrations of 500 μg/ml suramin blocks the infectivity of HIV in cultures of normal T4 lymphocytes and H9 cells. Higher drug levels, between 100–1,000 μg/ml, clearly protected against the cytopathic viral effect while not inhibiting normal T-cell growth [128].

Suramin is given by slow intravenous administration, as the drug is poorly absorbed by the oral route. When given by subcutaneous or intramuscular injection it causes intense local irritation.



SURAMIN

Figure 12. Structure of suramin.

Following intravenous injection, suramin combines with plasma proteins and may persist in the blood for up to six months [127]. The drug is cleared by the kidneys at a slow rate, with a half-life of about 40 days. Variable drug accumulation occurs, which may account for some therapeutic failures in the diseases for which the drug is indicated.

Acute toxicity encountered in suramin treatment include nausea, vomiting, abdominal pain, and urticarial eruptions. Very rarely, in 1/2,000 to 1/4,500 cases, neuromuscular irritability and cardiovascular collapse associated with coma may occur. Late reactions (usually within 24 hours) include fever, photophobia, lacrimation, palpebral edema, abdominal distension, and constipation. Cutaneous hyperesthesias of the soles and palms may begin within the first 24 hours after injection and may persist for a week or longer. The affected skin may desquamate. Renal damage is the most common delayed reaction noted several days after injection. Both the proximal tubules and the glomeruli can be affected and a self-limited, mild proteinuria is extremely common and generally not an indication for stopping therapy [127].

Preliminary reports of suramin therapy in patients with ARC and AIDS have been recently published. In one study, ten patients received a total dose of 6.2 gm over five weeks. All patients were HIV antibody seropositive, but positive viral cultures and reverse transcriptase activity were documented in only four cases. Viral replication was diminished in all four of these patients during the course of treatment and was actually undetectable in three. However, one week following discontinuation of treatment, viral growth resumed in all cases. Viral cultures in three patients who had been culture-negative at the start of therapy remained negative at the conclusion of treatment. There was no significant improvement in either the patients' clinical status or immunological parameters including total WBC, total lymphocytes, T4/T8 ratio, or delayed hypersensitivity skin tests to a routine panel of antigens [129].

A second study was conducted in Rwanda. Five patients with a clinical diagnosis of ARC were treated with 20 mg/kg of suramin intravenously every five days for 35 days. HIV antibody status was not documented, nor were viral cultures or reverse transcriptase activity measured. Quantification of T-cell subsets and delayed hypersensitivity skin tests were followed as parameters of immunologic competence. Objective clinical improvement was detected after the first dose — indicated by disappearance of lymphadenopathy arthralgias and diarrhea. Further improvement in opportunistic infections was also claimed in one patient with pulmonary tuberculosis and another with undocumented, presumptive pneumocystis pneumonia and nephrotic syndrome. Restoration of immune competence was claimed, in that three previously anergic patients mounted delayed hypersensitivity skin reactions to PPD and PHA after treatment, and T4/T8 lymphocyte ratios rose in four treated patients. In this nonrandomized, uncontrolled test of suramin in ARC, a positive antiviral effect was suggested [130].

A third group at the Goethe University in West Germany treated eight patients with ARC or AIDS. A total dose of 6.2 gm was administered over six weeks followed by a maintenance dose of 1 gm every two to three weeks, in order to maintain a serum suramin level of 100 μg/ml [131]. These investigators found no clinical or immunological improvement over a seven month follow-up, and indeed two patients actually deteriorated in a manner typical of ARC. Recent clinical data suggested that a trough serum concentration of less than 100 μg/ml allowed viral reactivation. In the United States, a total of 97 patients with AIDS were treated in a multicenter phase I suramin trial. Induction therapy included 0.5 gm, 1.0 gm, or 1.5 gm intravenously each week for six weeks. Maintenance therapy with 0.5 gm, 1.0 gm, or 1.5 gm followed for a maximum of one year. Of 92 patients with positive virus culture prior to suramin, 63 were evaluable for viral recovery. Of those, 19 (30%) became negative during treatment, including 50% on the 1.0 gm or 1.5 gm regimens. No immunologic improvement was observed. The only clinical response was a complete remission in a patient with Kaposi's sarcoma and AIDS-related non-Hodgkins lymphoma. Toxicities tended to occur during the first month of treatment and were generally reversible. Toxicities included fever (41%), malaise (31%), nausea and vomiting (25%), rash (31%), diarrhea (19%), and allergic reactions (12%). Suramin-induced neutropenia (less than 2,000/μl) was noted in 36%, thrombocytopenia (less than 50,000/μl) in 12%, creatinine greater than 2 mg% in 16%, and SGOT greater than 200 IU in 15%. Clinical and/or laboratory evidence of adrenal insufficiency was noted in 27 patients. Treatment was discontinued most often for disease progression or drug toxicity. In summary, although suramin could inhibit recovery of HIV in vivo in some patients, treatment was associated with little clinical activity and considerable toxicity [132]. Future trials may focus on combining suramin with other agents such as immune modulators.

Antimoniotungstate — (HPA-23)

HPA-23, a mineral condensed polyanion of ammonium 5-tungsto-2-antimoniate, is a competitive inhibitor of murine and human reverse transciptase [133, 134]. The drug is active in vivo against a wide variety of DNA and RNA viruses and has been used in humans in the treatment of scrapie [135]. It is active against retroviruses in vitro [136].

To date there has been limited clinical experience with this drug. A published report from Paris presented the results of intravenous infusion of HPA-23 in four AIDS patients. Although no detailed pharmacokinetics were performed, in one patient the serum half-life was less than 20 minutes. Patients received two courses of 200 mg/day for 15 days each. All four patients completed treatment. Viral replication and reverse transcriptase activity were measured prior to, during, and after treatment. HPA-23 was shown to inhibit both enzyme activity and viral reproduction. Anti-HIV antibodies persisted throughout treatment, and viral activity increased when treatment was discontinued. HPA-23 had no significant effect on either total T4 lymphocyte counts or on the T4/T8 ratio.

The primary toxicity of the drug was significant thrombocytopenia, to platelet counts of less than 50,000. This was a reversible side effect, with platelets rising to pretreatment levels 21–45 days after discontinuation of the drug. Mild elevations of hepatic transaminases were also noted. There was no renal toxicity [137]. Nineteen patients (12 AIDS, 3 ARC, and 4 healthy seropositive) were treated with a 50 mg daily bolus of HPA-23, five days every week. Long term HPA-23 was well tolerated but also associated with thrombocytopenia. Eighteen out of 19 patients showed initial HIV inhibition, as measured by culture and reverse transcriptase activity. A number of these patients, however, reverted to culture positivity while on therapy. T4 cells did not significantly increase [138]. Further studies of this drug in treating AIDS are ongoing.

Azidothymidine AZT, (compound S)

Azidothymidine is a thymidine analog (figure 13) which is converted by a cellular kinase enzyme to a competitive inhibitor of reverse transcriptase. AZT inhibits HIV replication in vitro without affecting normal uninfected cells. Adequate serum levels are obtained after oral administration, and the drug crosses the blood-brain barrier achieving concentrations 20–25% of those seen in the circulation [139–141]. Pharmacokinetic studies indicate a mean half-life of 1.1 hours, with the 5 glucoronide of AZT being the major plasma and urinary metabolite. The bioavailability after oral administration was 60%–70%; the incomplete bioavailability relates to first pass metabolism and not to poor absorption [142]. The renal excretion of AZT can be decreased by concommitant administration of probenecid. This, in turn,

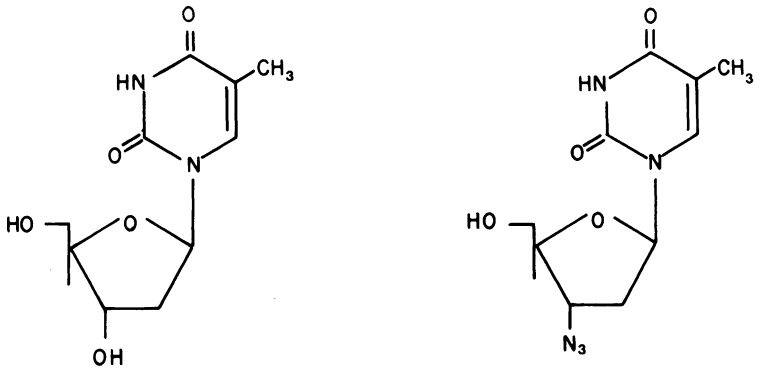


Figure 13. Structure of azidothymidine (AZT).

leads to a longer serum half-life [143]. Phase I/II studies have recently begun. Preliminary results are encouraging, with viral inhibition achieved at the highest dose regimens. Statistically significant increases in circulating T helper lymphocytes were seen in the majority of patients, indicating some immune reconstitution. Treatment-related toxicities included headaches and depression of white cell counts, which were not dose-limiting [144].

Conclusion

There has been a rapid expansion of antiviral agents over recent years. Several difficulties arise in treating viruses, mainly because of their obligatory intracellular parasitism and viral nucleic acid incorporation in host cell DNA. With further advances in molecular biology, it is anticipated that several new viral targets will be identified where specific treatment can be directed. For clinical trials, rapid diagnosis allowing early initiation of therapy, quantitative measurement of antiviral effect, and a better understanding of the pathogenesis and course of viral illness will all contribute to more effective treatment.

Acknowledgment

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10. The clinical horizon of deoxycoformycin

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Antimetabolites have enjoyed limited success as antitumor agents. Those that are clinically useful — methotrexate, cytosine arabinoside, and 5-fluorouracil, for example, have multiple sites of action and may have several mechanisms by which they are lethal to susceptible cells. On the other hand, several antimetabolites with a single, well-defined mechanism of cytotoxicity have been evaluated in clinical trials. PALA, for example, is a potent inhibitor of aspartate transcarbamylase. Pyrazofurin inhibits orotidylate synthetase irreversibly; both arrest de novo pyrimidine biosynthesis and are cytotoxic to several tumor models in vitro and in vivo. However, in extensive phase II clinical testing by the NCI in the past ten years, neither showed any reproducible activity.

Clearly, mere disruption of a metabolic pathway is an insufficient condition for antitumor activity; the pathway must be crucial to the survival of the tumor and must be of lesser importance to the normal tissues of the host. In the case of PALA and pyrazofurin, this type of selective effect was not obtained, probably because efficient salvage of pyrimidines in both tumor and normal tissues circumvented the induced inhibition. In animal models, Weber has demonstrated several examples of effective antimetabolite therapy based on biochemical differences between tumor and normal tissues [1]. To date, examples of such selectivity have not been demonstrated in man. However, there is now evidence that inhibitors of adenosine deaminase, a key enzyme in purine salvage, may fulfill the above requirements. Though data are preliminary as yet, the striking activity of deoxycoformycin (dCF) in a variety of lymphoid malignancies indicates that a highly sensitive biochemical target has been identified for this cell type.

Adenosine deaminase

Adenosine deaminase (EC 3.5.4.4, ADA) irreversibly deaminates adenosine and deoxyadenosine in the salvage pathway of purine metabolism. The highest ADA activities are found in lymphoid tissues — including circulating lymphocytes, spleen, and thymus — though significant activity occurs also in

gut and pancreas [2–3]. The activity of the enzyme has been shown to be elevated in stimulated lymphocytes and is even higher in the blast cells of patients with acute lymphocytic leukemia [4]. ADA levels are higher in normal circulating T-cells than in B-cells, a relationship which is also true for their malignant counterparts [5]. Though the activity is low in normal bone marrow, both acute and chronic myeloid leukemic blast cells also have high levels [5–6]. The ADA activity of solid tumors has not been characterized.

The important role of ADA in normal lymphocyte function was indicated by the finding that a congenital syndrome of severe combined immunodeficiency was associated with low or undetectable levels of the enzyme [7–8]. Features of this disorder include impairment of both T- and B-cell mediated functions (impaired cellular immunity, lymphopenia, and hypogammaglobulinemia), elevated levels of intracellular dATP, and markedly increased urinary excretion of deoxyadenosine [9–11]. These biochemical abnormalities are responsible for the observed lymphocyte dysfunction in ADA deficiency and explain many of the consequences of pharmacologic inhibition of ADA [12].

The simplicity of the ADA reaction (figure 1), the hydrolytic deamination of a single substrate without involvement of a cofactor, has held the interest of chemists and enzymologists. Agarwal has summarized the search for and characterization of large numbers of ADA inhibitors [13]. Coformycin, a heterocyclic purine analog of antimicrobial origin, was found to resemble the proposed transition state of the ADA reaction [14–16] and to inhibit ADA with a $K_i = 10^{-10}$ M [17–18]. In 1974, Woo et al. isolated 2′-deoxycoformycin from culture bottles of *Streptomyces antibioticus* [19]. Later work showed that it was the most potent inhibitor of ADA discovered to date, with a K_i of 2.5×10^{-12} M [20, 21]. In the same year, Schaeffer and Schwender reported the synthesis of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), a molecule rationally designed from extensive structure-activity studies to bind the hydrophobic, hydrophilic, and methyl-binding regions of ADA [22]. EHNA was found to have a $K_i = 1.6 \times 10^{-9}$ M for ADA and is therefore classified as a semi-tight binding inhibitor [20, 23].

Both deoxycoformycin and EHNA have been used extensively as inhibitors of ADA in laboratory models [24]. Though only dCF has been studied in man, the future clinical development of EHNA or other ADA inhibitors may be indicated.

Biochemical consequences of ADA inhibition

The major biochemical consequence of ADA deficiency is the inability to catabolize adenosine and deoxyadenosine. Hence, the accumulation of these nucleosides and their nucleotides in vivo would be anticipated from salvage of dying cells [25]. However, with rare exception [26], patients with ADA

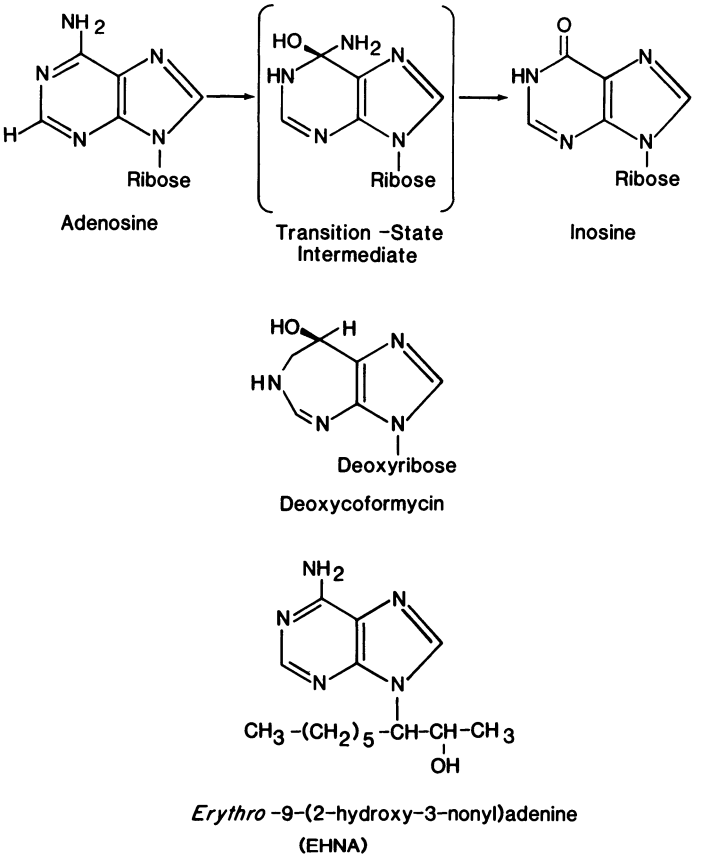

Figure 1. Schematic illustration of the hydrolytic deamination of adenosine, showing the transition state intermediate and the structures of the inhibitors 2′-deoxycoformycin and erythro-9-(2-hydroxy-3-nonyl-)adenine (EHNA).

deficiency or those treated with ADA inhibitors, accumulate only the deoxynucleosides and their phosphates: deoxyadenosine and dATP. ATP levels are normal (in congenital ADA deficiency) or depleted (following dCF treatment) in erythrocytes [27]. Barankiewicz and Cohen recently explained the difference in dATP versus ATP metabolism [28]. HGPRTase-deficient T-lymphoblasts incubated with dCF catabolize dATP to deoxyadenosine, but catabolize ATP to inosine and hypoxanthine; thus adenylate deaminase, not ADA, is responsible for ATP breakdown. Consistent with this, the major biochemical feature of ADA inhibition in vivo is deoxyadenosine and dATP accumulation [29–32]. Anabolism of deoxyadenosine to its higher phosphates is achieved by nonspecific deoxynucleoside kinases. Lymphocytes and erythrocytes have high levels of deoxynucleoside kinase activity, which accounts for the accumulation of dATP in those cells [33].

Incubation of lymphoctyes in vitro with ADA inhibitors and deoxyadeno-

Table 1. Putative mechanisms of cytotoxicity for deoxycoformycin and deoxyadenosine.

Elevated dATP levles	
Inhibit	Ribonucleotide Reductase
Inhibit	RNA Transcription
Deplete	NAD
Elevated deoxyadenosine levels	
Inhibit	S-Adenosylhomocysteine Hydrolase
Depletion of ATP	
Reduced	Cell Energy Level
Reduced	cAMP Formation
DNA incorporation of triphosphate of dCF	
DNA strand breaks	

sine, in physiologic concentrations, results in the accumulation of dATP and cell death; toxicity correlates with dATP formation [27, 34–40]. Lethality does not occur when the cells are incubated with dCF alone, but proliferation is inhibited through a mechanism independent of dATP elevation [41]. Similarly, lymphocytes exposed to deoxyadenosine (or other adenosine analogs) in the absence of ADA inhibitors do not form higher phosphates in significant amounts, and lethality does not result. Hence, the cytotoxicity of ADA inhibitors in vivo is a function of intracellular persistence of unmetabolized deoxyadenosine and its metabolites, probably derived from salvage of effete cells [25].

The relationship of the accumulation of deoxyadenosine and its triphosphate to cytotoxicity and lethality remains unestablished. dATP is an allosteric inhibitor of ribonucleotide reductase; the resulting inhibition of DNA synthesis was initially thought to be the major mechanism of cytotoxicity [9, 10, 12]. However, these experiments were conducted on cells in rapid growth phase and do not account for some important observations. First, dCF and deoxyadenosine are cytotoxic to nondividing as well as log-phase lymphocytes [42, 43]. Second, cytokinetic studies reveal G_1 arrest of replicating human leukemic T-cells, while S-phase arrest would result from ribonucleotide reductase inhibition [43, 44]. Finally, addition of deoxycytidine in vitro protects lymphocytes at lower levels of deoxyadenosine [43]. Thus several lines of evidence suggest sites of action other than ribonucleotide reductase.

Numerous additional sites of action have been proposed for dCF (table 1). Disruption of DNA was suggested by the demonstration of single-strand breaks in the DNA of cells treated with dCF and deoxyadenosine [45, 46]. DNA integrity in nondividing cells is maintained by a constant process of repair, the most important component of which is modification of nuclear proteins by addition of ADP-ribose units derived from NAD in the process of poly-ADP-ribosylation [47, 48]. Seto et al. demonstrated a dose- and

time-dependent depletion of NAD levels in lymphocytes exposed to dAdo and dCF [49]. These changes were preceded by loss of DNA integrity and decreased RNA synthesis, and followed by decreased cellular ATP levels and cell death. Addition of nicotinamide at high levels prevented the fall in NAD pools, and though strand breaks and dATP levels were unaffected, the cells survived.

This study confirmed the observations of Yu et al., which showed that dCF and deoxyadenosine inhibit RNA synthesis at the level of RNA transcription [50, 51], but suggested that this effect may be secondary to a primary DNA-directed mechanism of cytotoxicity. The attribution of lethality to NAD depletion as a 'terminal event' is novel and may be more generally applicable since several antimetabolites have been shown to cause DNA strand breaks.

The importance of dATP in mediating the cytotoxicity of dCF and deoxyadenosine has been questioned by Hershfield. He [52, 53] and other [54, 57] have shown that cytotoxicity correlates with inhibition of S-adenosylhomocyteine hydrolase following treatment of cells with deoxyadenosine or its analogs in the presence of an ADA inhibitor. Inhibition of this enzyme lowers the methylation index by reducing the S-adenosylmethionine to S-adenosylhomocystein ratio; cellular transmethylation reactions are inhibited as a result [58]. Studies in vitro showed that cytotoxicity from deoxyadenosine (with an ADA inhibitor) occurred even in adenosine kinase-deficient mutant cells which cannot form dATP [59,60]. In ADA-deficient patients [61] and in the erythrocytes and lymphoblasts of a patient treated with dCF [62], marked elevation of S-adenosylhomocysteine and inhibition of RNA methylation was observed. Others, however, did not relate cytotoxicity to S-adenosylhomocysteine hydrolase inhibition in rapidly dividing lymphoblasts [63, 64], while conflicting results were obtained with resting lymphocytes [63, 64]. Therefore, inhibition of methylation does not appear to be the major mechanism in rapidly dividing tissues. It may, however, contribute to the toxicity of dCF in organs such as liver, kidney, or CNS.

The major pharmacologic effects of dCF and EHNA appear to result from pertubation of adenine deoxynucleoside and deoxynucleotide metabolism. However, alteration of adenine ribonucleotide pools may also be important. Several studies have shown that ATP depletion results from pharmacologic inhibition of ADA [27, 65, 66], but not from ADA deficiency [9, 10, 67]. ATP does not accumulate in the presence of ADA inhibition because of its catabolism by adenylate deaminase [28], but it is not clear why levels are depleted. Koller et al. suggest that competition of dADP with ADP for phosphorylation in the glycolytic pathway may be responsible [68]. In mice, hemolysis following treatment with dCF is related to ATP depletion. Further study is warranted to explore this mechanism of toxicity in other tissues.

Finally in considering the cytotoxic effects of ADA inhibitors, direct drug effects have been reported. Siaw and Coleman found that dCF was metabol-

ized to higher phosphates in treated lymphoblastoid cells [69]. Significant DNA (but not RNA) incorporation of dCF triphosphate occurred. The enzymes responsible for dCF phosphorylation were not identified, since anabolism proceeded equally well in wild-type, adenosine kinase-deficient, and deoxycytidine kinase-deficient cells [70]. The relationship of these observations to cytotoxicity requires further study.

EHNA, on the other hand, has additional actions at drug levels higher than those required for ADA inhibition. Inhibition of cAMP phosphodiesterase causes impaired lymphocyte-mediated cytolysis [71]. In addition, several processes that are dependent upon intracellular mobility are inhibited by EHNA at millimolar levels, probably as a result of inhibition of dienin ATPase [72]. Differences between dCF and EHNA in these actions and in virus-inhibitory characteristics [73] suggest that the toxicity of EHNA in the clinic might differ from that of dCF.

In summary, the major cellular effects of ADA inhibitors appears to result from the accumulation of adenine deoxynucleotides. Elevated dATP pools correlate with cytotoxicity in resting and dividing lymphocytes. The mechanism of lethality from high levels of dATP is unestablished, but possibilities include depletion of nuclear NAD, inhibition of DNA synthesis, impairment of DNA integrity, inhibition of RNA synthesis, or disruption of cellular processes dependent on ATP. Cellular methylation reactions are inhibited by deoxyadenosine, and toxicity to nonproliferating organs may result. Actions of dCF and EHNA at sites remote from purine-dependent pathways have not been related to specific toxic effects, but merit further study.

ADA and immune function

Adenosine deaminase activity varies considerably in lymphocyte populations: T-cells have activity over tenfold higher than in B-cells [74], and less mature thymocytes have higher activity than their mature counterparts [75]. Studies of leukemic lymphocytes suggest that ADA levels vary with immunophenotype [5,76]. However, these biochemical observations have not yet been correlated with specific immunologic functions.

Somewhat paradoxically, T-cells are more susceptible than B-cells to the cytotoxic and growth inhibitory effects of ADA inhibition [36–38,77–80]; this difference correlates with greater dATP accumulation in T-cells [36–38,80]. It is postulated that B-cells have lower dATP levels because of the higher activity of triphosphate-catabolizing enzymes such ecto-5′-nucleotidase [78,79]. Others have attributed a similar role to the cytoplasmic nucleotidase [81] and to an ecto-(Ca++, Mg++)-ATP hydrolase [82]. However, none of these mechanisms fully accounts for differences in dCF-mediated cytotoxicity in vitro [77,80,83]. Studies in man are conflicting,

and have associated lymphocyte dATP accumulation with both ecto-5′-nucleotidase [84] and ecto-ATP-ase [85] activities.

Recent data suggest that observed differences between T- and B-cells may relate to culture conditions or to the source of the cells under study. In man, B-cells of peripheral [86] or tonsillar [87] origin and plasma cells [86] had ADA levels equal to or greater than T-cells isolated from the same subjects and accumulated and retained dATP to a similar extent [87]. These inconsistent results suggest that determinants other than dATP levels may be important in distinguishing T-cell from B-cell responses to ADA inhibition. For both dCF and EHNA, the value of pursuing such studies lies in their potential for describing the biochemical basis of their selective lymphocytotoxicity. This may facilitate and direct the antitumor and immunosuppressive applications of the drugs and suggest additional uses in other areas of investigation.

T-cell subsets which may be especially sensitive to ADA inhibition have been identified in a number of studies of lymphocyte function. Both dCF and EHNA are known to be immunosuppressive, as evidenced by their inhibition of such T-cell dependent phenomena as E-rosette formation [88, 89], phytohemagglutinin and pokeweed mitogen-induced proliferation [88, 90–92], and mixed-lymphocyte culture responses [92]. Cytotoxic functions of K and NK cells, which are independent of proliferation, are also inhibited [93]. Antibody production, on the other hand, has been reported to be suppressed [94–95] or stimulated [96] following dCF administration. Ratech et al. [97] showed that the timing of dCF administration (before or after antigen administration) determined whether suppression or enhancement was observed. Decreased antibody production was a consequence of B-cell suppression by dCF administered before antigen, while enhanced responses were a consequence of dCF following antigen administration and appeared to result from an inhibitory effect of dCF on a suppressor T-cell subpopulation [97]. Previous studies [96, 98] had suggested, but not established, that suppressor T-cells were susceptible and helper T-cells resistant to dCF action. The specificity of EHNA has not been investigated so extensively. These observations indicate that neoplastic and non-neoplastic disorders of suppressor lymphocytes may be appropriate targets of dCF action and should be exploited in the clinic.

Pharmacology

Cellular

It is established that the accumulation of dATP following dCF administration requires an exogenous source of deoxyadenosine [41], but that levels of deoxyadenosine in the physiologic range [10^{-6} to 10^{-4} M] cause maximal

accumulation of dATP in T-lymphoblasts by 16–24 hours of incubation [43]. Mitchell et al. suggest that a level of about 200 pM dATP/10^6 cells (i.e., a greater than tenfold increase) correlates with cytotoxicity in most human leukemia cells [84]. Persistence of dCF effect has been observed in human studies to last up to a week following a single dose of dCF [99], and the time course of ADA inhibition is clearly different in red cells and lymphocytes [Benjamin R, personal communication; Cummings FJ, personal communication]. Hence, the optimal plasma level of dCF required for ADA inhibition has not been established and may vary for different cell types.

EHNA, which binds the enzyme less tightly than dCF, was studied in mice [100]. A single oral dose of 50 mg/kg inhibited peripheral blood ADA completely for four hours, and recovery was complete by 12 hours. Substantial ablation of ADA activity was achieved only with frequent dosing, and cumulative effects were not seen. Much lower doses (3 mg/kg) were effective in reducing ADA activity by 50% for a similar period; this dose exhibited marked synergy with ara-A in a different experiment [101]. It was noteworthy that ribonucleotide pools were not perturbed even at high doses [100]. Further in vivo studies to observe deoxynucleotide pool changes in various cell types are warranted. The shorter duration of ADA inhibition resulting from EHNA, as opposed to dCF, administration may confer a selective advantage in the setting of acute leukemia and in combination regimens.

An important determinant of selectivity of dCF may be its cellular accumulation. Influx of dCF has been characterized in red blood cells. Inactivation of ADA in intact RBCs occurs 300–500 times more slowly than in hemolysates [102], so transport may be limiting to intracellular enzyme inhibition. Coincubation with NBMPR, dilazep, and dipyridamole antagonizes dCF action; thus, dCF enters cells by the nucleoside transporter(s) [102]. Adenosine competes for binding to the transport protein [103]. Efflux of dCF from cells has not been characterized, though it is likely to be less important than dissociation from the enzyme. It is not clear in nucleated cells if recovery from ADA inhibition occurs by elimination of dCF or by new enzyme synthesis.

Pharmacokinetics — preclinical

The pharmacokinetics of dCF have been studied preclinically in mice, rats, and dogs. McConnell et al. [104] reported biphasic elimination of radiolabelled 2′-dCF from the plasma of mice following a single IP administration of drug. After doses of 0.25 and 2.5 mg/kg, the half-lives for the alpha and beta phases were 17 and 64 minutes and 18.5 and 104 minutes, respectively. Peak plasma levels of 0.24 μg/ml were obtained within 15 minutes after an injection of 0.25 mg/kg. Urinary excretion accounted for more than 90% of the 2′-dCF within two hours [104]. In the rat, rapid distribution of radiolabelled dCF to all body tissues follows an IM injection of 2 mg/kg [105]. More than 80% of the drug, mostly unchanged, was

Table 2. Pharmacokinetics of dCF in man.

Study (ref)	Dose	# Patients	Terminal Half-Life (h)	Urine Recovery (%)
Smyth (114) et al	0.1–0.25 mg/kg	5	5–15	32–48%
Malspeis (109) et al	2–10 mg/M^2	24	3.0–9.4	96%
Major (110) et al	10–30 mg/M^2	6	4.9–6.4	50–82%
Schneider (111) et al	0.1–0.25 mg/M^2	9	—	>90%

excreted in the urine. In dogs, a plasma half-life of 75–120 minutes followed IV administration of dCF 0.1–1.0 mg/kg [106].

EHNA pharmacokinetics have been studied in the mouse and the monkey [107–108]. In the monkey one cannot reliably estimate the plasma half-life, however, plasma levels at six hours are reduced to 7.5% of those immediately following injection. Significant metabolism occurs in both species; parent compound represents less than 0.3% of EHNA-derived urine radioactivity following administration of radiolabelled EHNA to monkeys. The metabolites are similar in both species; at least four hydroxylated derivatives have been identified, all of which inhibit ADA but with less potency than EHNA. The rapid clearance of parent and metabolites (as assessed by clearance of radiolabel) suggests that on kinetic grounds too, the behavior of EHNA is sufficiently different from dCF to warrant a more complete evaluation.

Pharmacokinetics in man

The clinical pharmacokinetics of dCF were initially characterized by Smyth et al. [4] as part of a phase I study. In five patients who received single doses of 0.1 and 0.25 mg/kg, spectrophotometric analysis showed biexponential plasma decay curves with an alpha phase range of 30–85 minutes and a beta of 5–15 hours. Urinary recovery in the first 24 hours in these five patients ranged from 32%–48%.

Pharmacokinetic studies were also components of the phase I studies sponsored by the NCI [109–112]; results are shown in table 2. These studies reported a greater percentage of drug recovered in urine at 24 hours (50%–96% versus 32%–48%), though half-lives were similar to those reported by Smyth. Malspeis et al. [110] showed a positive correlation between total body clearance of dCF and creatinine clearance. Hence, renal function is a critical determinant of dCF's pharmacologic behavior and an important variable in assessing which patients should be treated with the drug.

Malspeis et al. [110] demonstrated that the mean apparent volume of distribution was 41.7 liters and the steady state volume of distribution

36.1 liters (20.1 l/m^2); thus it appears that dCF does not bind to tissues extensively.

Plasma levels following dCF administration varied. Peak plasma levels drawn immediately after administration of dCF (0.25 mg/kg/dx 4–5) contained dCF concentrations of 12–36 mM in six of the seven patients, with the seventh at 120 nM [4]. Similar peak values were obtained by Malspesis [110]. Venner et al. [109] obtained plasma samples one hour after the administration of dCF at 0.25 mg/kg as a 30 minute infusion and measured a mean dCF plasma concentration of 1.5 μM. These investigators report that plasma levels increased as dose increased, with a mean dCF level of 4.7 μM one hour following a dose of 1.0 mg/kg. Reported levels are generally lower than those used in the in vitro studies quoted; however, ADA inhibition provides evidence of the adequacy of the doses.

ADA inhibition in man

The degree of ADA inhibition is both dose- and schedule-dependent [4, 29]. Only modest inhibition of lymphocyte ADA was detected following single administration of 0.1 mg/kg, but more pronounced inhibition resulted from 1.0 mg/kg. Repeated daily administration of dCF results in progressive inhibition of ADA, although the associated lymphopenia may impede investigation of lymphocyte enzyme activity beyond 72 hours from the start of treatment. The degree of ADA inhibition also correlated with depletion of circulating lymphocytes [4].

Although assessment of ADA activity has not been standardized, the majority of studies show significant ADA inhibition over a wide range of doses and schedules [29,11,113]. Doses as low as 0.1 mg/kg inhibited ADA from mycosis fungoides tissue by 93% at 24 hours. Serial measurements of ADA activity in mononuclear cells from patients treated by Poplack et al. [113] at 0.25 mg/kg/day showed that ADA activity was not completely inhibited until 72 hours after beginning treatment. Though it is not possible to derive a time course of ADA inhibition, Grever et al. noted a patient variability in both the dose and the time required to completely ablate enzyme activity [29]. Twelve patients treated with single doses of 4 mg/m^2 of dCF exhibited a mean ADA inhibition of 87% at 24 hours, with the range of inhibition being 52%–100%. The duration of inhibition was similarly variable; in the seven patients whose ADA was completely inhibited at 24 hours, the duration of inhibition ranged from two to seven days.

This variability of ADA inhibition reflects interpatient differences in determinants of enzyme inactivation and recovery that have yet to be characterized. For example, the transport of dCF and its intracellular accumulation has not been described in man. In addition, the intracellular concentration of dCF required for maximal ADA inhibition is not known, nor is the relationship of plasma drug level to enzyme inhibition established. Initial

studies in mice [114] have shown that recovery of ADA activity is slower in the anucleate red blood cell than in L1210 cells; this may indicate that enzyme turnover plays a significant role. The effect of dCF on the *rate* of ADA turnover is also in question, as is the selectivity for tumor tissue of its inhibitory effect. Finally, the relationship of ADA inhibition to the antitumor activity of dCF has not been fully described.

Results from phase I pharmacology studies illustrate the lack of a clear relationship between ADA inhibition and dCF-induced cytotoxicity. Therapeutic responses were seen in some, but not all, of the patients who manifested ADA depletion [113]. In addition, both Poplack [113] and Grever [115] show evidence of therapeutic responses in patients whose ADA activity was reduced, but not completely ablated, by dCF. These considerations must be viewed in the context of adenine nucleotide pool changes, the extent of which has not yet been correlated with the degree of ADA inhibition.

Adenine nucleotides

In all phase I trials, elevated plasma levels of deoxyadenosine (dAdo) followed administration of dCF, but a clear dose-relationship was not evident in most studies [21, 29, 109, 111, 112, 116]. Moreover, there was considerable variation in the levels both of deoxyadenosine and adenosine among individuals receiving the same dose of dCF [29, 109, 111, 112]. The reason for the variability is unclear and cannot be explained by differences in ADA inhibition, since ADA was completely inhibited in many of the patients who exhibited variable adenosine and dAdo levels [19, 109].

There was no clear correlation between plasma concentration of adenosine or dAdo and either toxic or therapeutic responses to dCF administration [109]. Koller and Mitchell [117], however, noted that erythrocyte nucleotide levels were markedly altered following therapy with dCF and suggested that toxicity was related to red cell dATP/ATP ratios. In their phase I studies, Grever et al. [116] confirmed an increase in dATP levels with concomitant decreases in ATP levels and noted that clinical toxicity seemed to appear when the erythrocyte dATP/ATP ratio exceeded a value of 0.50. Murphy et al. [118] also demonstrated a statistically significant relationship between the peak erythrocyte dATP/ATP ratio and systemic toxicity in children. There is, therefore, a test which might predict for dCF-induced toxicity. However, the complexity of the test, requiring immediate availability of high performance liquid chromatography, limits its general applicability. As a research tool, the ratio may help in the application of dCF to other malignancies. Support for the application of the ratio comes from the studies of Yu, which show that the dATP/ATP ratio in malignant lymphoblasts incubated with radiolabelled deoxyadenosine correlates with patients' responses to dCF therapy [119]. The altered dATP/ATP ratio may explain why pharmacologic

inhibition of ADA has produced clinical sequelae significantly different from the syndrome of hereditary deficiency of enzyme, since red cell ATP depletion is not a manifestation of inherited ADA deficiency [9].

Clinical trials

Phase I

Extensive phase I evaluation showed considerable toxicity from dCF administration, especially at high doses given for five days. Significant renal, pulmonary, and central nervous system toxicity was noted in many of the adult studies [120–122]. Pediatric patients, on the other hand, tolerated dCF in doses higher than adults; toxicity appeared to be more reproducible and less severe [113]. A major concern in the adult studies was that this toxicity was unpredictable; some patients tolerated doses up to 30 mg/m^2/dx5 with minimal adverse effects, while others succumbed to toxicity at a fraction of that dose. The red cell dATP/ATP ratio was found to predict for toxicity in both adults and children, but was limited in applicability for reasons noted above.

Grever et al. [123] approached a dose-seeking study by reducing the size and number of doses administered, with the goal of administering only the amount of dCF required to inhibit ADA. By careful measurement of ADA activity and nucleotide pools in normal and malignant cells, they established that doses as low as 4 mg/m^2 could inhibit lymphocyte and red cell ADA and produce responses in refractory lymphoid neoplasms. The majority of studies now use a maximum daily dose of 4 mg/m^2.

Toxicity in phase I studies was related both to the dose of dCF and to the disease under treatment. Patients with solid tumors and indolent lymphoid malignancies demonstrated lymphopenia and tumor regression at doses which were associated with little toxicity. Patients with higher tumor burdens (such as those with acute leukemias), on the other hand, required considerably higher doses for total ADA inhibition and experienced greater systemic toxicity. A recent study by Smyth using dCF at 10 mg/m^2/dx5 for ALL and lymphoblastic lymphoma yielded a 10% incidence of drug-related deaths [124]. This stands in marked contrast to the low dose regimens (5 mg/m^2/dx2 every one or two weeks) in which minimal toxicity is observed, provided treatment is restricted to a selected group of patients.

Definition of the patient characteristics which predict for unexpected dCF toxicity was largely accomplished by Grever. Over the course of a number of studies, he demonstrated that impaired renal function and poor performance status placed a patient at high risk for toxicity even at low doses of the drug [123, 125, 116]. The safe and effective administration of dCF has been a result of restriction of eligible patients to those with normal creatinine clearance and performance status <2 (ECOG). Studies are ongoing to

define suitable dose modification criteria for poor-risk patients who might benefit from dCF.

The organs most affected by severe dCF-induced toxicity are the kidneys (acute renal failure) and the central nervous system (lethargy and seizures). Patients who have impaired renal function before dCF administration may experience life-threatening renal and other organ toxicities even at low doses (4 mg/m^2) of dCF. Patients who develop renal failure, or those treated at very high doses of dCF, may have seizures and coma as terminal events [118]. However, dCF appears to cause schedule-dependent central nervous system toxicity even at low doses in patients with normal renal function. Grever [128] and Johnson [127] report that repeated weekly dosing causes moderately severe lethargy which increases with each dose. Accordingly, dCF for chronic or indolent malignancies should be administered at 4 mg/m^2 every other week, or 4 mg/m^2/week for three doses followed by a rest period.

Side effects of lesser severity include reversible hepatitis (rare at low doses), keratoconjunctivitis, anorexia, nausea and vomiting (easily controlled with antiemetics), and myelosuppression (at high doses). These effects are usually well tolerated and resolve with interruption of therapy.

Finally, early clinical trials also made us aware that pharmacologic inhibition of ADA may also increase a patient's risk of infection during the period of treatment [128]. Infections associated with immunosuppression, especially disseminated herpes zoster, have been frequent during the course of dCF treatment. These infections have occurred predominantly in the *absence* of myelosuppression. Further characterization of the possible increase in susceptibility to infection during dCF treatment should accompany future clinical trials. Patients who are suspected of infection during treatment with dCF should be worked-up vigorously and thoroughly.

Phase II

Responses to dCF have been observed in several chronic lymphoid neoplasms. Most notable among these has been its effect in hairy cell leukemia (HCL). This variant of chronic lymphocyte leukemia, described by Bouroncle [129], was refractory to chemotherapy until some three years ago, when it was reported that interferon-alpha could control its progression and yield complete remissions in some 10%–20% of patients [130]. In phase I studies, a number of patients with HCL were observed to enter complete remission with dCF, stimulating further clinical trials. Results to date indicate that, as with interferon-alpha, almost 100% of patients with HCL respond to dCF; of these, at least 50% have a complete remission [127, 131, 132]. Several patients who have failed to respond or who have relapsed on IFN-alpha have gone into remission with dCF [131, 134]. Because these studies are early, there are insufficient data to assess the duration of remission following

dCF treatment. However, there are now two agents with at least comparable activity in the treatment of HCL.

It is now appropriate to compare these two treatment modalities to each other. The National Cancer Institute will support phase III studies in two population groups: those who have relapsed from standard therapy (splenectomy) and those who have never been treated for HCL. These studies will help to answer some important biological as well as therapeutic questions, e.g., can one obviate the necessity for splenectomy in this disease? Does survival depend on achieving complete (as opposed to partial) remission? Will single agent therapy suffice for the majority of patients or will combinations be required? Large-scale studies involving the collaboration of several groups and institutions across the country will begin to address these issues within the next few months. It is likely that these and other studies will assign dCF a permanent role in the treatment of HCL.

Other chronic lymphoid malignancies are also responsive to dCF, though less dramatically so. Very heavily pretreated patients with CLL have a response rate of 20% [135]. Remissions have been noted in both B-cell and T-cell disease. Trials to evaluate dCF at an earlier stage of treatment and in combination with standard agents are planned. Similarly, in mycosis fungoides, a response rate of 40% is obtained with dCF [125]. Studies of dCF in combination with interferon are in progress here. Finally, responses have been noted in both favorable and unfavorable histology nonHodgkin's lymphoma, and confirmatory trials are in progress [134].

In the acute leukemias, based on the higher doses required to achieve remission and the severe toxicity often encountered, dCF is not being pursued aggressively despite remissions in pediatric and adult ALL [113, 124]. It seems more prudent to seek to achieve the same effect with less toxicity. One means of doing so could involve the adoption of an alternative inhibitor of ADA; the preclinical data discussed above suggest that EHNA may be a suitable candidate in this regard. The clinical development of EHNA or alternative ADA inhibitors holds some promise for the treatment of acute hematologic malignancies.

dCF in combination

dCF was originally introduced to protect adenosine analogs from rapid inactivation. Inhibitors of ADA potentiate the cytotoxicity of adenine arabinoside (ara-A) and other analogs against murine tumors in vitro and in vivo [91, 136–141]. In the case of ara-A, this appears to be related to the increased intracellular levels of the triphosphate (ara-ATP) [142], previously shown to be the major determinant of ara-A's antileukemic activity [143]. These authors warn, however, that elevation of dATP following dCF administration may antagonize ara-ATP-induced inhibition of DNA synthesis

in vivo by competition for their common binding site on DNA polymerase [144]. Others relate the synergistic interaction of dCF and ara-A to the inhibitory effects of both drugs on S-adenosylhomocysteine hydrolase [145], while inhibition of RNA synthesis [146] and processing [147] are additional targets.

The combination of dCF and ara-A has been studied in man. Detailed characterization of their pharmacology has been published [148–150], and a tolerable dose and schedule described for use in pediatric patients [151]. Hepatic and central nervous system side effects predominated. Completion of this study coincided with the development of another adenosine analog by the NCI. Fludarabine (2-fluoro-ara-AMP) is highly active in murine tumors and is *not* a substrate for ADA. For this reason, the drug development effort has shifted to this compound, which is currently in phase I/II trials. Further investigation of dCF in combination with ara-A will await results of these studies.

Conclusion

dCF is the first ADA inhibitor to be investigated therapeutically in man. Though inactive in murine prescreens, its in vitro lymphocytotoxicity appears to translate to significant cytotoxicity to lymphoid tumors in man. The clinical development of dCF has been associated with significant toxicity, as we have detailed. However, through the efforts of a few investigators, a well-tolerated and effective regimen has emerged. This work represents an example of how detailed pharmacologic studies have a favorable impact on early drug studies. The integration of biochemical, pharmacologic, and clinical information was crucial to refining the use of dCF in the clinic.

The role of dCF in the treatment of chronic lymphoid malignancies will be defined by current studies. The very promising results in HCL are expected to make dCF a standard agent in th treatment of this disease. In other disease, such as mycosis fungoides and CLL, dCF may have an important therapeutic role. Studies of its possible antitumor effect in other tumors would seem to be indicated.

Its potential as an immunosuppressive agent is clear from recent work in normal animals. Experiments in models of autoimmune disease are needed to support clinical studies in both aggressive and indolent rheumatic diseases. An area in which both cytotoxic and immunosuppressive effects might usefully be explored is in bone marrow transplantation.

In like manner, results obtained in the clinic may feed back to basic science. The basis for the extreme sensitivity of certain malignant lymphocytes to dCF may be of importance in clarifying the relationship between purine metabolism and immune response. Two patients with leukemia treated with dCF experienced phenotypic conversion from lymphoid to

myeloid morphology, surface markers, and cytoplasmic enzyme activities [152, 153]. Such examples provide models which may be of value in the study of cellular differentiation.

The development of new drugs in cancer or in other areas benefits from the close association of laboratory and clinic at each level, as has been elegantly shown by Elion [154]. The example of dCF confirms the value of this relationship.

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