CANCER DRUG DISCOVERY AND DEVELOPMENT

Antifolate Drugs in Cancer Therapy

Edited by

Ann L. Jackman

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PREFACE

Antifolates are an important class of anticancer drugs originally developed as antileukemic agents, but now used, usually in combination with other drugs, for the treatment of a wide range of tumors, notably carcinomas of the head and neck, breast, germ cell tumors, non-Hodgkin's lymphoma, acute lymphoblastic leukemia, and osteogenic sarcomas. 5-Fluorouracil and its prodrugs also target, in part, the folate-dependent enzyme, thymidylate synthase. Furthermore, folate supplementation in the form of leucovorin, modulates 5-fluororuacil activity. 5-Fluorouracil is widely used in the treatment of colorectal and gastric cancer and in combination for other solid tumors such as breast and head and neck cancers. Ongoing clinical trials with the newer antifolates suggest that the range of solid tumors where these agents will be of use may broaden further.

Half a century ago, interesting scientific and clinical discoveries suggested that folic acid was a vitamin involved in vital cellular metabolic processes. The folate analogs, aminopterin and methotrexate, were synthesized by the American Cyanamid Company in an attempt to interfere with these processes and were shown to have anticancer activity by Farber and his colleagues. Hence, the principle of antimetabolite therapy for the treatment of cancer was established. Biomedical research over the following years led to a deeper understanding of the complex biochemical pharmacology of folates and antifolates. Selective antimicrobial agents were discovered, but more tumor-selective anticancer agents did not immediately emerge. However, advances in drug development practice in recent years has led to the discovery of novel antifolates with encouraging clinical anticancer activity. As the new millenium approaches, it is timely to assess progress so that future research will expand on these promising foundations. Importantly, it is necessary to embrace and exploit exciting new technologies and knowledge relating to the oncological process and selective approaches to therapy.

The contributors to *Antifolate Drugs in Cancer Therapy* are largely drawn from researchers highly regarded in the field of folate biochemistry and antifolate drug development. However, there is the deliberate inclusion of some laboratory and clinical scientists whose work has only recently encompassed the antifolate area or is peripheral to their main research areas. I believe this has led to a book that provides a contextual review and exciting new avenues for future research.

Antifolate Drugs in Cancer Therapy is divided broadly into five areas. First, an historical and future perspective, along with an overview of folate biochemistry, are given. This is followed by the preclinical and clinical pharmacology of methotrexate, other dihydrofolate reductase inhibitors, and 5-fluorouracil. Eight chapters review the preclinical development and clinical activity of the new generation of antifolates, the thymidylate synthase and glycinamide ribonucleotide formyltransferase inhibitors. The fourth area draws together experience from all of the above and reviews in depth subjects such as folate and antifolate transport mechanisms, modulation of antifolate drugs, polyglutamation, resistance, and drug combinations. Finally, the rapidly expanding topics of pharmacogenomics, pharmacodynamics, regulation of gene expression, and

mechanisms of cell death bring this volume to a close. The wide and progressive scope of *Antifolate Drugs in Cancer Therapy* makes it an important point of reference for basic scientists and clinicians and provides a platform on which to build further reading in areas of interest. Editing this volume has been an exciting project, and I am very grateful to all the contributors for their participation.

Ann L. Jackman

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1 Antifolate Drugs

Past and Future Perspectives

Robert C. Jackson

CONTENTS

1. INTRODUCTION

The antifolates remain a topic of continuing fascination to pharmacologists. This interest is not entirely theoretical. Recent years have seen two new antifolate drugs approved for marketing: trimetrexate (Neutrexin), a lipophilic inhibitor of dihydrofolate reductase (DHFR) for treatment of the life-threatening fungal infection, *Pneumocystis carinii* pneumonia; and the thymidylate synthase (TS) inhibitor, raltitrexed (Tomudex), for colorectal cancer. In addition to their importance as drugs, however, the antifolates have taught us some important lessons about general principles of pharmacology—how to use drugs optimally, how to design improved selectivity into next-generation compounds, how cells become drug resistant and how to use biochemical modulation ap-

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proaches. They have also been valuable probes for exploring basic biology. These points will be made using 10 examples of topics in antifolate pharmacology that have sparked major debates over the years. Several of these areas will be dealt with very briefly, because they are covered in more detail in subsequent chapters. The final section touches on some unanswered questions, which are areas of current debate, and which are likely to be the focus of ongoing research.

Much of the work discussed in this volume had its origins in programs of analog development, often considered to be uncreative "fine tuning," rather than innovative research, and thus an area in which it is difficult to obtain funding, both from government agencies and from pharmaceutical companies. In fact, as this book demonstrates, the antifolate field continues to be a productive source not only of new drugs, but of new therapeutic strategies, and important findings in basic biology.

2. DHFR INHIBITORS AND THE CONCEPT OF THYMINELESS DEATH

The antimetabolite drugs arose from two distinct lines of research. The first of these approaches, which used inhibitors of enzymes involved in essential biosynthetic processes, emerged from the studies of D. D. Woods *(1)* on the mechanism of action of the sulfa group of antibacterial drugs. Sulfa drugs are structural analogs of *p*-aminobenzoate in which the carboxylate group has been replaced by a sulfonamide function. These agents act as potent competitive inhibitors of the dihydrofolate synthetase reaction (which occurs in many bacteria but not in eukaryotic cells) in which *p*-aminobenzoate is condensed with a pteroate to form dihydrofolate. It is characteristic of this kind of antimetabolites that they are structural analogs of normal metabolites, that they are potent inhibitors of biosynthetic reactions, that they are competitively antagonized by the corresponding normal substrate (which generally accumulates behind the block), and that the product of the inhibited reaction will give a noncompetitive reversal of the antimetabolite effect. There is another kind of antimetabolite (exemplified by 6-thioguanine) that is a substrate analog that is incorporated into macromolecules, producing, in this case, defective or misfunctional RNA and DNA molecules, a process known as "lethal synthesis." Whereas many analogs of purines and pyrimidines exert both types of effect, antifolates are not incorporated into macromolecules, and their effects must therefore be understood in terms of depletion of purine and pyrimidine precursors. It was originally suggested that antimetabolites of the first class must be cytostatic, rather than cytotoxic, since they were believed to act by starving cells of synthetic precursors. Although antifolates are sometimes cytostatic, in other cases they are clearly cytotoxic. The question thus arose: Why should precursor depletion be lethal? Seymour Cohen, working with bacteria, formulated the concept of unbalanced growth, that if cells could not synthesize DNA, but could still synthesize RNA and protein, then giant cells would result (as he observed experimentally) which would be nonviable. He proposed that thymineless death represented a form of unbalanced growth. Cohen showed that, though selective thymine starvation was often lethal to bacteria, simultaneous depletion of thymine and purines was simply cytostatic, which, he claimed, was because unbalanced growth could not occur in the absence of purines *(2)*. The development of the early DHFR inhibitors, e.g., aminopterin, methotrexate (MTX), pyrimethamine, and trimethoprim, indicated that their cellular effects on bacteria were similar to those of sulfa drugs,

and, in the case of aminopterin and methotrexate, that similar effects were seen in mammalian cells. The unbalanced growth hypothesis prompted much fruitful thought and experiment, but although it explained many of the experimental data, it did not give any insight into drug selectivity. The antibacterial selectivity of sulfa drugs is explained by the fact that mammalian cells do not possess dihydrofolate synthetase, obtaining their folates from the diet. The antitumor selectivity of methotrexate, although quantitatively much less than the selectivity of sulfa drugs, nevertheless exists, and could not be easily explained, since the DHFR enzymes of normal and transformed cells were identical, and were inhibited by MTX to a similar extent. Current explanations of thymineless death are discussed in Chapter 21.

3. MTX AND TIGHT-BINDING INHIBITION

Straus and Goldstein *(3)* pointed out that inhibitors whose binding constants for their target enzyme were of the same order of magnitude as the molar concentration of enzyme in the system, or lower, could not be assumed to follow Michaelis-Menten kinetics. Puzzling early observations reported that aminopterin and methotrexate, despite being close structural analogs of folic acid, appeared to be noncompetitive inhibitors of DHFR. This conclusion was a consequence of inappropriate kinetic analysis; when methods appropriate for tight-binding inhibitors were used, the inhibition was shown to be competitive. Most new inhibitors of TS and of glycinamide ribonucleotide formyltransferase (GARFT) are sufficiently potent that tight-binding kinetic analysis is also appropriate in these cases. However, many investigators continue to report inhibition constants obtained with conventional kinetic analysis. These reported K_i values are not in fact constants, but will depend upon the concentration of enzyme used in the assay system, since for a tight-binding inhibitor the apparent K_i will approximate [E]/2.

Having grasped the tight-binding nature of inhibition of DHFR by methotrexate, some investigators then made the opposite error and assumed that it was virtually irreversible. In fact, MTX has an off rate constant from human DHFR that corresponds to a half-life for the complex of approx 15 min. In a cell-free system in which the concentration of the competing substrate, dihydrofolate (actually as polyglutamates), may be very low, a newly dissociated molecule of MTX may rapidly rebind to DHFR, so that it may appear that the inhibition is functionally irreversible. However, in the cell the system shows more complex behavior: DHFR activity is often in 10- to 50-fold excess over that of TS, which is the ratelimiting enzyme in the cycle of dihydrofolate oxido-reduction (DHFR, serine hydroxymethyltransferase, TS). Thus the steady-state concentration of dihydrofolate is very low, typically below 0.1 μM. When DHFR is inhibited, dihydrofolate is now being produced faster than it is re-reduced, so that dihydrofolate accumulates until the flux through the three enzymes of the cycle again becomes equal. In this way, the dihydrofolate concentration may increase by as much as three logs, at which point it represents a significant fraction of total cellular folate cofactors. At some point, the system can no longer generate enough additional dihydrofolate to overcome the DHFR inhibition, DHFR then becomes rate limiting for the cycle, and the flux through the cycle will fall. The pool size of methylenetetrahydrofolate polyglutamates will now be depleted, and biosynthesis of thymidylate and of purine ribonucleotides will decrease. The kinetics of the system are such that by the time this point is reached, there will be free MTX (i.e., MTX that is not bound to DHFR) in the cell. In early studies it

was sometimes concluded that the need for free MTX to be measurable in the cell before a growth-inhibitory effect was observed indicated that there must be a second site of action of MTX, other than DHFR, that was required for its pharmacological effect. Computer simulation of the biochemical pathways showed that the observed kinetics were consistent with inhibition of DHFR being the primary site of action of MTX, and that the necessity for free MTX was an inherent consequence of the kinetics of this cyclic multienzyme system.

4. TRANSPORT AS A DETERMINANT OF SELECTIVITY AND RESISTANCE

The early antifolates that were developed as anti-infective drugs (e.g. pyrimethamine, trimethoprin) were lipophilic compounds that entered cells by passive diffusion. This is an advantageous property since some microorganisms are unable to transport folic acid and its analogs. However, the early anticancer folate analogs, such as MTX are polyanions, and thus require facilitated or active transport to get across cell membranes. It was observed that some transformed cells (and embryonic cells in general) tended to have relatively high rates of folate and antifolate transport, and it was thus suggested that transport was a determinant of antifolate selectivity. Studies with mouse tumors indicated that experimental mouse leukemias often had high levels of MTX transport, and were MTX-sensitive, whereas mouse carcinomas frequently were relatively inefficient at transporting MTX, and tended to be MTX-insensitive. It was thus widely believed for a time that antifolates should be regarded as antileukemic drugs, without much potential against solid tumors. One line of approach to developing more active DHFR inhibitors was to optimize transport parameters (to increase V_{max} and decrease K_m) for the reduced folate carrier, and this was one of the principles that guided development of 10-ethyl-10-deazaaminopterin, which did indeed possess better activity than MTX against murine carcinomas *(4)*. A second line of evidence that transport was a major determinant of the antifolate response of tumors was the observation that acquired resistance of tumor cells to MTX was frequently associated with decreased MTX transport. The current view is that, although the selectivity of antifolates and cellular resistance to them are multifactorial, transport is an important determinant of therapeutic effect and toxicity. This subject is discussed in detail in Chapter 14. Another view is that transport is not necessary for antitumor selectivity, since lipophilic antifolates such as trimetrexate and Thymitaq have shown clinical anticancer activity, but that being a good substrate for the carrier confers potency upon a drug.

This topic was made more interesting, and more complex, by the discovery that in addition to the high capacity, low-affinity carrier that transports MTX, leucovorin, and 5-methyltetrahydrofolate (the reduced folate carrier, or RFC) some tissues possessed a high-affinity, low-capacity membrane folate-binding protein (mFBP) whose physiological function appeared to be binding and uptake of folic acid. When lometrexol was developed, it was found to be an excellent ligand for mFBP, and a subject of ongoing research is whether this contributes to lometrexol's broad antitumor spectrum, whether it contributes to lometrexol's severe delayed toxicity, and whether mFBP binding is a desirable attribute for an antitumor drug or not. The discovery (discussed in Chapter 13) of closely related compounds with very different affinity for mFBP will help to resolve these questions.

5. HOMOGENEOUSLY STAINING REGIONS, DOUBLE MINUTES, AND GENE AMPLIFICATION

Early studies on resistance of tumor cells to methotrexate elicited two frequent mechanisms: transport defects as discussed above, and overproduction of the target enzyme, DHFR. Whereas in principle enzyme overproduction might be achieved by increasing the expression level of the DHFR gene, it was found that MTX-resistant cells often had multiple copies of the DHFR gene. Gene amplification is an aspect of the genetic instability of transformed cells, and has been reported for several other enzymes, including TS. The additional genetic material may either occur as pairs of small additional chromosomes (double minutes) or as a large piece of extra DNA in one of the normal set of chromosomes, referred to as a homogeneously staining region (HSR). In cells that have extra DHFR gene copies, the expression level of active enzyme roughly parallels the gene copy number, and the amount of tight-binding inhibitor required to inhibit the enzyme is somewhat more than proportionately greater, so that a cell with a 10-fold gene amplification will have an IC_{50} that is increased by more than 10-fold relative to the wild-type. Kinetic analysis indicates that, regardless of whether an enzyme is normally rate limiting in its pathway or not, and regardless of whether the inhibitor exhibits conventional or tight-binding kinetics, increased expression of target enzyme will always tend to confer increased resistance to the inhibitor. Recent clinical studies that relate response rates to expression levels of target enzyme are discussed in Chapter 18 which reports that tumors with high levels of TS were less likely to respond to 5-FU than the subgroup with lower TS expression.

6. ANTITHYMIDYLATE AND ANTIPURINE EFFECTS OF MTX

An early debate concerned the issue of whether the antithymidylate effect or the antipurine effect of MTX was the primary cause of its antitumor effect. The work of Cohen *(2)* in bacteria, and of Borsa and Whitmore *(5)* with murine cells appeared to implicate thymidylate depletion as the primary cause of cytotoxicity, since addition of a purine to methotrexate-treated cultures decreased the amount of cytotoxicity. Opposing this viewpoint was the work of Hryniuk *(6),* who studied the L5178Y leukaemia in mice, and found that the primary lesion caused by MTX in this system was purine depletion. A possible explanation of this discrepancy was suggested by Jackman and colleagues *(7),* who found that mice have relatively high concentrations of circulting thymidine, whereas the plasma thymidine concentration is much lower in humans. As a result, mice tend to underpredict for the activity of TS inhibitors in humans; with DHFR inhibitors responses are seen in certain murine tumor systems, but it is likely that the effect in mice is primarily a consequence of purine depletion (as suggested by Hryniuk) rather than of thymidylate depletion as may be the case in humans.

The suggestion that the antitumor activity of DHFR inhibitors, at least in humans, is primarily an antithymidylate effect, and that the antipurine effect actually limits the degree of cytotoxicity, suggests that a pure TS inhibitor should be a more effective drug than a DHFR inhibitor. It is still not clear whether this is, in fact, the case and since this subject cannot be studied in mice it is difficult to design definitive in vivo experiments to address this question. What is clear is that antifolate drugs that have a pure antithymidylate effect (the TS inhibitors) or a pure antipurine effect (the GARFT in-

hibitors) have clinical anticancer activity, despite having different cell cycle effects. The present indications are that the effect of DHFR inhibitors in humans is primarily a consequence of thymidylate depletion.

7. POLYGLUTAMYLATION IN RELATION TO SELECTIVITY AND RESISTANCE

It has been known from the early days of folate biochemistry that cellular folate cofactors existed primarily as poly-gamma-glutamates, and that enzymes—initially termed conjugases, now more formally termed folylpolyglutamate hydrolases (FPGH)—existed in plasma and intestine that could hydrolyze these polyglutamates to the corresponding monoglutamates. It was subsequently shown that FPGH existed within most cells, as did the enzyme folylpolyglutamate synthetase (FPGS) that formed polyglutamates from folate cofactors, glutamate, and ATP. It then became clear that classical antifolates, as well as natural cofactors, existed as polyglutamates, and that MTX, for example, exerted most of its pharmacological effect as a polyglutamate. Antifolate polyglutamates are pharmacologically important for two reasons: In some cases the long-chain polyglutamates (e.g., heptaglutamate) may be hundreds of times more potent as enzyme inhibitors than the parent monoglutamate (though this is not generally the case with DHFR inhibitors), and secondly, since polyglutamates above diglutamate cannot readily efflux from cells, they represent a long-acting cellular repository of drug, and have a profound effect on the cellular pharmacokinetics of the antifolate drug. Thus all classical antifolates (i.e., drugs that are close structural analogs of natural folates, and that have a glutamate function) must be regarded as prodrugs, requiring cellular activation by FPGS to exert their full effect. It follows from this that if the FPGS activity of a tumor cell is decreased, or if its active site is mutated in a way that decreases the substrate affinity of the antifolate, some degree of drug resistance will result. This subject is treated in detail in Chapter 16. It also follows that the FPGS activity of a tissue will be a major determinant of drug selectivity for a classical antifolate drug. Cancer cells, and embryonic tissues, tend to have high activity of FPGS, and this probably contributes to the antitumor selectivity and teratogenic activity of classical antifolates. Another consequence of polyglutamylation of classical antifolates is to increase their potency, since the trapping effect within the cell of polyglutamylation greatly increases the area under the curve (AUC, the concentration $\times$ time integral) of the drug.

It was mentioned above that some enzymes are more sensitive than others to the degree of polyglutamylation of their reduced folate cofactors, or to that of antifolate inhibitors. DHFR appears to be almost indifferent to polyglutamate chain length, and TS is also relatively insensitive to chain length. GARFT appears to have a marked preference for longer chain length, with cofactors or inhibitors increasing in binding affinity up to a chain length of seven glutamates. 5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT) is even more affected by chain length. These differences have given rise to speculation that folate cofactors may be functionally compartmented within the cell according to their number of glutamate residues. There does not appear to be any strong evidence for this; however, in the case of an antifolate that inhibits, for example, both GARFT and AICARFT, it is probable that the latter effect may become relatively more important as the polyglutamate chain length increases,

so that the partitioning of the inhibitor between its two targets may change with increasing chain length.

From the drug-design perspective, susceptibility to polyglutamylation by FPGS has generally been considered a positive attribute, endowing a molecule with some degree of antitumor selectivity, with long-acting cellular pharmacokinetics, and with high dose potency. However, it confers vulnerability to an additional mechanism of drug resistance. A few counter-examples are emerging that suggest we may sometimes have too much of a good thing: The long-chain polyglutamates of lometrexol appear to turn over so slowly (if at all) that the drug is effectively permanently trapped within the cell. It is possible that the severe, delayed thrombocytopoenia caused by lometrexol may be related to too-effective retention of its polyglutamates within megakaryocytes. Perhaps analogs of lometrexol whose polyglutamates are better substrates for FPGH may be safer drugs.

8. LIPOPHILIC ANTIFOLATES

Having made the case that transport and polyglutamylation of classical antifolates contribute to their antitumor selectivity, one must question whether the effort to develop nonclassical, lipophilic antifolates has been fundamentally misdirected. In the anti-infective arena, the rationale is clear: Many microorganisms cannot transport folate-like molecules, so that the lipophilic nature of the antimalarial, pyrimethamine, or the antibacterial, trimethoprim, was a desirable, even essential, attribute. But what is the justification for the development of the lipophilic anticancer DHFR inhibitor, trimetrexate, or the lipophilic anticancer TS inhibitor, Thymitaq? The development of trimetrexate was certainly influenced by the observation that mouse carcinomas tend to transport folates poorly, and tend to be relatively insensitive to MTX. Broome et al. *(8)* showed that the M5076 murine sarcoma, and several murine colon carcinomas, were responsive to trimetrexate but not to MTX. Thus, for murine solid tumors, avoiding the necessity for facilitated transport seems to confer a broader antitumor spectrum to a drug. It is not at all clear whether this argument can be directly extrapolated to human solid tumors, since some of these are undoubtedly responsive to MTX and to other classical antifolate drugs. However, it seems likely that there is a subset of human solid tumors with relatively low activity of the RFC, and against such cancer cells a lipophilic antifolate should be a better drug. In theory, lipophilic antifolates should have a broader spectrum than classical antifolates; against mouse tumours, there is considerable evidence that this is the case. Clinically, there are suggestions that lipophilic inhibitors of DHFR or TS may have a different antitumour spectrum than their classical counterparts. A price is paid for this putative increase in spectrum, in two ways: Since transport and polyglutamylation contribute partially to antitumor selectivity, removing these two factors could make lipophilic antifolates less selective, i.e., more toxic. This is an example of the drug designers' maxim that broader spectrum is usually bought at the price of greater toxicity. Second, comparing trimetrexate with MTX, or Thymitaq with Tomudex, it is clear that removing the capacity for polyglutamylation results in a marked loss of dose potency.

One special case in which lipophilic drugs will clearly have an advantage as antitumor agents is when a tumor has acquired resistance to a classical antifolate though a deletion either of transport or of FPGS, both established mechanisms of resistance of hu-

man cancer cells to classical antifolates. On the down side, lipophilic antifolates are themselves vulnerable to some resistance mechanisms that do not affect response to classical antifolates, notably the p170 glycoprotein-mediated form of multidrug resistance. The fact is that the target mechanism of action of a drug is only one of its determinants of activity, and cellular pharmacokinetic factors—routes of cellular uptake and cellular activation and retention, are at least as important as the nature of the molecular target in determining a drug's properties. The lipophilic antifolates are very different drugs from their classical counterparts, with differences in spectrum, toxicity profiles, and susceptibility to resistance. So far as antitumor selectivity is concerned, the nonclassical antifolates undoubtedly sacrifice the contributions to selectivity that could be made by exploiting a tumor cell's generally high activity of RFC and FPGS. However, the major determinant of the antifolate drugs' anticancer selectivity is probably the changes in cell-cycle control in transformed cells that make a cancer cell more likely to respond to depletion of thymidylate or purine by triggering apoptosis; a glutamate sidechain is not required to take advantage of this.

Finally the work of Allegra and his colleagues *(9)* in developing trimetrexate as an agent for treatment of *Pneumocystis carinii* pneumonia deserves mention as an elegant example of biochemical modulation. The fungus that causes this infection does not transport reduced folates, but the lipophilic molecule trimetrexate is able to enter cells of both the fungus and the host. The trimetrexate treatment is then followed by leucovorin, which rescues the host cells, but not those of the fungus, which it is unable to penetrate.

9. INHIBITORS OF GLYCINAMIDE RIBONUCLEOTIDE FORMYLTRANSFERASE

There are numerous antimetabolites that inhibit purine biosynthesis, and some of them have anticancer activity. Until recently these drugs fell into two classes: Several were analogs of purine bases or nucleosides (e.g. 6-mercaptopurine, methylmercaptopurine riboside, MMPR). Although these compounds (or their nucleotide derivatives) are often inhibitors of *de novo* purine biosynthesis (the 5'-phosphate of MMPR, for example, is a potent inhibitor of PRPP amidotransferase), their pharmacological effects are complicated by their incorporation into RNA or DNA or both, so that it is impossible to determine whether their therapeutic and toxic effects are a direct consequence of purine depletion. The second class of antipurines is the glutamine antagonists, such as azaserine, diazooxonorleucine, and acivicin; since two enzymes of the purine *de novo* pathway are glutamine-dependent amidotransferases, these compounds are certainly antipurine agents. However, in this case the interpretation of their effects is complicated by the fact that these glutamine analogs inhibit numerous other glutamine-requiring enzymes. The development of lometrexol (5,10-dideazatetrahydrofolate), which was shown to act on GARFT was thus of great interest as the first antimetabolite that unequivocally acted through depletion of purine ribonucleotide formation. Lometrexol and other GARFT inhibitors clearly had experimental antitumor activity in mice, and were active against tumors that were unresponsive to MTX. However, there was a debate as to whether GARFT inhibitors were cytotoxic or cytostatic. More recent work *(10,11)* indicates that GARFT inhibitors are indeed cytotoxic to certain cell lines, but take much longer to kill cells (72 or more) than do inhibitors of TS. Moreover, the GARFT inhibitor AG2034

was only cytotoxic at low concentrations of folic acid; in standard tissue-culture medium, containing 2.2 μ*M* folate, it was cytostatic. Also, AG2034 was much more cytotoxic to cells that lacked a functional G1 checkpoint; in cells that had a functional checkpoint, purine starvation appeared to induce a G1 cell-cycle block in which cells could remain arrested for many days without losing viability.

Despite showing promising preliminary clinical anticancer activity, lometrexol's usefulness was compromised by severe delayed thrombocytopoenia; for a discussion of this, and of the use of folic acid supplementation to prevent it, *see* Chapter 12). The biochemical basis for the thrombocytopoenia is still under investigation. Since platelets have a higher requirement for ATP than any other cell of the body, it seems likely that it is an antipurine effect. It is delayed because of the long maturation time of megakaryocytes (which is probably further extended under conditions of purine shortage), and it may be irreversible because the polyglutamate forms of lometrexol turn over so slowly that, once formed, they are effectively impossible to eliminate.

Whether these disadvantages of lometrexol can be designed out may be clarified by studies with two newer GARFT inhibitors; these compounds are reviewed in Chapters 12 and 13. It seems clear, however that GARFT inhibitors have clearly distinct properties from those of TS inhibitors, and as a class they have therapeutic potential, and perhaps the unusual property of selectivity against p53-defective cells.

10. ANTIFOLATES IN COMBINATION CHEMOTHERAPY

The most useful applications of antifolates as anticancer treatments involve using the antifolate as part of a combination chemotherapy regimen; a well-known example is the combination of methotrexate with cytoxan and 5-fluorouracil (CMF) for treatment of breast cancer. This topic is discussed in Chapter 17, and the present chapter will be confined to a couple of general observations on the design of antifolate-containing combinations.

Several investigators have explored the combination of DHFR inhibitors with inhibitors of TS, sometimes under the impression that inhibition of sequential sites in a cyclic pathway results in an inherently synergistic combination. In fact, this combination when studied (e.g. with Thymitaq and methotrexate) in cell culture generally results in additivity. Theoretically, such combinations may give interactions that are synergistic, additive, or antagonistic *(12,13)*. If the TS inhibitor is 5-fluorouracil (5-FU), the situation is complicated by the fact (first shown by Bertino, ref. *14*) that DHFR inhibitors cause accumulation of PRPP within cells, which results in more efficient conversion of 5-FU to its active TS-inhibiting metabolite, 5-FdUMP, so that MTX and other DHFR inhibitors generally do make 5-FU (but not antifolate TS inhibitors) more effective.

One kind of antifolate combination that gives a very marked synergistic interaction is the use of a lipophilic DHFR inhibitor (e.g., trimetrexate) in combination with a classical antifolate that inhibits TS, GARFT, or AICARFT. The very pronounced synergism observed with this kind of combination is referred to as the "Kisliuk effect," after the investigator who first demonstrated the phenomenon in bacteria. The mechanism of the effect has been elucidated by Galivan, Greco, and others *(15,16)*. Briefly, trimetrexate depletes cellular levels of tetrahydrofolate polyglutamates, and results in formation of much higher levels of polyglutamates of the classical antifolate drug than would otherwise be the case. This postulated mechanism is supported by the fact that the Kisliuk ef-

fect is not seen in mutant cells that lack FPGS, and also that trimetrexate does not potentiate the effect of nonclassical inhibitors of TS or GARFT. So far, most studies of the Kisliuk effect have been in vitro, but trimetrexate has been shown to be synergistic with the classical GARFT inhibitor, AG2034, against a number of in vivo tumor models, and there is great interest in exploring the therapeutic utility of this class of antifolate combinations.

Another kind of combination regimen that frequently gives therapeutic synergism is the use of an antifolate with a DNA-damaging drug. The CMF combination is an example of this kind. In this case, it does not seem to matter whether the antifolate component of the combination is classical or lipophilic, and synergism is obtained with several kinds of DNA strand-breaking drugs. Two possible explanations have been advanced for this synergistic interaction. Since antifolates deplete cellular pools of deoxyribonucleoside 5'-triphosphates (dNTP), it has been proposed that this inhibits repair of the DNA damage. However, in some cases the synergism seems to be greatest when the antifolate precedes the DNA-damaging agent, which seems inconsistent with potentiation of DNA damage by repair inhibition. In this case it has been proposed that antifolates may have a cell-synchronizing effect that maximizes the number of tumor cells in the part of the cell cycle where they are most sensitive to DNA damage. The study of cell-cycle changes induced by TS inhibitors has been facilitated by the availability of Thymitaq, which can be rapidly washed out of cells, so that the timing of cell-cycle perturbations can be precisely correlated with drug effects. It is possible that combinations of antifolates with DNA strand-breaking drugs draw their efficacy from a combination of these two mechanisms. In any event, further mechanistic studies of these effects should make possible the design of optimal clinical combinations of this type.

11. DNA STRAND BREAKS, APOPTOSIS, CHECKPOINTS, AND ANTIFOLATE SELECTIVITY

Antifolates deplete cellular pools of thymidylate or purines or both, they cause cell-cycle arrest, which may or may not be followed by programmed cell death (apoptosis); and in the case of inhibitors of DHFR and of TS, but not GARFT, they cause DNA strand breaks. The relationship of these various effects to drug efficacy and selectivity, and the extent to which antifolate selectivity is mediated by altered cell-cycle checkpoint function in cancer cells, is one of the hottest topics in the current investigation of antifolate drugs. In certain cell lines, inhibitors of DHFR and of TS cause an imbalance in the ratio of dTTP to dUTP (largely mediated by release of dCMP deaminase from feedback inhibition by dTTP) that results in misincorporation of uracil into DNA. Uracil bases are excised, resulting in apyrimidinic sites, and a futile cycle of misincorporation and misrepair results that can lead to DNA strand breaks and irreparable DNA damage. The extent to which this process is a primary cause of cell death in antifolate-treated cells is discussed in Chapter 20. Do these DNA strand breaks result in a cell-cycle block by the p53-associated checkpoint mechanism? In the case of inhibitors of DHFR and TS, this seems possible, and there is evidence that cells that lack a functional p53 checkpoint are less sensitive to these antifolates than cells that have such a checkpoint. However, some flow-cytometric studies seem to indicate that TS inhibitors cause arrest in very early S-phase, rather than at the G1:S boundary, as seen with DNA-damaging agents. This is what would be expected if the cells are arrested because of lack of dTTP for DNA syn-

thesis, rather than because unrepaired DNA damage has triggered the G1 checkpoint. After this cell-cycle arrest has been sustained for a certain period of time (often approx 12h), the arrested cells move into apoptosis. Some cells are more apt to do this than others, so that, for example, bcl-2 overexpression makes cells less subject to antifolate-induced apoptosis (*see* Chapter 22). The expression or not of those genes that make cells sensitive to programmed cell death are thus important discriminants of response to antifolates.

GARFT inhibitors kill cells much more slowly than do inhibitors of TS or of DHFR *(10)*. Unlike TS and DHFR inhibitors, GARFT inhibitors do not cause DNA strand breaks within 24h of drug treatment (though DNA breaks appear much later when the purine-starved cells undergo apoptosis). Unlike inhibitors of TS and DHFR, which seem to block cell-cycle progression in early S-phase, GARFT inhibitors block in late G1, or at the G1:S transition. This is consistent with the metabolite depletion hypothesis of Wahl and his collaborators, who claim that depletion of cellular ribonucleotide pools can trigger the p53-dependent G1 checkpoint in the absence of DNA strand breaks *(17)*. The cells that are arrested in this way by the GARFT inhibitor AG2034 do not die, at least for several days, but remain arrested, and can move back into the cell cycle if a purine source is added to the medium. However, this nonlethal arrest state appears to require a functional G1 checkpoint: Cells that lack such a checkpoint, when treated with AG2034, progress slowly through S-phase, and die, either in S-phase or in G2 (*see* Chapter 13). Thus p53-competent cells are less sensitive to GARFT inhibitors than p53-defective cells; this pattern of selectivity is the reverse of the situation for inhibitors of TS and DHFR.

12. CONCLUSIONS: WHERE NEXT WITH ANTIFOLATES?

The antifolate field remains a rich source of new drugs, new mechanistic approaches, and new therapeutic ideas. Investigators in this field have a wide choice of compounds to study, including potent, selective inhibitors of DHFR, TS, GARFT, and AICARFT, as well as compounds that inhibit more than one of these targets to varying degrees. We can choose between classical antifolates that are subject to facilitated transport and to polyglutamylation, lipophilic compounds that are neither transported nor polyglutamylated, or compounds that require transport but are not polyglutamylated (as well as some intermediate situations such as that of GW1843, which is converted to a diglutamate, but no further; *see* Chapter 9).

Many questions remain, some of which can only be answered by extensive clinical trials. Are selective TS inhibitors, such as Tomudex, in fact better drugs than 5-FU? Can novel GARFT inhibitors reproduce the clinical activity of lometrexol without its serious delayed toxicity? Will selective AICARFT inhibitors show antiarthritic activity; as may be the case if the hypothesis of Cronstein (*18*) is correct, that this enzyme is the target for the anti-inflammatory activity of methotrexate?

Studies to date have clearly established the major influence of cellular pharmacokinetics on drug efficacy, selectivity, and potency, but many interesting questions remain unanswered: would a classical antifolate that was transported exclusively by the mFBP have a radically different spectrum from an otherwise similar compound that was transported exclusively by the RFC? Would drug polyglutamates that are good substrates for FPGH be less toxic without compromising potency and selectivity? Are there circum-

stances in which the rapid loss of a lipophilic antifolate from the cell could be turned to therapeutic advantage, e.g., by giving a tightly synchronized target cell population whose response to a second agent acting later in the cell cycle would thus be optimized? The availability of a group of inhibitors designed against a set of related target molecules, and with a wide range of physicochemical and pharmacokinetic properties, provides us with the opportunity to tailor chemotherapy rationally against tumors with particular biochemical profiles. Antifolates are thus a class of drugs in which the prospects for designing more efficacious and more selective chemotherapy remain unusually promising.

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2 Folate Biochemistry in Relation to Antifolate Selectivity

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CONTENTS

1. INTRODUCTION

This review will deal with advances in folate biochemistry related to antifolate toxicity and selectivity. Because of the interrelatedness of reactions of folate metabolism, alterations in the activity of any folate enzyme, cellular transport system, as well as the concentration of any folate metabolite may be relevant to antifolate cytotoxicity and selectivity. Therefore, it is difficult to predict the results of inhibiting a given folate enzyme on antifolate selectivity. For example, in many experimental systems, the *cytotoxicity* of methotrexate is caused by its ability to inhibit dihydrofolate reductase, resulting

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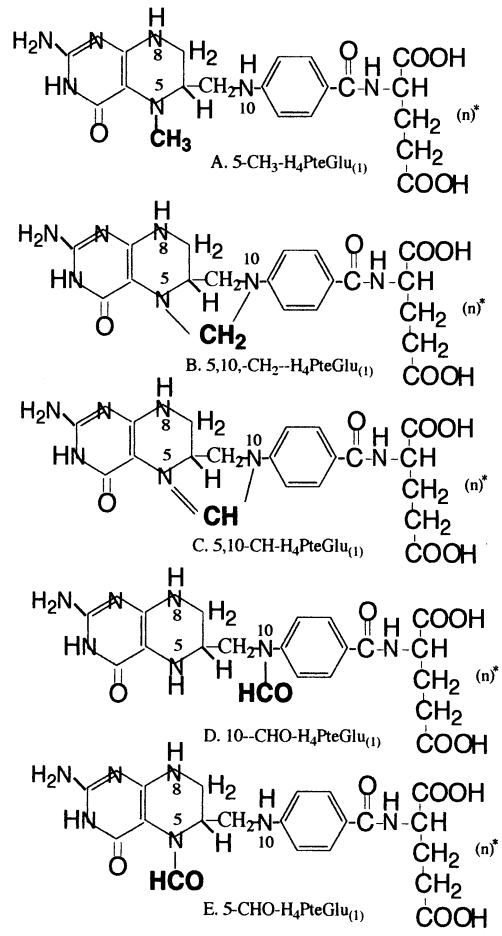

Fig. 1. Structures of tetrahydrofolic acid (THF) derivatives: **(A)** 5-methylTHF; **(B)** 5,10-methyleneTHF; **(C)** 5,10-methenylTHF; **(D)** 10-formylTHF; **(E)** 5-formylTHF (also called folinic acid, leucovorin or citrovorum factor). Pte stands for pteroic acid (p-[(2-amino-4-oxy-6-pteridyl-methyl)amino]benzoic acid).*n refers to the total number of glutamate residues attached to pteroic acid. All additional Glu residues are joined by amide bonds to the γ-carboxyl group of Glu(1).

in lowered thymidylate formation, leading to lethal defects in DNA. However, its *selectivity* is often dependent on differential cellular uptake and polyglutamylation. Favorable clinical results with aminopterin, the forerunner of methotrexate, in acute leukemia in children were reported by Farber et al. *(1)* in 1948. This work depended on knowledge generated at the American Cyanamid Company, Pearl River, NY, on the structure of folic acid and the chemical synthesis of analogs in addition to the insightful clinical observations of the Farber group *(1)*. This work was done before the role of tetrahydrofolates in the metabolism of single carbon units was known. The present discussion of the current literature on folates is offered in the hope that, given the powerful analytical, structural, molecular genetic, and synthetic methods now available, new approaches to selective toxicity can be generated.

We focus on the metabolic interconversions and enzymology of three areas of folate metabolism, areas related to the essential metabolites methionine, thymidylate, and purine