

Resistance to antifolates

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The antifolates were the first class of antimetabolites to enter the clinics more than 50 years ago. Over the following decades, a full understanding of their mechanisms of action and chemotherapeutic potential evolved along with the mechanisms by which cells develop resistance to these drugs. These principals served as a basis for the subsequent exploration and understanding of the mechanisms of resistance to a variety of diverse antineoplastics with different cellular targets. This section describes the bases for intrinsic and acquired antifolate resistance within the context of the current understanding of the mechanisms of actions and cytotoxic determinants of these agents. This encompasses impaired drug transport into cells, augmented drug export, impaired activation of antifolates through polyglutamylation, augmented hydrolysis of antifolate polyglutamates, increased expression and mutation of target enzymes, and the augmentation of cellular tetrahydrofolate-cofactor pools in cells. This chapter also describes how these insights are being utilized to develop gene therapy approaches to protect normal bone marrow progenitor cells as a strategy to improve the efficacy of bone marrow transplantation. Finally, clinical studies are reviewed that correlate the cellular pharmacology of methotrexate with the clinical outcome in children with neoplastic diseases treated with this antifolate.

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Introduction

The antifolates were the first antimetabolites developed and brought into clinical use in the late 1940s (Farber *et al.*, 1948). While these agents, aminopterin and methotrexate (MTX), were known to be inhibitors of dihydrofolate reductase (DHFR), an enzyme that plays a key role in folate metabolism, a clear understanding of the mechanism of action of these drugs evolved only slowly and long after MTX became an established component of many clinical regimens. Indeed, it was not until the 1970s that

the kinetics of the interaction between MTX and DHFR was fully understood, and not until the late 1970s and early 1980s when polyglutamate derivatives of MTX were detected and their pharmacologic importance clarified. Likewise, an understanding of tumor cell resistance to antifolates evolved slowly, often paralleling the emergence of new molecular concepts. As the mechanisms of resistance to antifolates were characterized, this provided insights and principles that were broadly applicable to other antineoplastics. Ultimately, this knowledge led to the development of a new generation of antifolates, in the late 1980s and 1990s, which are potent direct inhibitors of tetrahydrofolate (THF)-cofactor-dependent enzymes. Several of these drugs are now in clinical trials, and the activity of one, pemetrexed, has been confirmed in a large Phase III trial (Vogelzang *et al.*, 2003).

Over the past 45 years, an understanding of the cellular pharmacology of antifolates also evolved slowly, encompassing in particular the mechanisms of transport, a critical determinant of activity and resistance. Indeed, it was only in 1989 that membrane folate-binding proteins that mediate transport by endocytosis were cloned, and in the mid-1990s that a major folate/antifolate transporter, the reduced folate carrier (RFC), was cloned. Moreover, new transport routes for antifolates continue to be discovered – most recently, the family of multidrug resistance-associated proteins (MRPs) that export antifolates from cells.

To understand the mechanisms of resistance to antifolates requires a clear understanding of: the mechanisms by which these drugs are transported into and out of cells and concentrated within cells; the kinetics of the interactions between drugs and their target enzymes and the enzymes that mediate their polyglutamation and deglutamylation in cells; and the impact of physiological cellular folate pools on these processes. This review will analyse drug resistance within this context. It will focus on processes that are perturbed and that alter the cellular pharmacology of these drugs and their target enzymes. It will cite the most important literature that has elucidated these phenomena.

Metabolism of folates, and the interactions among folates, antifolates, and folate-dependent enzymes in cells

Interconversions of folate-cofactors in cells and the role of these cofactors in biosynthetic processes

Folates are a class of physiological substrates present in mammalian cells at concentrations in the range of 10^{-8}

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to $>10^{-5}$ M (Jackson and Harrap, 1973; Moran *et al.*, 1976; White and Goldman, 1976; Seither *et al.*, 1989). While folic acid is the most familiar term for this family of vitamins, folic acid is an oxidized form of folate that is reduced by bacteria in the gut and is not present in significant amounts in the blood or cells of humans or rodents. Folic acid is, however, the major formulation available as a dietary supplement because of its chemical stability. In fact, the predominant plasma folate is 5-methyl tetrahydrofolate (5-CH₃-THF), and cellular folates consist of this and other THF-cofactors that carry a one-carbon group at different oxidation states at the N5 or N10 positions, or shared by both nitrogen atoms. These THF-cofactors play a key role in the provision of one-carbon moieties for a variety of biosynthetic reactions within cells (Stokstad, 1990). Hence, as indicated in Figure 1: (i) 5,10-methylene tetrahydrofolate (5,10-CH₂-THF) provides a carbon for the synthesis of thymidylate from deoxyuridylate mediated by thymidylate synthase (TS), an initial step in the synthesis of DNA. (ii) 10-formyl tetrahydrofolate (10-CHO-THF) provides two carbons for the synthesis of the purine ring in reactions mediated by phosphoribosylaminoimidazolecarboxamide transformylase (AICARFT)- and glycinamide ribonucleotide transformylase (GARFT). (iii) 5-CH₃-THF provides a methyl group for the vitamin B-12-dependent synthesis of methionine from homocysteine mediated by methionine synthase, which is followed by the synthesis of S-adenosylmethionine. To the extent to which the products of these reactions (i.e. thymidine, purines) are not available in the blood for delivery into cells, these THF-cofactor-dependent reactions are critical to the survival of mammalian cells.

When a carbon group is utilized in the synthesis of purines or methionine with the generation of THF, the

THF-cofactor pool is rapidly replenished through the reaction of THF with a formate molecule to regenerate 10-CHO-THF mediated by formyl THF synthetase, or serine donates a carbon to THF to form 5,10-CH₂-THF and glycine, a reaction mediated by serine hydroxymethyltransferase. On the other hand, in the synthesis of thymidylate, 5,10-CH₂-THF is oxidized to dihydrofolate (DHF), and DHFR is required for the regeneration of THF. The critical role of DHFR is reflected by its high level ($\sim 1 \mu\text{M}$) in tumor cells (Goldman *et al.*, 1968). When DHFR activity is abolished, THF-cofactors rapidly interconvert to 5,10-CH₂-THF that, in turn, is rapidly oxidized to DHF. This is followed by a rapid decrease in THF-cofactors that, while often incomplete (this is considered in more detail below), is associated with the cessation of THF-cofactor-dependent reactions within a few minutes (White and Goldman, 1976; Seither *et al.*, 1989). It is this crucial role of DHFR that has made it an attractive target for the development of chemotherapeutics and is the basis for the efficacy of MTX and other 4-amino antifolates.

Polyglutamation of antifolates – a key determinant of antifolate activity

Folates in the blood (primarily 5-CH₃-THF) are present as monoglutamates. However, virtually all cellular folates exist as long-chain polyglutamate derivatives (Baugh *et al.*, 1973; Moran *et al.*, 1976). In this biochemical transformation mediated by folylpolyglutamate synthetase (FPGS), glutamate molecules are added one by one to the γ -carboxyl acid moiety of the pteroylglutamate molecule. This change results in the retention of these polyglutamyl derivatives in cells, because these congeners are not substrates for folate transport systems (McBurney and Whitmore, 1974;

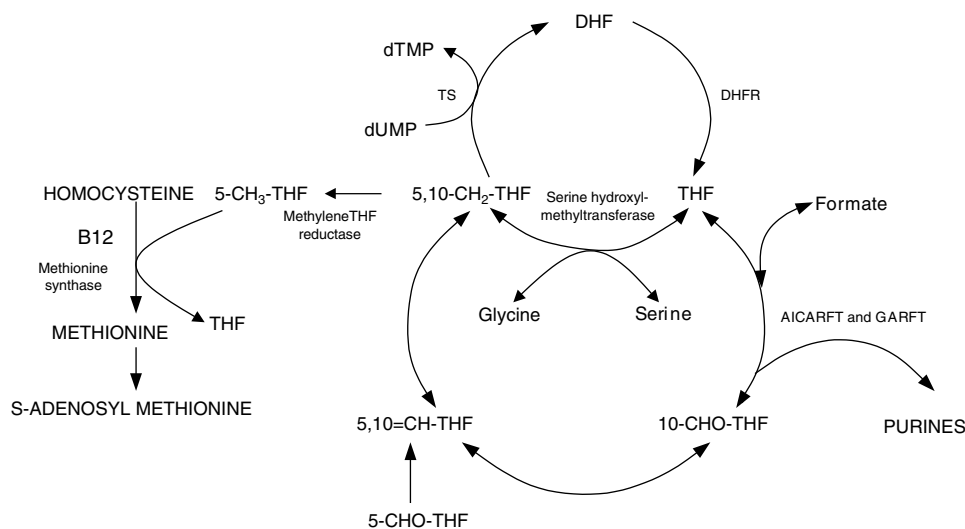


Figure 1 Biochemical interconversions of the folates, their role in folate-dependent metabolic reactions. The abbreviations are: DHFR, dihydrofolate reductase; TS, thymidylate synthase; GARFT, glycinamide ribonucleotide transformylase; AICARFT, phosphoribosylaminoimidazolecarboxamide transformylase; 5-CH₃-THF, 5-methyl tetrahydrofolate; 5-CHO-THF and 10-CHO-THF, 5-formyl- and 10-formyl tetrahydrofolate, respectively; 5,10-CH₂-THF, 5,10-methylene tetrahydrofolate; 5,10-CH-THF, 5,10-methenyl tetrahydrofolate; DHF, dihydrofolate; THF, tetrahydrofolate; dTMP, deoxythymidylate; dUMP, deoxyuridylate

Moran *et al.*, 1976). The polyglutamyl derivatives of most, but not all, natural folates are better substrates than their monoglutamyl form for THF-cofactor-requiring enzymes, due to an increase in V_{max} , a fall in K_m , or changes in both parameters. These are the active forms of folate-cofactors in cells (Schirch and Strong, 1989). In the case of MTX, only a fair substrate for FPGS, polyglutamyl forms have the same, or only a small increase in, affinity for DHFR (Whitehead, 1977; Domin *et al.*, 1982; Appleman *et al.*, 1988; Kumar *et al.*, 1989). MTX polyglutamates accumulate at different rates in a variety of tumor cells and ultimately become the predominant species and the form of antifolate bound to DHFR (Whitehead, 1977; Rosenblatt *et al.*, 1978a; Schilsky *et al.*, 1980; Matherly *et al.*, 1983). The major impact is the retention and buildup of appreciable levels of polyglutamyl derivatives of the drug in cells (Fry *et al.*, 1982b; Jolivet *et al.*, 1982; Jolivet and Chabner, 1983) with sustained inhibition of DHFR (Rosenblatt *et al.*, 1978b). The level and rapidity of formation of MTX polyglutamates in tumor cells is an important determinant of the pharmacologic activity of this drug (Fabre *et al.*, 1984; Curt *et al.*, 1985). For other antifolates, this biochemical transformation results not only in retention but also in a marked increase in affinity for their target enzyme(s). Agents that form polyglutamate derivatives can be administered in protocols with long intervals (i.e. weeks) between dosing, due to the prolonged retention of these congeners. On the other hand, for antifolates that do not form polyglutamates and are cleared rapidly from cells, such as AG337, ZD9331 (TS inhibitors), or trimetrexate (DHFR inhibitor), optimum activity requires frequent administration (i.e. daily, infusions, etc) (Jackson *et al.*, 1984; Webber *et al.*, 1996; Jackman *et al.*, 1997).

The rate and extent of polyglutamation in cells is determined by a variety of factors. First, the folate must be transported into the cell and a free component generated, the substrate for FPGS. The concentration of free monoglutamyl folate is critical, as polyglutamates accumulate and feedback inhibit FPGS (Andreassi and Moran, 2002). Natural folates utilize this enzyme to add glutamate residues; and the higher their level is in cells, the lower is the rate of polyglutamylation of the monoglutamate that is present at much lower concentrations. At some point, the level of polyglutamates in cells is high enough to suppress the entry of monoglutamates into the polyglutamate pool completely. At that point, the THF-cofactor pool is at steady state (Andreassi and Moran, 2002); the rate of monoglutamyl glutamylation is equal to the rate of hydrolysis mediated by gamma glutamyl hydrolase (γ -GH) and the very low rates of leakage from cells. The steady-state level varies among different tissues. For instance, tumor cells utilize folates rapidly as they divide. Other tissues, such as liver and kidney, serve mainly as storage depots for folates. FPGS is found in two isoforms. The higher polyglutamates are weaker inhibitors of the FPGS isoform found in the liver and kidney than the isoform expressed in murine L1210 leukemia cells (Andreassi and Moran, 2002). This may be an important basis for of the high

levels of folates that accumulate in the liver and kidney. Similarly, the cellular THF-cofactor pool is also an important determinant of the polyglutamation of antifolates, limiting the rate and extent of formation of these active derivatives. This is an important determinant of antifolate activity and is considered in detail in the section impact of alterations in cellular THF-cofactors.

Nature of the interaction among folates, antifolates, and DHFR in mammalian cells – understanding the mechanisms of action of 4-amino-folates

MTX and related 4-amino-antifolates are the most potent enzyme inhibitors within the armamentarium of pharmacologic agents used to treat human cancers. Indeed, the PT523 analog and some of its derivatives have affinities for DHFR that are an order of magnitude greater than that of MTX (K_i 's as low as 10^{-13} M (Rosowsky *et al.*, 2000; Wright *et al.*, 2000). As indicated above, DHFR plays a critical role in maintaining THF-cofactor pools at normal levels within the cells irrespective of the rate of synthesis of thymidylate. When studied in cell-free preparations, MTX inhibits DHFR with a K_i of ~ 5 pM (Domin *et al.*, 1982; Appleman *et al.*, 1988). The early view of drug action was that this agent is a 'stoichiometric' inhibitor of its target enzyme, that is, as the drug enters the cell, there is a mole-for-mole inhibition (titration) of the enzyme with a proportional decrease in activity until the enzyme is saturated and at that point catalytic activity is abolished (Werkheiser, 1961). According to this paradigm, only trivial amounts of free drug should be sufficient to abolish enzyme activity completely. While this formulation is essentially the case in cell-free systems, subsequent studies showed that the interaction of MTX with DHFR in cells is entirely different. When the level of drug in the cell is indistinguishable quantitatively from the DHFR-binding capacity, THF-cofactor pools are unchanged, and THF synthesis and THF-cofactor-dependent reactions are unperturbed. Rather, free drug levels in excess of $1 \mu\text{M}$, 5–6 orders of magnitude higher than the K_i for this enzyme, are required to suppress these processes completely (Goldman, 1974; Sirotnak and Donsbach, 1974; White *et al.*, 1975; White and Goldman, 1976).

The basis for the difference in the kinetics of inhibition of the isolated enzyme versus the enzyme in cells was clarified experimentally and with the application of computer modeling systems (Jackson and Harrap, 1973; Jackson *et al.*, 1977; White, 1979; White and Goldman, 1981). Unlike the cell-free system, folate metabolism in cells is a dynamic process in which the levels of THF-cofactors and DHF vary with the activities of enzymes and flows in this system. In the resting cell, DHFR activity is very high in comparison to TS activity. This maintains cell DHF at very low levels, that is, in the range of 10^{-8} M (Moran *et al.*, 1976). Since the DHF K_m for DHFR is 10^{-7} – 10^{-6} M (Domin *et al.*, 1982), this enzyme operates at a very low level of saturation; only a very small fraction of DHFR activity (<5%) is sufficient to sustain normal rates of THF

synthesis. As MTX enters the cell and binds to DHFR, DHF builds up behind the block to react with enzyme unassociated with the drug to maintain an unchanged rate of THF synthesis. As the DHF concentration increases several orders of magnitude to in excess of 10^{-5} M, it competes with MTX for the very small percentage of enzyme sites critical for sustaining THF synthesis. Owing to this, achieving complete suppression of this small fraction of DHFR requires levels of drug far in excess of the K_i . Inhibition is, in fact, competitive with DHF (Jackson *et al.*, 1977; White, 1979; White and Goldman, 1981).

Likewise, when intracellular MTX levels are sufficient to suppress THF synthesis completely (prior to the buildup of MTX polyglutamates), following which cells are placed in a drug-free environment, free drug rapidly leaves the cells at a time when DHF levels are high (the latter is present as polyglutamates that do not exit the cell). The ratio of DHF:MTX is high, and this results in net displacement of a small amount of MTX from DHFR by DHF as the drug cycles on and off the enzyme. Following this, free MTX leaves the cell and the enzyme is reactivated (Jackson *et al.*, 1977; White, 1979). As this occurs, DHF is consumed, normal rates of THF synthesis are restored, DHF returns to its usual very low levels, and the remaining MTX remains essentially irreversibly bound to $\sim 95\%$ of the enzyme, a level that cannot be distinguished experimentally from the total enzyme-binding capacity. This sequence of events, many of which are too minute to be measured, can be illustrated by computer simulations, one of which, based on a network thermodynamic model, is indicated in Figure 2. Hence, a highly potent enzyme inhibitor functions in cells as a rapidly reversible, competitive inhibitor of its target enzyme. The formation of MTX polyglutamates that occurs relatively slowly alters this interaction, because these congeners are not exported and build to high levels within the cell, changing the drug from a rapidly reversible to an essentially irreversible inhibitor of the target enzyme. Accordingly, important determinants of resistance to 4-amino antifolate inhibitors of DHFR are:

(i) *Increased DHFR expression*: The higher the level of DHFR the smaller is the fraction of enzyme required to sustain normal rates of THF synthesis, and the higher is the degree of saturation required to suppress this reaction (Jackson and Harrap, 1973; White and Goldman, 1981). For instance, under physiological conditions, if 5% of the enzyme is required to meet cellular demands for THF and if the DHFR level increases by a factor of 10, the degree of saturation required to suppress activity increases from 95 to 99.5%. Owing to the severe limitation on concentrative transport of MTX, the free levels of drug necessary to achieve this degree of inhibition are very difficult to achieve and require very high extracellular drug concentrations.

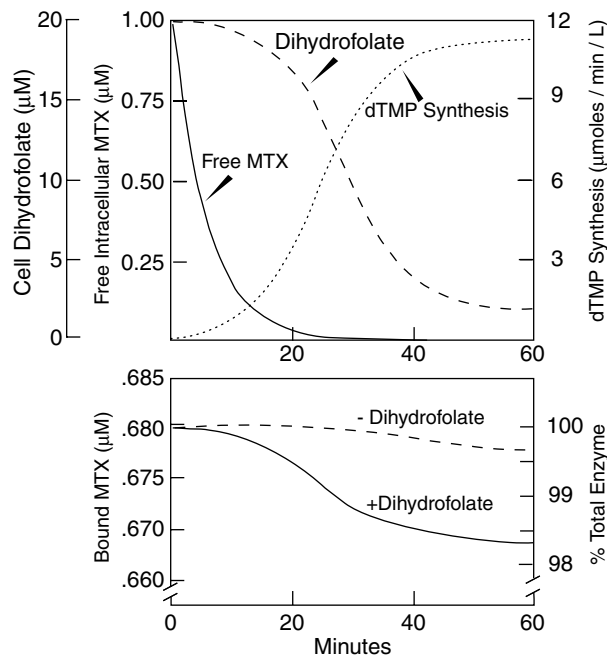


Figure 2 Computer simulations of the sequence of events that occurs when cells are exposed to sufficient MTX to suppress DHFR activity completely, following which the cells are separated and resuspended into a large volume of MTX-free medium at time zero. The upper panel simulates changes in the levels of free MTX, dihydrofolate, and the rate of dTMP synthesis. The lower panel indicates DHFR that is free or associated with MTX with an expanded ordinate scale that encompasses only 2% of the enzyme. At the time of resuspension, intracellular free MTX and DHF are high, cell THF is low, thymidylate synthesis (dTMP synthesis) is absent, and DHFR is saturated with MTX. Following resuspension, free MTX rapidly leaves the cell; and a series of events begin that result in the net displacement of only a trivial amount of MTX from DHFR, but a level sufficient to restore DHF to its usual low level and return cell THF and thymidylate synthesis to their usual rates. The simulation was created with a network thermodynamic model from White (1979)

- (ii) *Decreased affinity of DHFR for MTX*: Mutations in DHFR that decrease the K_i for MTX but retain sufficient activity for DHF substrate result in drug resistance.
- (iii) *Impaired membrane transport*: Transport mechanisms regulate the influx and efflux of drug across the cell membrane, and the net effect of these processes determines the level of free MTX achieved in the intracellular water. The free drug level is the critical parameter in the interactions between the drug and its target enzyme and between most 4-amino antifolates and FPGS. Alterations in membrane transport are a frequent basis for drug resistance.
- (iv) *Low rates of thymidylate synthesis*: The requirement for DHFR activity is based on the need to regenerate THF following the oxidation of 5,10- CH_2 -THF to DHF. When TS activity is low as in cells in G_0 , there is little need for DHFR and there is relative resistance to MTX. When TS activity is suppressed as with a fluoropyrimidine or one of the new-generation antifolates (ZD1694, ZD9331, or

pemetrexed), DHF generation is blocked and THF-cofactor pools are preserved. Under these conditions, inhibitors of DHFR have no pharmacologic activity beyond the late, direct suppression of purine synthesis as polyglutamate derivatives of some 4-amino antifolates accumulate in cells.

- (v) Impaired polyglutamylation. This might be due to alterations in the expression of FPGS or to mutations that affect catalytic activity.
- (vi) *Expansion of the THF-cofactor pool size in cells:* As THF-cofactors interconvert to DHF when DHFR is suppressed by MTX, the level of THF-cofactors in cells will determine the concentrations of DHF generated when DHFR is blocked. DHF will compete with MTX at the level of DHFR, and this increases the IC_{50} for this agent. The higher the affinity of the antifolate for DHFR, the lesser the importance of this phenomenon. Also, as the THF-cofactor pool size is increased, the rate and extent of formation of MTX polyglutamates is decreased.
- (vii) *The availability of thymidylate and purines:* These are key end products of THF-cofactor-dependent reactions. When they are present in sufficient quantity, DHFR activity is not necessary and suppression of this enzyme is irrelevant.

The basis for the preservation of THF-cofactors after treatment of cells with 4-amino antifolates

THF-cofactor-dependent reactions cease rapidly after cells are exposed to inhibitors of DHFR, and this parallels the rapid rise of cell DHF and decline in cell THF-cofactors. However, often this occurs under conditions in which there is substantial preservation of THF-cofactor pools *in vitro* (Baram *et al.*, 1987; Chu *et al.*, 1990; Trent *et al.*, 1991b) and *in vivo* (Priest *et al.*, 1989). Why then do THF-cofactor-dependent reactions stop? It has been shown that DHF polyglutamates inhibit TS; and it has been proposed that as DHF builds to high levels, the inhibition is of a sufficient magnitude to shut off this enzyme and block further oxidation of 5,10- CH_2 -THF (Chu *et al.*, 1990). Likewise, DHF polyglutamates are also inhibitors of AICARFT; and this has been proposed as a basis for the cessation of purine synthesis (Allegra *et al.*, 1985b, 1986, 1987; Baggott *et al.*, 1986). However, there are a number of observations that do not support this hypothesis: (i) 5,10- CH_2 -THF is a better substrate for TS than is DHF an inhibitor even in their higher polyglutamyl forms (Rhee *et al.*, 1990). Hence, the magnitude of inhibition that DHF could achieve, even with a fall in cellular 5,10- CH_2 -THF, would not be sufficient to produce the inhibition required to abolish enzyme activity. (ii) A variety of other reduced folates in cells are inhibitors of TS, so that as DHF is generated these THF-cofactors are diminished. For instance, the K_i for 10-CHO-THF heptaglutamate is 100 nM, and this folate is present at a concentration of $\sim 5 \mu M$ in hepatoma cells (Balinska *et al.*, 1991). (iii) In some cell lines with usual levels of THF-cofactors, there is total depletion of THF-cofac-

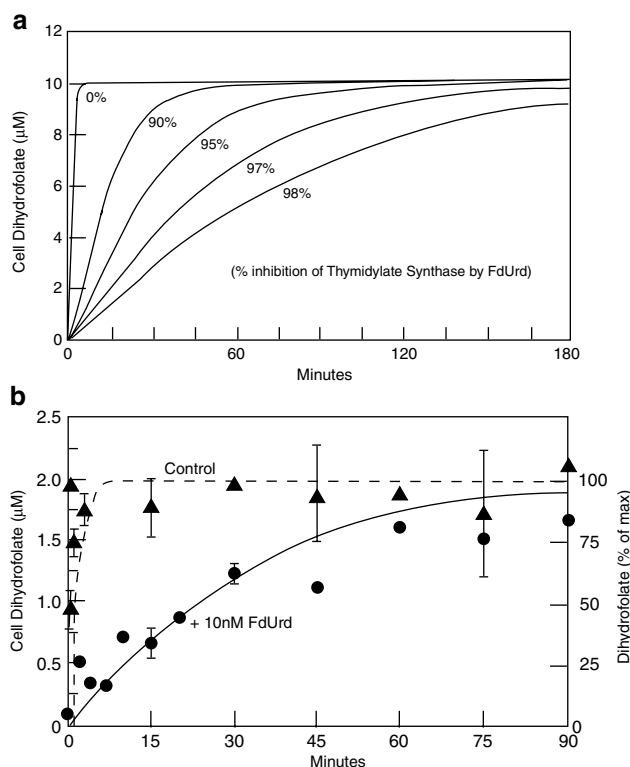


Figure 3 Panel a shows a computer simulation of the rate of rise of cell DHF as it is generated from THF-cofactors when DHFR is completely inhibited by a 4-amino-antifolate. The figure depicts this interconversion when TS activity is normal (0% suppression) and as TS activity is increasingly suppressed (90–98%). It can be seen that the model predicts that the decrease in the rate of interconversion is relatively small, in comparison to the high degree of TS inhibition; and even with 98% TS inhibition, the extent of interconversion is not changed. Panel b illustrates data that are the average (\pm sem) of five experiments in which the generation of DHF was assessed after exposure of cells to trimetrexate under control conditions (\blacktriangle), and after cells were exposed to fluorodeoxyuridine (FdUMP) (\bullet), to achieve $\sim 95\%$ suppression of TS. The lines represent computer simulations of the expected changes based upon a network thermodynamic model from Seither *et al.* (1989)

tors despite the fact that DHF reaches high intracellular levels (Rhee *et al.*, 1990).

Figure 3, panel a is a network thermodynamic simulation that brings together all these elements and shows the predicted rise in DHF and fall in THF-cofactors as TS is increasingly inhibited. Along with this is an actual interconversion of DHF to THF after TS was suppressed by $\sim 95\%$ by fluorodeoxyuridine depicted in panel b (Seither *et al.*, 1989). It can be seen that the model predicts and the data confirm that marked inhibition of the enzyme ($>90\%$) is required to slow the rate of DHF generation appreciably. Even with 98% inhibition of TS, while the rate of interconversion is slowed, the extent of interconversion to DHF does not change. Based on the K_i for DHF polyglutamate inhibition of TS and the levels that accumulate in cells, the predicted decrease in the rate of interconversion would be trivial, and as seen there would be no preservation of the THF-cofactor pool. Finally, another

study assessed the flows of radiolabeled one-carbon from 5-formyl tetrahydrofolate (5-CHO-THF) into thymidylate and purines, under conditions in which DHFR was inhibited with trimetrexate and cells had been depleted of endogenous folates (Matherly *et al.*, 1987). Incorporation of the one-carbon into thymidylate was unchanged, despite the rise in DHF ruling out inhibition of TS. There was, however, inhibition of the one-carbon flow into purines, indicative of a direct inhibitory effect of DHF polyglutamates at AICARFT, consistent with the DHF K_i for this enzyme and the lack of buildup of 10-CHO-THF behind the block (Allegra *et al.*, 1985b, 1986, 1987). On the other hand, after the accumulation of MTX polyglutamates in cells, carbon flows into both thymidylate and purines were suppressed, consistent with the high affinity of long-chain MTX congeners at the levels of TS and AICARFT (Allegra *et al.*, 1985a; Baggott *et al.*, 1986).

How then are THF-cofactor pools partially preserved in many cell lines after antifolate treatment? First, when DHFR is not *completely* suppressed by 4-amino antifolate (i.e. >5% residual activity), interconversion will not be complete, particularly when there is simultaneous suppression of TS (Seither *et al.*, 1991). Beyond this, the data support the concept that there is compartmentation of a portion of THF-cofactors, varied among cells, which makes them unavailable for oxidation to DHF. Hence, when cells become increasingly stationary, there is an increasing fraction of THF-cofactors that are not oxidized to DHF (Trent *et al.*, 1991b). This compartmentation likely represents cells in the G_1 or G_0 phase of the cell cycle when thymidylate synthesis is low or absent. Further, THF-cofactors within mitochondria are not oxidized to DHF when DHFR is suppressed, representing compartmentation within an intracellular organelle (Trent *et al.*, 1991a). Also, THF-cofactors in cells are present free or bound to cellular proteins. It is possible that differences in these components may be another determinant of the availability of folates for interconversion and oxidation (Matherly *et al.*, 1990; Matherly and Muench, 1990; Appling, 1991).

Leucovorin 'rescue' phenomenon and the role of polyglutamation of 4-amino antifolates

Leucovorin rescue is a therapeutic approach in which 5-CHO-THF is administered at low doses following, or long after (24–42 h), treatment with very high doses of MTX. This results in the provision of reduced folates, bypassing the block at DHFR, with rescue of normal susceptible host cells of the bone marrow and gut while the antitumor effect is sustained (Bertino, 1977). The effectiveness of this approach was well established in animal systems and was brought into the clinics in the mid-1960s (Bertino, 1977; Sirotnak *et al.*, 1978). High-dose MTX continues to be applied in clinical regimens (Pisters *et al.*, 1996). While the efficacy of this approach is generally accepted, the basis for its selectivity has never been well established. The generally accepted

rationale is as follows: the high MTX blood levels achieved are likely to facilitate diffusion of the drug into poorly perfused solid tumors and into tumor-containing body compartments with low permeability to this drug. Likewise, high blood levels of MTX would overcome a variety of mechanisms of resistance (i.e. decreased affinity for RFC, DHFR, FPGS) and generate high free intracellular levels through passive diffusion. At the much lower levels of 5-CHO-THF achieved during rescue, net diffusion into tumors would be slow; but this folate would be readily available to normal host tissues. Likewise, tumors with defective transport via RFC would have low transport of 5-CHO-THF since both are substrates for this carrier.

Beyond this, there are substantial data to support the concept that the biochemical basis for the selectivity of leucovorin rescue is related to a greater formation of MTX polyglutamates in tumor versus susceptible host cells and the ability of these congeners to block the utilization of added folates. While the polyglutamylation of MTX does not produce a pharmacologically important change in its affinity for DHFR, it does change the selectivity of binding and the spectrum of enzymes inhibited by this drug. The higher polyglutamate derivatives of MTX are potent inhibitors of both TS and AICARFT ($K_i \sim 50$ nM) (Allegra *et al.*, 1985a,b; Baggott *et al.*, 1986). As these reactions cease rapidly after cells are exposed to MTX due to THF-cofactor depletion, and because MTX polyglutamates accumulate slowly, there is little initial pharmacologic importance with respect to the direct inhibition of these sites. Rather, the impact is more likely to be the suppression of subsequent utilization of the added 5-CHO-THF. In normal regenerating cells of the bone marrow and GI tract, there is only low-level formation of MTX polyglutamates (Poser *et al.*, 1981; Fry *et al.*, 1983; Fabre *et al.*, 1984; Koizumi *et al.*, 1985); and the added 5-CHO-THF during rescue should be readily utilized for one-carbon reactions (Fabre *et al.*, 1984; Koizumi *et al.*, 1990). On the other hand, in tumor cells that accumulate high levels of MTX polyglutamates, rescue is impaired because there is direct suppression by these congeners of the utilization of the added folates at the level of the THF-cofactor-requiring enzymes (Matherly *et al.*, 1987; Koizumi *et al.*, 1990). The presence of 4-amino antifolate polyglutamates in cells also blocks the reactivation of DHFR that occurs with the administration of 5-CHO-THF (Matherly *et al.*, 1983, 1986). When only MTX monoglutamate is present in cells, the added natural folate and/or the folate-cofactors it generates cause the net displacement of MTX bound to DHFR with subsequent efflux from the cell. This cannot be attributed to a buildup of DHF, since it occurs when TS is blocked by fluorouracil (Matherly *et al.*, 1986). However, net displacement of bound antifolate does not occur when MTX polyglutamates are present at high levels in cells (Matherly *et al.*, 1983, 1984, 1986). Hence, it is the presence of MTX polyglutamate derivatives in tumor cells that appears to be the biochemical basis for the selectivity of leucovorin rescue.

Table 1 Representative antifolates used in preclinical and clinical studies

| Antifolate | Target enzyme (K_i) | Murine liver FPGS (K_m) |
|---------------------|--|-----------------------------|
| MTX | DHFR, mono (4.1 pM), penta (4 nM) | 166 μ M |
| TMQ (TMTX) | DHFR, 43 pM | NS |
| PT523 | DHFR, 0.35 pM | NS |
| ZD1694 (Tomudex) | TS, mono (65 nM), tetra (1.0 nM) | 1.37 |
| ZD9331 | TS, 0.4 nM | NS |
| AG337 (Thymitaq) | TS, 11 nM | NS |
| DDATHF (lomotrexol) | GARFT | 9.3 μ M |
| | Mono (5.6 nM), hexa (0.12 nM) | |
| LY309887 | GARFT | 6.0 (hog liver) |
| | Mono (0.6 nM) | |
| AG2034 | GARFT, mono (28 nM) | 6.4 μ M (rat liver) |
| Pemetrexed | TS, mono (109 nM), penta (1.3 nM) | 0.80 μ M |
| | GARFT, mono (9.3 μ M), penta 65 nM | |
| | DHFR, mono (7.0 nM), penta (7.2 nM) | |

Affinity of antifolates for target enzymes was cited from the following references: MTX (Jackson *et al.*, 1984; Chabner *et al.*, 1985); TMQ (Jackson *et al.*, 1984); PT523 (Rosowsky *et al.*, 1998); ZD1694 (Jackman *et al.*, 1991); ZD9331 (Jackman *et al.*, 1997); AG337 (Webber *et al.*, 1996); DDATHF (Sanghani and Moran, 1997); LY309887 (Sanghani and Moran, 1997); AG2034 (Boritzki *et al.*, 1996); pemetrexed (Shih *et al.*, 1997). Affinity of the antifolates for FPGS was obtained from Habeck *et al.* (1995) except for AG2034 (Boritzki *et al.*, 1996). NS: not a substrate

New-generation antifolates

The recognition that polyglutamation of MTX altered the spectrum of enzymes inhibited by this agent led to the identification of a variety of new antifolates that, in their polyglutamyl forms, are direct inhibitors of TS and/or GARFT. These agents include the TS inhibitors ZD1694 and pemetrexed (which is also a weaker inhibitor of GARFT), and the GARFT inhibitors DDATHF, LY-309887, and AG-2034 and AG-2037. There are other novel antifolate agents that are modified at the glutamate moiety so that they cannot form polyglutamates such as PT-523 (and its analogs), some of which are more potent inhibitors of DHFR than MTX, and the thymidylate inhibitors ZD9331 and AG337 (Table 1). All these agents have been, are, or will be in clinical trial. Some of their properties are indicated in Table 1.

Resistance to agents that require polyglutamation for activity may occur under a variety of circumstances: (i) When there are alterations in the catalytic activity or expression of FPGS. (ii) When there are changes in membrane transport that depress the levels of free antifolate substrate available to react with FPGS. (iii) When there are increased levels of natural folates in cells. This results in feedback inhibition of FPGS to depress synthesis of antifolate polyglutamates and/or competition with the drug at the level of the target enzyme. This can occur because of increased availability of folates, enhanced transport of folates into, or decreased folate export from cells. (iv) When there is increased activity of γ -GH, which hydrolyses the polyglutamyl forms to their monoglutamates. These will be considered in detail in the following sections.

Role of membrane transport of antifolates in drug resistance

Like the natural folates, most antifolates (with some exceptions) carry two negative charges in the glutamate

moiety of the molecule at physiological pH. They are hydrophilic and enter cells very slowly by passive diffusion. Hence, their internalization requires utilization of specific membrane transporters. There are a variety of mechanisms by which folates are transported across mammalian cell membranes. Some are bidirectional; others are unidirectional importers or exporters. The level of free antifolates within cells, the critical pharmacologic parameter, is therefore determined by the relative activities of all these processes. The reduced folate carrier (RFC) and folate receptors (FRs) are the most intensively studied. However, the cycling rate of RFC is two orders of magnitude faster than that of the FRs (Spinella *et al.*, 1995; Sierra *et al.*, 1997). Hence, FRs may be important only when the RFC function is very low, and/or there is very high receptor expression, and the drug has a moderate or high affinity for the receptor. Recently, several organic anion carriers, expressed in the liver and kidney, have been shown to transport MTX, some with affinities comparable to that of RFC (Saito *et al.*, 1996; Masuda *et al.*, 1999; Sun *et al.*, 2001; Russel *et al.*, 2002). There is no evidence as yet indicating that these carriers are widely expressed in malignant cells and play a role in drug resistance. However, LST-2 has been shown to be highly expressed in a variety of GI cancers (Abe *et al.*, 2001). The physiological role of these carriers with respect to folates, beyond mediating transport in liver and kidney, is not clear. Pathways relevant to the transport of antifolates in most tumor types will be described in this section, along with their role in drug resistance.

The reduced folate carrier

RFC is a typical member of the major facilitator superfamily located on human chromosome 21 (21q22.2–q22.3) with a predicted secondary structure characterized by 12 transmembrane domains (TMDs) with the N- and C-termini, and the large central loop between the sixth and seventh TMDs, inwardly directed

(Dixon *et al.*, 1994; Williams *et al.*, 1994; Moscow *et al.*, 1995; Prasad *et al.*, 1995; Williams and Flintoff, 1995; Wong *et al.*, 1995). The validity of this secondary structure has been strengthened by hemagglutinin epitope insertion analysis (Ferguson and Flintoff, 1999) although there remains a question regarding the topology of TMDs 9-12 (Liu and Matherly, 2002). A His27Arg polymorphism has been identified in the human carrier (Chango *et al.*, 2000), but appears to have only minor functional importance (Whetstine *et al.*, 2001). There are three recent reviews that encompass the biology of RFC (Sirotnak and Tolner, 1999; Matherly, 2001; Matherly & Goldman, 2003).

Transport mediated by RFC is highly sensitive to the anionic composition of the extracellular compartment, and influx is inhibited by a wide range of inorganic and organic anions (Goldman, 1971; Henderson and Zevely, 1981, 1982a, b, 1983). This carrier system appears to generate small transmembrane chemical gradients through an exchange with organic phosphates, in particular the adenine nucleotides that are synthesized and accumulate to high levels within cells. This ability of structurally unrelated anions to exchange with folates and augment concentrative folate and antifolate transport was confirmed by studies with membrane vesicles (Yang *et al.*, 1984). Recently, thiamine mono- and pyrophosphate were shown to utilize RFC in murine leukemia cells (Zhao *et al.*, 2000d, 2002). The interaction between these organic phosphates and RFC at the cytosol-membrane interphase was supported by the demonstration that the level of RFC activity modulates the level of thiamine pyrophosphate in cells by modulating its efflux. Hence, in cells transfected to high RFC levels, the net level of thiamine pyrophosphate was suppressed due to enhanced RFC-mediated efflux. The highest thiamine phosphate levels were detected in cells in which RFC was mutated and inactive.

Owing to the high membrane potential in tumor cells and the divalent nature of the folate molecule, transmembrane chemical gradients achieved by RFC are small in leukemic and other cells (Goldman *et al.*, 1968; Zhao *et al.*, 1997; Sharif *et al.*, 1998). Another factor in limiting concentrative transport is the activity of exporters that pump monoglutamyl folates and antifolates out of cells (see below). However, when charge and membrane potential are taken into consideration, this transport system is shown to generate transmembrane *electrochemical-potential differences* for MTX across cell membranes.

Resistance associated with impaired RFC-mediated transport

Soon after the clinical introduction of MTX, impaired transport was recognized as an important mechanism of resistance to this agent. A number of studies with tumor systems, largely *in vitro* but *in vivo* as well, characterized the mechanisms by which transport capacity is impaired (Sirotnak *et al.*, 1968; Niethammer and Jackson, 1975; Hill *et al.*, 1979; Sirotnak *et al.*, 1981b). These included increased K_m , decreased V_{max} , or changes in both

parameters. Whether changes in V_{max} were due to impaired mobility of the carrier versus depressed expression was difficult to determine prior to cloning of the gene. However, specific binding to the cell membrane was one measure of carrier protein expression (Henderson and Zevely, 1984a; Schuetz *et al.*, 1988). With the cloning of RFC, it became possible to understand the molecular basis for these defects. An L1210 leukemia cell line with a markedly decreased V_{max} due to impaired mobility was shown to harbor an A130P mutation in the third TMD (Brigle *et al.*, 1995). Several human CEM-CCRF MTX-resistant cell lines, generated in different laboratories, with decreased MTX influx V_{max} , were subsequently shown to have a Glu45Lys mutation in the first TMD (Jansen *et al.*, 1998; Drori *et al.*, 2000), the latter homologous to a mutation identified in murine leukemia cells selected for MTX resistance under chemical mutagenesis (Zhao *et al.*, 1998a). However, this mutation has not been detected in cells obtained directly from patients with leukemia (Whetstine *et al.*, 2001; Gifford *et al.*, 2002). The intrinsic resistance of sarcoma 180 cells to MTX as compared to L1210 leukemia cells was associated with a single amino-acid difference at codon 297 (Asn in the latter, Ser in the former) resulting in an increase in the influx K_t without a change in V_{max} (Roy *et al.*, 1998). This mutation was in, or closely adjacent to, the external loop between the seventh and eighth TMDs of RFC. Alterations in transport also play a role in acquired resistance to new antifolates such as ZD9331, a TS inhibitor, and PT523, a DHFR inhibitor, which utilize this carrier but cannot form polyglutamate derivatives. Likewise cell lines with impaired RFC-mediated transport acquired in the presence of other antifolates are crossresistant to ZD9331 (Mauritz *et al.*, 2002). Complicated studies that relate carrier expression with drug resistance include observations in K562 cell lines transfected with hRFC. There was a good correlation between photoaffinity labeling of carrier and message but not with the level of transport. This suggested that a portion of the carrier within or adjacent to the membrane was not functional (Wong *et al.*, 1997). While alterations in the regulation of RFC expression are a basis for resistance particularly under clinical conditions (Matherly *et al.*, 1995; Gorlick *et al.*, 1997), the mechanism(s) involved are not clear. Impaired transport of MTX with absence of RFC message in an MDA-MB-231 breast cancer cell line was associated with methylation of a CpG island in the promoter. 5-aza-2'-doxycytidine restored RFC expression but not sensitivity to the drug. The latter was attributed to increased efflux of drug associated with increased expression of MRP1-3 (Worm *et al.*, 2001). Recently, impaired RFC expression in human leukemia cells was associated with altered expression of transcription factors that regulate this gene (Rothen *et al.*, 2003).

Recent studies that assess RFC-based mechanisms of resistance, selectivity of resistance, and structure/function

A variety of recent studies have added to our understanding of the molecular changes that accompany

RFC-mediated resistance to antifolates. Of special interest has been the selectivity of some of these changes. Particularly intriguing was the selection of a cell line resistant to DDATHF, in which transport of this drug was unchanged. Instead, these cells had marked augmentation of folic acid transport shown to be due to mutations in the first, Ile48Phe, and third, Trp105Gly, TMDs (Tse *et al.*, 1998). Each mutation alone, and cumulatively, markedly decreased the influx K_t for this folate. Since these cells were grown in folic acid, this resulted in expansion of the cellular THF-cofactor pool, and this in turn caused feedback inhibition of FPGS and impaired formation of DDATHF polyglutamates (Tse and Moran, 1998; Andreassi and Moran, 2002). The study provided an important insight into the critical role that natural folates within cells can play in modulating the activity of antifolates, in particular those that require polyglutamation for activity.

Figure 4 illustrates the spectrum of mutations that accompanied murine L1210 leukemia cell resistance to MTX due to impaired RFC function following selection with this drug with chemical mutagenesis (Zhao *et al.*, 1999b). It can be seen that virtually all the mutations were located in, or at the boundaries of, TMDs. Mutations within these domains could alter V_{max} , presumably by perturbing carrier mobility, decrease the affinity of carrier for inhibitor, disrupt the tertiary structure of the RFC, or impair its trafficking to the cell membrane. These changes can be highly selective as was seen for DDATHF (see above). These studies indicated the impact of the folate source in the growth medium during selection. When cells are grown in folic acid, for which RFC has a very low affinity, folic acid is transported to a large extent by other pathway(s) (Sirotnak *et al.*, 1987). However, when cells are grown in 5-CHO-THF, which best mimics the physiological folate 5-CH₃-THF, transport is almost exclusively via RFC. Hence, mutations in the carrier that impair drug transport must be sufficiently selective to permit entry of enough 5-CHO-THF to sustain growth. For instance, the E45K mutation in the first TMD of RFC in cells

selected in folic acid produced a global decline in carrier mobility along with a decreased affinity for MTX. However, while the affinity for folic acid was markedly increased, the affinity for 5-CHO-THF was increased to a lesser extent, and the affinity for 5-CH₃-THF was unchanged (Zhao *et al.*, 1998a). This pattern of change in K_t for folic acid and 5-CHO-THF was the same in human leukemia cells in which RFC was amplified with the same mutation, but the K_t for MTX influx was unchanged (Jansen *et al.*, 1998). When these cells were grown in folic acid, cellular THF-cofactors increased resulting in marked crossresistance to antifolates that require polyglutamation for activity. In cells selected for resistance to MTX with 5-CHO-THF as the folate growth source, a mutation in the adjacent residue Ser46Asn in murine RFC did not alter the influx K_t for MTX and the natural folates (Zhao *et al.*, 1998b). Rather, it produced a marked (40-fold) decrease in the V_{max} for MTX but a lesser (~7–8-fold) decrease in V_{max} for 5-CHO-THF and 5-CH₃-THF. Hence, the selectivity of the change in carrier mobility preserved sufficient transport of 5-CHO-THF to sustain normal growth. Recently this mutation was identified in human osteogenic sarcoma tissues (Gang *et al.*, 2003). In general, most point mutations associated with acquired MTX resistance under these conditions are associated with changes in V_{max} and to a varying extent with changes in K_t as well. For instance, an MTX-resistant L1210 cell line with a Ser309Phe mutation in the eighth TMD associated with impaired MTX transport and a decreased V_{max} , differentiated between binding of reduced folates (Zhao *et al.*, 1999a). While the K_t for 5-CH₃-THF and folic acid influx were minimally changed, the K_t for MTX and 5-CH₃-THF influx increased by a factor of 4–5. The first TMD appears to be a favored locus of mutations in acquired resistance to antifolates, further supported by the mutations at this site in a variety of CCRF-CEM cell lines selected for resistance to antifolates in the absence of a mutagen (Jansen *et al.*, 1998; Drori *et al.*, 2000; Rothem *et al.*, 2002). Beyond single point mutations within the open reading frame, a variety of other mutations have been detected in MTX-resistant cell lines including mutation of the ATG start codon, insertions and frameshifts, truncated proteins, deletions, mutations resulting in RFC instability, and loss of RFC alleles due to translocations (Wong *et al.*, 1999; Zhao *et al.*, 1999b; Ding *et al.*, 2001; Rothem *et al.*, 2002).

Mutations in RFC that result after exposure to one antifolate need not necessarily produce comparable or any resistance to another antifolate that utilizes the same carrier. For instance, a Val104Met mutation in the third TMD further illustrates the role of cellular THF-cofactor pools in modulating antifolate activity (Zhao *et al.*, 2000b). These cells were selected with MTX, using 25 nM 5-CHO-THF in the growth medium, and had markedly impaired transport of MTX and 5-CHO-THF. Despite the fact that the cellular THF-cofactor pool was substantially decreased, growth was sustained consistent with the low levels of folate required for normal growth. This cell line turned out to be

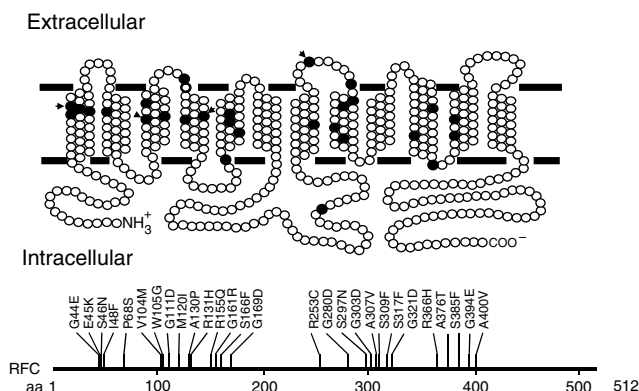


Figure 4 Pattern of mutations detected after murine L1210 leukemia cells were selected for MTX resistance with chemical mutagenesis. The specific amino-acid substitutions are indicated in the lower section. Arrows indicate reported mutations in other cell lines that were not generated by chemical mutagenesis as described in the text. From Zhao *et al.* (1999a)

collaterally sensitive to DDATHF. The basis for this finding was related to three factors. First, the mutation was, in part, selective in that influx of DDATHF was not reduced as much as MTX and 5-CHO-THF influx. Second, there was relative preservation of the free DDATHF level that was unexplained, but might be related to decreased MRP-related export of this drug in this cell line. Third, because the decrease in 5-CHO-THF transport resulted in a marked reduction in the THF-cofactor pool, feedback inhibition of DDATHF polyglutamation was diminished, compensating for the decline in DDATHF transport. The result was that the level of DDATHF polyglutamates was largely preserved in these cells along with the activity of the drug. On the other hand, when these cells were grown in folic acid, the THF-cofactor pool was only minimally perturbed, there was a decline in the level of DDATHF polyglutamates, and these cells had low-level resistance to this drug. Relative preservation of DDATHF activity was also observed in MTX-resistant CCRF-CEM cells with a substantial transport defect due to the preservation of accumulation of polyglutamyl derivatives of this drug (Matherly *et al.*, 1993).

This phenomenon was also seen with other mutations and other drugs. For instance, sensitivity to pemetrexed due to the Ala130Pro or Ser46Asn mutations described above was partially preserved when cells were grown in 5-CHO-THF, but the activity was very low when cells were grown in folic acid (Zhao *et al.*, 2000a). This is unlike what was observed with MTX when high-level resistance persisted with either folate source. These data indicate that cells can become resistant to MTX or possibly other antifolates due to impaired RFC-mediated transport, and yet can retain partial or full sensitivity to other antifolates that utilize the same transport mechanism to enter cells. The extent to which this can occur clinically is unclear, but there is some evidence based on studies on cell preparations from patients to suggest that this phenomenon may be relevant to the clinical setting (Rots *et al.*, 1999). These observations point to the need for physiologically reduced folate as the growth source when resistance mechanisms are studied *in vitro*. The impact of the THF-cofactor pool on antifolate activity is considered in detail (see below).

Of interest was the demonstration that in a cell line resistant to MTX due to a mutation in the fourth TMD of hRFC (Arg133Leu), the induction of a second mutation in the second TMD (Asp88Val), which alone also impaired function, restored the activity of the carrier and drug sensitivity (Liu and Matherly, 2001). This phenomenon was attributed to charge pairing (Sahin-Toth *et al.*, 1992; Merickel *et al.*, 1997) between residues in the two domains, when the carrier is oriented in its tertiary membrane structure. With the first mutation, the interaction between the two domains is disrupted and function is impaired. The second mutation neutralizes the incompatibility, allowing the mutated residues and their TMDs to come into their usual alignment. An interaction between the E45 and K404 residues in the murine carrier has also been noted (Zhao *et al.*, 2003).

RFC is an anion exchanger, and an influx of folates via this mechanism is highly sensitive to the anionic milieu. Hence, a variety of organic anions added, or substitutions of chloride with other inorganic anions, inhibit the transport of folates associated with an increase in influx K_t (Goldman, 1971; Henderson and Zevely, 1983; Zhao *et al.*, 1998a). The removal of chloride enhances the influx of folates associated with a decrease in K_t . Mutations in RFC can profoundly alter anion sensitivity. For instance, the E45K mutation made mRFC influx anion dependent. In the absence of chloride the influx V_{max} was markedly decreased. Function was restored by the addition of chloride, nitrate, or fluoride (Zhao *et al.*, 1998a). Similarly, the same mutation in human leukemia cells also resulted in anion dependence (Jansen *et al.*, 1998). Subsequent studies using site-directed mutagenesis indicated that it is the introduction of the positive charge, rather than the loss of the negative charge, at this site that results in loss of function. Hence, the requirement for anions is likely due to the neutralization of the positive lysine charge (Zhao *et al.*, 2000c). Ser309Phe (eighth TMD) and Glu404Leu (11th TMD) mutations also produced changes in anion sensitivity. Influx was independent of chloride over a broad range of chloride concentrations with a stimulatory effect below 50 mM (Zhao *et al.*, 1999a; Sharina *et al.*, 2001).

Folic acid receptor-mediated transport

Two human membrane FRs, folate receptor- α (FR- α) and folate receptor- β (FR- β), have been cloned and mediate the transport of folates (Ratnam *et al.*, 1989; Sadasivan and Rothenberg, 1989). Both murine homologs have been cloned as well (Brigle *et al.*, 1991). The folate receptor genes map to chromosome 11q13.3–3.5 (Ragoussis *et al.*, 1992). A third, FR- γ , is a secretory form (Shen *et al.*, 1995). FR- α and FR- β are anchored to the cell membrane by a glycosyl-phosphatidylinositol (GPI) tail. Membrane FRs have a very high affinity for folic acid (~ 1 nM). There are differences in the stereo specificities of the two receptors for the reduced folates, and the affinities for 5-CHO-THF and CH₃-THF are lower than for folic acid (Wang *et al.*, 1992; Brigle *et al.*, 1994). Both FRs have a relatively low affinity for MTX (~ 300 nM) but a high affinity for new-generation antifolates such as DDATHF, ZD1694, ZD9331, and pemetrexed (Westerhof *et al.*, 1995). FRs are internalized within membrane vesicles; the vesicles are then acidified with the release of folate ligand, following which the folate enters the cytosol – a process termed ‘potocytosis’ (Kamen *et al.*, 1988). Receptors are generally located within caveolae, although there is evidence that they cluster in cell membranes in other sites as well including clathryn-coated pits (Wu *et al.*, 1997). Current concepts of the mechanism of potocytosis have been reviewed recently (Mineo and Anderson, 2001).

Transport of folates mediated by FRs is slow, with cycling rates about one – one hundredth that of RFC

(Spinella *et al.*, 1995; Sierra *et al.*, 1997). When FR expression is increased in cells with functioning RFC, the impact on antifolate activity and folate growth requirement depends on the relative expression of the transporters and the relative affinities of the folate for the two processes (Westerhof *et al.*, 1991). With sufficient expression of FR, even drugs such as MTX, with low affinity for these proteins relative to folic acid, can be delivered into cells at rates comparable to those mediated by RFC (Dixon *et al.*, 1992; Spinella *et al.*, 1995); and growth inhibition can be enhanced (Chung *et al.*, 1993). The human KB nasopharyngeal epidermoid line with high-level expression of FR- α is highly sensitive to MTX, and transport is mediated largely by this process (Deutsch *et al.*, 1989). On the other hand, CCRF-CEM human leukemia cells that lack RFC but have increased FR- α expression have markedly diminished transport of MTX relative to wild-type cells and are highly resistant to the drug (Van der Veer *et al.*, 1989). In the case of folic acid with high affinity for FRs but low affinity for RFC, the level of FR expression is a major determinant of the rate of transport and the receptor is upregulated by growth in media with low folate levels (Henderson *et al.*, 1988; Jansen *et al.*, 1989). Likewise, growth inhibition by antifolates with high affinity for FRs and low affinity for RFC is markedly increased by overexpression of receptor even when RFC function is absent (Henderson and Strauss, 1990a; Jansen *et al.*, 1990). The impact of FR expression on the activity of antifolates is influenced by the folate present and its concentration *in vitro* or *in vivo* and the relative affinities of the folate and antifolate for this transporter. Hence, in standard media with folic acid levels of 2.3 μM , transport of MTX via FRs is markedly suppressed, with a comparable decrease in growth inhibition by this agent due to the 300-fold higher affinity of folic acid for the receptor (Kane *et al.*, 1986; Chung *et al.*, 1993). Likewise, activity of the nonpolyglutamatable TS inhibitor, CB300638 which has a very high affinity for folate receptor but a low affinity for RFC, was markedly suppressed when 1 μM folic acid was added to medium containing either 1 or 20 nM 5-CHO-TFH (Theti *et al.*, 2003).

FR- α is highly expressed in normal tissues including among others, choroid plexus, lung, thyroid, kidney, and female reproductive organs (Weitman *et al.*, 1992a, b). FR- α is highly expressed in tumor cell lines including among others, those of ovarian, kidney (MA104), colon (Caco-2), and epidermoid (KB) origin (Coney *et al.*, 1991; Weitman *et al.*, 1992a). FR- α is overexpressed in a variety of human neoplastic tissues, such as carcinomas of the ovary, uterus, brain, and squamous cell carcinoma of the head and neck (Campbell *et al.*, 1991; Coney *et al.*, 1991; Weitman *et al.*, 1992b, 1994; Ross *et al.*, 1994; Miotti *et al.*, 1995; Wu *et al.*, 1999). In contrast, FR- β expression is much more restricted. It appears to be present in some normal cells of myeloid lineage but is highly expressed in myeloid but not lymphoid leukemias (Shen *et al.*, 1994; Reddy *et al.*, 1999; Ross *et al.*, 1999b; Pan *et al.*, 2002). FR- β may have little function in normal myeloid progenitor cells,

since receptors on these cells do not appear to bind or transport folate, possibly due to a defect in the GPI membrane anchor (Reddy *et al.*, 1999; Pan *et al.*, 2002).

There is little information on the role of alterations in membrane FR expression in acquired resistance to antifolates. For tumors with high-level FR expression, such as KB cells, selection with MTX can result in decreased expression of the protein, decreased transport, and an increase in the IC₅₀ for this drug (Saikawa *et al.*, 1993). In one case, a decreased level of FR- α protein and message associated with acquired MTX resistance in a KB cell line was attributed to methylation of the gene (Hsueh and Dolnick, 1994).

FRs have been targeted as selective pathways for the delivery into tumor cells of molecules coupled to folic acid. This strategy is being explored for the delivery of cytotoxins or radiolabeled molecules for tumor imaging (Leamon and Low, 2001; Lu and Low, 2002). The relatively restricted expression of FR- β to myeloid leukemias and its upregulation by retinoids has been identified as a potentially important approach for the selective delivery of antineoplastics, coupled to folic acid for the treatment of these diseases (Reddy *et al.*, 1999; Ross *et al.*, 1999b; Wang *et al.*, 2000; Pan *et al.*, 2002). As these approaches evolve, it is likely that down-regulation, mutation of FRs expression, or alterations in function will emerge as mechanisms of tumor cell resistance.

Transport routes for folates that operate optimally at low pH

It has been recognized for decades that absorption of folates in the small intestine is favored by a low pH with minimal activity at pH 7.4 (Selhub *et al.*, 1983). This has also been observed for folate transport into brush-border membrane vesicles (Dudeja *et al.*, 1997). The mechanism by which folates are transported in the intestine at low pH is not entirely clear. Supporting a role for RFC in this process are the observations that the protein is present at the apical brush-border membrane (Chiao *et al.*, 1997; Wang *et al.*, 2001), activity at low pH is enhanced by transfection of the cDNA into intestinal cells (Kumar *et al.*, 1998; Rajgopal *et al.*, 2001), and an antibody to RFC inhibited 5-CH₃-TFH transport into intestinal cells (Chiao *et al.*, 1997).

However, there are a variety of observations suggesting that this phenomenon is more complex. First, if the N- and C-termini of the carrier are directed into the cytoplasm as indicated by epitope insertion studies (Ferguson and Flintoff, 1999), it is unclear as to why antibodies to these domains should inhibit transport. Second, recent studies indicate that transfection of RFC enhances transport activity at *both* low and physiological pH (Rajgopal *et al.*, 2001). Third, transport activity in intestinal cells for folic acid and MTX is comparable at low pH, inconsistent with the low affinity of RFC for folic acid (Rajgopal *et al.*, 2001). Fourth, while energy inhibitors slightly stimulate the MTX influx via RFC at physiological pH, inhibition of energy metabolism

suppresses influx into intestinal epithelial cells at low pH (Rajgopal *et al.*, 2001).

These data suggest at least three possibilities: (i) There are post-translational modifications of RFC in the intestine that alter its substrate specificity, pH sensitivity, and possibly its energetics. (ii) The basal folate transport activity at low pH is due to an entirely different protein with distinct characteristics, a process that is augmented by a protein–protein interaction with RFC that is enhanced when RFC expression is increased. (iii) There is a protein–protein interaction that alters the function and pH sensitivity of RFC, which is the sole mediator of transport.

Low pH transport routes for folates have been detected in other normal and malignant cells. For instance, transport routes with low and physiological pH optima and substrate specificities, similar to what has been observed in intestinal cells, are present in NIH 3T3 cells. Each activity appeared to be regulated independently (Kuhnel *et al.*, 2000). A low pH transport activity for MTX far exceeds activity at physiological pH in PC-3 human prostate cancer cells (Horne and Reed, 2001) and is present in hepatocytes (Horne, 1990) and retinal pigment epithelial cells (Huang *et al.*, 1997; Chancy *et al.*, 2000). This activity is unchanged in tumor cells in which RFC function is markedly impaired or not present, suggesting that transport is mediated at least in part by some other genetically distinct process (Henderson and Strauss, 1990b; Sierra and Goldman, 1998). The importance of a transport route that functions at a low pH to tumor cells is unclear. The extracellular milieu of solid tumors is acidic due to poor oxygenation and the high level of anaerobic glycolysis. It is possible that low-pH routes could play a role in mediating the transport of both folates and antifolates under these conditions. There are no data to implicate loss of transport activity at low pH as a factor in antifolate drug resistance. In fact, in a CHO cell line resistant to antifolates due to the loss of folate exporter(s), a low pH influx activity was enhanced (Assaraf *et al.*, 1998).

Transport mechanisms that pump folates out of cells

RFC is a facilitative, bidirectional carrier that mediates export as well as influx of folates, a mechanism that is not directly energy dependent. However, the presence of a second, ATP-dependent folate export process was recognized in the earliest studies on the transport properties of MTX. Energy inhibitors were shown to enhance net transport and slow the efflux of MTX from sarcoma 180 and murine L1210 leukemia cells, consistent with the presence of energy-dependent export (Hakala, 1965; Goldman, 1969). Studies with inside-out membrane vesicles confirmed the presence of an ATP-dependent, low-affinity, high-capacity export mechanism (Schlemmer and Sirotnak, 1992, 1995). Subsequent studies using a variety of selective inhibitors separated efflux into several different pathways (Henderson and Zevely, 1984b; Saxena and Henderson, 1995, 1996). It now appears that these pathways reflected transport mediated

by members of the family of MRPs as well as other exporters, as described in the next section.

MRPs and their role in antifolate resistance

Impact of MRP overexpression on membrane transport and acquired resistance to antifolates

MRPs are a family within the superfamily of ATP-binding cassette (ABC) transport proteins that link the release of free energy in the hydrolysis of ATP to the unidirectional export of a broad range of neutral as well as anionic compounds out of cells. Nine members of this family (MRP1–MRP9) have been identified to date (Borst *et al.*, 2000; Borst and Elferink, 2002).

The linkage between MTX transport and MRPs was established in 1997, when impaired clearance and secretion of MTX was noted in hyperbilirubinemic rats that lack MRP2. ATP-dependent MTX transport was also shown to be absent in canalicular membranes prepared from the liver of these animals (Masuda *et al.*, 1997). Subsequent studies established that THF, 5-CH₃-THF, 5-10-CH₂-THF, and 10-CHO-THF are also substrates for MRP2 (Kusuhara *et al.*, 1998).

It is now clear that at least four MRP proteins transport folates and antifolates. Transfection of MRP1 or MRP2 into human ovarian 2008 carcinoma cells increased ATP-dependent MTX uptake into inside-out membrane vesicles obtained from these cells and conferred resistance to short-term exposure to MTX, ZD1694, and GW1843U89 (Hooijberg *et al.*, 1999). In recent studies, site-directed mutagenesis of the highly conserved Trp¹²⁵⁴ of MRP2 established this residue as a major determinant of MTX transport (Ito *et al.*, 2001). Overexpression of MRP3 also resulted in high-level resistance to MTX and augmented transport of 5-CHO-THF, folic acid, and MTX into inside-out membrane vesicles (Hirohashi *et al.*, 1999; Zeng *et al.*, 2000, 2001). MRP1-, MRP2-, MRP3-, and most recently MRP4-mediated MTX uptake into inside-out membrane vesicles recapitulated an important feature of folate transport in intact cells in that MTX monoglutamate, but not the diglutamate, are substrates for these transporters (Zeng *et al.*, 2001; Chen *et al.*, 2002). Based on studies with membrane vesicles, MRP4 appears to have a higher affinity for MTX (~0.2 mM) than MRP3 (~0.7 mM), or MRP1 (~2 mM) with a similar trend for folic acid and 5-CHO-THF (Zeng *et al.*, 2000, 2001; Chen *et al.*, 2002). Expression of MRP4 in NIH 3T3 cells conferred ~fivefold resistance to MTX after short-term exposure (Lee *et al.*, 2000) and was recently shown to enhance ATP-dependent MTX, folic acid, and 5-CHO-THF transport into inside-out membrane vesicles prepared from insect cells transfected with MRP4 cDNA (Chen *et al.*, 2002). Transfection of MRP1, MRP2, or MRP3 into ovarian cancer cells resulted in increased folate growth requirements (Hooijberg *et al.*, 2003). The observation that each of these transporters export a broad range of antineoplastics and other agents could be a basis for collateral resistance to MTX

associated with primary resistance to other antineoplas- tics with entirely different structures and mechanisms of action. However, this has not been demonstrated as yet.

It is of interest that in several cases, overexpression of MRPs was associated with MTX resistance only when the duration of exposure was brief (~4 h). However, resistance did not persist when the interval of exposure was longer (>24 h) (Hooijberg *et al.*, 1999; Kool *et al.*, 1999; Lee *et al.*, 2000). This occurred despite the fact that the accumulation of drug in MRP-overexpressing cells, although increasing over this interval, was always less than that in the wild-type cells. This was also the case for TS inhibitors that require polyglutamylation for activity (Hooijberg *et al.*, 1999). The amplification of the inhibitory effect of short-term exposure to these drugs reflects the critical role that polyglutamylation plays in drug retention and activity. Hence, high MRP activity will decrease free drug levels achieved and thereby decrease the *rate*, and likely the extent, of formation of polyglutamate congeners. For MTX, the polyglutamate derivatives are not much more potent inhibitors of the target enzyme than the monoglutamate, and the levels of these derivatives are not of pharmacologic importance when exposure to the drug is continuous. For agents like ZD1694 and pemetrexed that require this bioconversion for activity, these derivatives play a crucial role in the suppression of the target enzyme and exposure must be sufficiently long to allow high levels to accumulate in cells.

Unlike the P-glycoprotein (Pgp) family of exporters (as discussed below), MRPs appear to interact with their substrates at the membrane-cytosol boundary. Owing to this, MRPs may have little inhibitory effect on unidirectional drug influx. The influx of MTX in murine leukemia cells is stimulated to only a small extent by inhibitors of energy metabolism (Goldman, 1969; Fry *et al.*, 1980). This suggests that only a small fraction of MTX was captured and ejected by exporters before entering the cytosol in these cells. The extent to which influx might be augmented will depend on the relative rates of transport mediated by RFC versus MRPs and the specific characteristics of the exporters that are expressed and their distribution within the cell membrane.

MRPs as a factor in the limited concentrative transport of, and intrinsic resistance to, antifolates

Current studies that evaluate transport of folates by MRPs and the role of MRPs in drug resistance are focused on transfection and *overexpression* of these genes. This approach has been utilized to establish that folates and antifolates are substrates for these exporters and to characterize their properties, in particular substrate specificities and impact on drug sensitivities. No tumors have been selected for *acquired* resistance to an antifolate due to *enhanced* activity of these exporters or any undefined energy-requiring exit process. However, the opposite has been observed. For instance, a tumor cell line selected for pyrimethamine resistance (a lipid-soluble antifolate) has been described with impaired exporter function that is crossresistant to a

variety of antifolates. This is due to decreased folate export, with expansion of the cellular folate-cofactor pool resulting in impaired antifolate polyglutamylation, and the buildup of high cellular levels of DHF after 4-amino antifolates (Assaraf and Goldman, 1997; Jansen *et al.*, 1999). Recently, this loss of folate export function was shown to be due to the loss of MRP1 expression (Stark *et al.*, 2003).

However, one or more MRPs must always be operational in tumor cells, as demonstrated by the markedly enhanced net uptake of MTX on exposure to energy inhibitors and specific inhibitors of MRPs (Goldman, 1969; Fry *et al.*, 1980; Dembo *et al.*, 1984). Hence, MRPs always oppose the concentrative transport mechanisms for folates such as RFC; and therefore, their functional expression is an important factor in the very low concentrations of free antifolates that accumulate in cells. In fact, a variety of agents, such as probenecid, vincristine, VM-26, VP-16, and PGA₁ depress the efflux and augment the free level of MTX in cells (Fyfe and Goldman, 1973; Sirotiak *et al.*, 1981c; Fry *et al.*, 1982a; Yalowich *et al.*, 1982; Assaraf *et al.*, 1999). This has been associated with enhanced accumulation of antifolate polyglutamates (Fry *et al.*, 1982a; Yalowich *et al.*, 1982) and in some cases enhanced antifolate activity *in vivo* (Sirotiak *et al.*, 1981a, 2000; Khokhar *et al.*, 2001). The inhibition of MRPs, as with probenecid, remains a potentially useful approach to enhance the activity of antifolates, although the inhibitory effect of this and other similar agents on renal excretion of antifolates is a confounding factor (Kates *et al.*, 1976).

The consequence of the limitation in concentrative antifolate transport is a reduction in the rates of polyglutamylation, since it is the free monoglutamyl antifolate that is the substrate for FPGS. Hence, the activity of these exporters may be an important factor in intrinsic resistance to these agents. Different affinities of MRPs for different antifolates may also be a determinant of differences in concentrative transport. For instance, the free pemetrexed level in murine leukemia cells is more than twofold that of MTX. This is associated with a comparable decrease in the efflux rate constant. Further, augmentation of pemetrexed uptake by prostaglandin A₁, an MRP inhibitor, is far less than observed for MTX (Zhao *et al.*, 2000a). These observations suggested that pemetrexed may be a lesser substrate for MRP exporters operational in these cells relative to MTX. Likewise, the preservation of DDATHF levels, in contrast to the greater decline in influx observed in a cell line with the V104M mutation (Zhao *et al.*, 2000b) and in CEM/MTX cells (Matherly *et al.*, 1993), may reflect an associated decrease in MRP-mediated drug export that tends to preserve activity. Clearly, differences in antifolate substrate specificities among the MRPs, suggested by earlier studies (Schlemmer and Sirotiak, 1995), will be of considerable interest, along with studies on tissues from patients that correlate the expression and function of RFC (and other inward or bidirectional transporters) and the MRP family of exporters with treatment outcome.

The activity of the MRP family of exporters in mammalian cells is also a critical factor in the limitation of augmentation of cell antifolate levels as the extracellular concentration of drugs is increased. RFC is a much higher affinity transporter than the MRPs – with K_t 's for many antifolates and reduced folates in the 1–5 μM range. MRP K_t 's are at least one to two orders of magnitude higher. This means that as the drug level is increased, the influx mechanism saturates long before the export system; and this results in a progressive decline in the ratio of free intracellular to extracellular drug (Goldman *et al.*, 1968; Zhao *et al.*, 1997). When the primary resistance mechanism is a decrease in affinity of a target enzyme for drug (i.e. TS, DHFR) or amplification of DHFR (see below), it can be readily seen how this suppression of concentrative transport limits the ability to circumvent resistance, without undue toxicity, by increasing the dose of the drug. This is in addition to limitations in concentrative transport related to the bianionic nature of many antifolate molecules and the transmembrane electrical-potential differences in tumor cells. These are reasons for the need for high-dose MTX regimens with leucovorin rescue in order to generate sufficient free intracellular drug levels to achieve a pharmacologic effect.

Another manifestation of MRP export of folates is their impact on concentrative transport when RFC expression is altered. Consider the hypothetical situation in which transport of folates into and out of cells is mediated by RFC alone. As the level of expression of RFC is increased, the bidirectional fluxes of RFC will increase and the rate at which the system comes to steady state will increase. However, the steady-state level achieved will not change if the energy source driving the system (i.e. the organic anion gradient) does not change. On the other hand, if in addition to the carrier there is an energy-driven exporter, which accounts for a large component of the efflux of the drug, there will be a substantial depression of the steady-state drug level. Under these conditions, overexpression of RFC will increase the steady-state level as the carrier becomes the dominant transport pathway. When MTX influx was increased ninefold in L1210 murine leukemia cells by transfection of RFC, the efflux rate constant increased by a factor of five, resulting in only a twofold increase in the free drug level achieved (Zhao *et al.*, 1997). In contrast, in MTX^r-ZR-75 breast cancer cells, transfection of RFC increased steady-state free drug by a factor ~half that of the increase in influx (Sharif *et al.*, 1998). In both cases, the concentrative effects were greater as the drug level was increased. The data suggest that high expression of RFC increases the rate of cycling of the carrier, but that this is not necessarily translated into proportionately higher transmembrane gradients.

Role of Pgp in antifolate resistance

Pgp, encoded by the MDR1 gene, is an ATP-dependent transporter that exports a wide spectrum of hydro-

phobic drugs, thereby rendering cells resistant to these agents (Juliano and Ling, 1976; Gottesman and Pastan, 1993; Litman *et al.*, 2001). It has been proposed that Pgp can recognize, capture, and eject its substrates as they diffuse across the lipid bilayer of the plasma membrane before reaching the cytosol (Higgins and Gottesman, 1992; Stein, 1997). This is supported by the observation that overexpression of these pumps reduces the unidirectional flux of substrates into the cell, although this may not always be the case, and can vary among substrates in the same cell lines (Stein, 1997). Likewise, when free drug has accumulated in cells that overexpress Pgp, the rate of drug efflux is increased so that the overall impact of these changes in the bidirectional fluxes is to reduce the level of free drug achieved. This has been considered in detail (Stein, 1997). This suppression of drug influx is in contrast to what is observed with the MRP family of exporters, as described in the previous section, in which the influx process may be minimally changed as the substrate released from the carrier associates with the MRP at the cell membrane–cytosolic interface (Fry *et al.*, 1980). Presumably, in this case, the hydrophilic substrate complexed to the carrier is less accessible to the MRP protein.

Among antifolate drugs developed, only a few such as trimetrexate, a DHFR inhibitor, and AG337, a TS inhibitor, are hydrophobic. Neither contains a glutamate moiety and, hence, neither forms polyglutamate derivatives. MTX has not been considered to be a substrate for the MDR family due to its hydrophilicity and carrier-mediated transport into and out of cells. This is consistent with the observation that cell lines that are selected for resistance to trimetrexate and that overexpress the Pgp are crossresistant to a variety of structurally dis-similar antineoplastics, which are substrates for this exporter, but not to MTX (Arkin *et al.*, 1989). Likewise, cells selected for resistance to doxorubicin due to overexpression of Pgp were crossresistant to trimetrexate but not to MTX (Assaraf *et al.*, 1989).

However, when cells are subjected to very high concentrations of MTX, so that passive diffusion is an important component of uptake, resistance has been associated with the multidrug-resistance phenotype and crossresistance to agents that are substrates for Pgp (Norris *et al.*, 1996). MDR1-mediated resistance is more likely to occur when RFC-mediated transport is impaired, requiring high concentrations of drug during the selection. For instance, increased expression of Pgp was identified in a human leukemia CCRF-CEM line with a premature stop mutation in the RFC gene, which was subsequently selected with high levels of MTX (Gifford *et al.*, 1998). This was further clarified by transfection of MDR1 into murine 3T6 fibroblasts with and without functional RFC (De Graaf *et al.*, 1996). The expression of this gene augmented MTX resistance only in those cells already resistant due to a defect in RFC-mediated MTX transport. Hence, when high doses of MTX or other antifolates are employed and there is a high

passive diffusional component, Pgp can be a factor in drug resistance.

Role of the breast cancer resistance protein (BCRP) in antifolate resistance

As indicated in the previous section, a variety of MRPs transport antifolates, with the induction of resistance upon transfection of these exporters into tumor cells. It would be expected then that cells resistant to anti-neoplastics, such as vincristine, doxorubicin, and VP-16 that are substrates for MRPs, would show cross-resistance to antifolates. This has not been demonstrated as yet. There is, however, evidence that overexpression of BCRP, the ABC half-transporter (Doyle *et al.*, 1998; Ross *et al.*, 1999a), detected in the MCF7/MX breast cancer cell line selected for resistance to mitoxantrone results in crossresistance to MTX (Volk *et al.*, 2000). In this cell line, the net transport of MTX was markedly decreased as compared to wild-type cells, there was substantial reduction in the level of polyglutamate derivatives, in particular those of high chain length, but no alteration in γ -GH or FPGS protein or activities. Neither were there changes in MTX influx kinetics nor in RFC protein. The data suggested that resistance was due to enhanced efflux of MTX, resulting in lower free monoglutamate levels and reduced accumulation of polyglutamate derivatives. Yet, Western blot indicated no difference in the expression of MRP1-5 between the two cell lines, and transfection of BCRP cDNA into breast cancer cell lines conferred resistance to mitoxantrone but not to MTX. A subsequent study suggested an explanation for these findings – amino-acid differences at position 482 that affect BCRP substrate specificity (Honjo *et al.*, 2001; Allen *et al.*, 2002). In the MCF7/MX line with BCRP Arg482, suppression of MTX accumulation was greater than in cell lines with Thr482 or Gly482; and both the depression in MTX accumulation and resistance were reversed with the BCRP inhibitor GF120918 (Volk *et al.*, 2002). The original transfection utilized the Thr482 cDNA. Recently, this explanation was confirmed by transfection of the variant BCRP forms in HEK 293 cells which demonstrated that, in contrast to the wild-type R482 transporter, the T482 and G482 variants do not transport MTX (Chen *et al.*, 2003).

The prevalence of BCRP in human tumors and the level of expression after clinical administration of agents, such as doxorubicin, topotecan, VP-16, and irinotecan, which are substrates for this exporter, will be of considerable interest. Of further interest will be the frequency and extent of crossresistance to antifolates in addition to MTX; in particular, the new-generation TS inhibitor, pemetrexed, which will be a component of multidrug regimens that are likely to include agents that are substrates for this exporter. It is of interest, however, that crossresistance in the MCF7/MX line did not extend to either ZD1694 or DDATHF, suggesting that these

antifolates might be poor substrates for this efflux route (Volk *et al.*, 2000).

Impaired accumulation of antifolate polyglutamates as a basis for drug resistance

Alterations in FPGS

As described above, similar to the natural folates, the majority of the classical antifolates and many antifolates currently under development are converted to polyglutamated derivatives in tumor cells. The addition of multiple glutamate residues, one by one, at the γ -carboxyl group of the folate molecule is catalysed by FPGS, which is present in both the cytosol and mitochondria. Polyglutamation is a key determinant of drug activity: (i) These derivatives are retained and accumulate to substantial levels in cells, since they are not substrates for RFC or MRPs. (ii) Polyglutamation generally enhances affinities for THF-cofactor-requiring enzymes. For example, the affinity of ZD1694 and pemetrexed pentaglutamate for TS is increased a 100-fold over the monoglutamates, and for the latter there is a similar increase in affinity for GARFT (Shih *et al.*, 1997). The affinity of DDATHF for GARFT is increased by polyglutamation, although the monoglutamate is itself a potent inhibitor of this enzyme (Sanghani and Moran, 1997). LY309887 is a very potent inhibitor of this enzyme in the monoglutamyl form; and the major impact of polyglutamation is to ensure retention and concentration within the cell (Sanghani and Moran, 1997; Zhao *et al.*, 2001).

Impaired polyglutamation of antifolates can be due to changes at the level of FPGS and/or changes in transport. Hence, either decreased influx or increased export will decrease the free monoglutamyl substrate available for FPGS, and will decrease the rate and possibly the extent of antifolate polyglutamate formation. Alterations in the influx mechanism, such as RFC, are relatively easy to identify by measuring initial uptake rates, and when influx is impaired, it is expected that free drug levels will decline as well. Identifying an additional alteration at the level of FPGS may be difficult to discriminate in the intact cell and will require assay of enzyme activity or protein. These parameters may change without a change in FPGS message – see below, or transport and FPGS activity might be unchanged or minimally changed, but polyglutamation suppressed due to increased cellular THF-cofactors with feedback inhibition of this enzyme.

The impact of impaired polyglutamylation on the level of drug resistance will depend on the specific antifolate and the duration of exposure to the drug. For instance, under conditions of continuous exposure of cells to MTX, decreased polyglutamation has little effect on cytotoxicity because MTX and its polyglutamates are comparable inhibitors of DHFR (Barnes *et al.*, 1999; Zhao *et al.*, 2000e). On the other hand, when polyglutamation is required for activity, even prolonged exposure to the drug cannot circumvent the lack of

formation of these derivatives. Brief exposure of tumor cells to drug in *in vitro* systems best resembles the clinical setting when the agent is administered as a pulse and the monoglutamate is cleared rapidly from the blood. Only those tumors that accumulate substantial levels of polyglutamylated drug will have sustained inhibition of target enzyme, as the monoglutamates exit the cell with the decline in blood levels.

Decreased polyglutamation has been identified as an important element in MTX resistance, although the specific defect(s) that were the basis for this phenomenon were not clearly identified in earlier studies (Cowan and Jolivet, 1984; Pizzorno *et al.*, 1988). Reduced accumulation of MTX polyglutamates contributed to acquired and intrinsic MTX resistance in several human cell lines. These included breast cancer (MTX^R-ZR-75), CCRF-CEM leukemia, soft-tissue sarcoma, and squamous carcinoma cell lines (Pizzorno *et al.*, 1989; McCloskey *et al.*, 1991; Li *et al.*, 1992a; Barnes *et al.*, 1999; Mauritz *et al.*, 2002). In several cases, other changes, such as decreased membrane transport or amplification of DHFR, were also detected in resistant cells. A decrease in FPGS activity was noted in murine leukemia L1210 cells resistant to ZD1694 (Jackman *et al.*, 1995), in human CCRF-CEM leukemia cells resistant to MTX with crossresistance to DDATHF (McCloskey *et al.*, 1991; Pizzorno *et al.*, 1991), and in colon carcinoma cells resistant to ZD1694 (Lu *et al.*, 1995; Drake *et al.*, 1996). Low FPGS activity was associated with rapid recovery of TS activity, consistent with the role that polyglutamylation plays in sustaining the inhibition of target enzymes (Lu *et al.*, 1995). In general, resistance due to decreased FPGS activity affects all antifolates that require polyglutamation for activity, although the degree of resistance can vary among the drugs. The lower levels of MTX polyglutamates (with lower chain lengths) in T-lineage ALL, as compared to B-lineage ALL, is associated with both a decrease in transport and FPGS activity. Lower polyglutamate levels may be the basis, in part, for the poor response of T-cell ALL to chemotherapies that include MTX (Barredo *et al.*, 1994; Galpin *et al.*, 1997).

With the cloning of FPGS, it became possible to discriminate between loss of activity due to decreased expression versus mutations in the enzyme, and to correlate alterations in message with enzyme activity. FPGS mutations were identified in both alleles in DDATHF-resistant murine leukemia L1210 cell lines (Zhao *et al.*, 2000e). These cells were crossresistant upon continuous exposure to LY308887, ZD1694, and pemetrexed, but not to ZD9331, the latter an agent that does not undergo polyglutamation. There were no alterations in membrane transport and in the activities of GARFT and γ -GH. Several of the alleles in different lines had similar mutations. All were in residues conserved across species and clustered in four distinct regions either in, or close to, the binding sites for ATP, glutamate, or folate, based on the *L. casei* FPGS crystal structure (Sun *et al.*, 1998) and an analysis of structure-function of the human enzyme by site-directed mutagenesis (Sanghani *et al.*, 1999). These mutations

markedly impaired the polyglutamylated of natural folates, yet there was sufficient accumulation to sustain growth. Cell lines with acquired resistance to MTX (McGuire *et al.*, 1995; McGuire and Russell, 1998), ZD1694 (Drake *et al.*, 1996), edatrexate (Roy *et al.*, 1997), or DDATHF (Pizzorno *et al.*, 1995) had marked reductions in FPGS activity and protein, without associated changes in FPGS message consistent with the alterations in post-transcriptional regulation of this gene. Likewise, loss of FPGS expression, often without a decrease in message, was noted to be a frequent basis for resistance to a variety of polyglutamatable antifolates in human leukemia cells. This was associated with collateral sensitivity to AG377 and trimetrexate attributed to the contraction of cellular folate pools (Liani *et al.*, 2003). A decrease in both FPGS and RFC message, associated with decreased transport and polyglutamation, was noted with acquired resistance to pemetrexed in murine leukemia cells (Wang *et al.*, 2003).

The impact of FPGS on antifolate activity has also been assessed by transfection of the cDNA into tumor cells. Overexpression of hFPGS in CHO cells increased sensitivity to a brief exposure to MTX (Kim *et al.*, 1993). Rat gliosarcoma cells infected with this gene had increased sensitivity to 4 h pulses of MTX or edatrexate. Animals inoculated with these tumors survived longer with bolus antifolate treatment (Aghi *et al.*, 1999). This was proposed as a gene therapy approach to overcome the intrinsic resistance of certain tumors to antifolates due to low endogenous FPGS activity.

Alterations in γ -GH activity

γ -GH are lysosomal and secreted glycoproteins that hydrolyse folate and antifolate polyglutamates (Galivan *et al.*, 2000). Functionally, the role of γ -GH is opposite to that of FPGS. Owing to this, it is expected that when γ -GH activity is high, the intracellular levels of antifolate polyglutamates achieved will be decreased; and the rate at which these congeners are cleaved and the monoglutamate drug leaves the cells will be increased after pulse administration of the drug. This turned out to be the case in several studies on acquired resistance to antifolates. For instance, a human H35 hepatoma cell line selected for resistance to DDATHF had a sevenfold increase in γ -GH activity, but no change in FPGS activity (Rhee *et al.*, 1993). The loss of γ -GH activity resulted in resistance to 10-propargyl-5,8-dideazafolate for which transport was not impaired. Low-level increase in γ -GH was also detected in DDATHF-resistant cells (Pizzorno *et al.*, 1995). In a clinical correlative study, the ratio of γ -GH to FPGS was the best predictor of the extent of MTX polyglutamate formation in acute leukemia cells, suggesting that the activity of γ -GH may be an important factor in drug activity (Longo *et al.*, 1997).

On the other hand, when human γ -GH cDNA was transfected into human HT1080 fibrosarcoma and MCF-7 breast carcinoma cells, although the γ -GH activity was markedly increased in the stable transfectants, the cells were not resistant to MTX after a brief

exposure to the drug (Cole *et al.*, 2001). While there was no change in the MTX polyglutamate level in HT1080 cells, there was ~50% decrease in MTX polyglutamates in the MCF-7 cells accompanied by a comparable decrease in the 5-CH₃-THF level. The latter may have compensated for lesser accumulation of antifolate.

Impact of alterations in cellular THF-cofactors

It is now well established that intracellular THF-cofactor pools modulate the activity of antifolates. This is based in large part on the impact of these cofactors on the polyglutamation of antifolates (Nimec and Galivan, 1983; Johnson *et al.*, 1988; Van der Wilt *et al.*, 2001; Zhao *et al.*, 2001). Cell lines grown with low folic acid concentrations are much more sensitive to a variety of antifolates than cells grown in higher concentrations of folic acid (Jansen *et al.*, 1998). Augmentation of the intracellular folate pool, either due to decreased efflux of folic acid or increased influx of folic acid due to mutations in RFC, render cells resistant to antifolates, particularly those that require polyglutamylation for activity (Assaraf and Goldman, 1997; Tse and Moran, 1998; Jansen *et al.*, 1999). Modulation of antifolate activity *in vivo* is likely to be more complex, in that folate depletion in some tumors has been associated with increased expression of TS and thymidine kinase (Van der Wilt *et al.*, 2001). In tumor-bearing animals, folate deficiency markedly increases the toxicity of DDATHF

and pemetrexed (Alati *et al.*, 1996; Worzalla *et al.*, 1998). The therapeutic efficacy of DDATHF is improved in folate-replete animals, although the therapeutic window is narrow. Pemetrexed activity is also increased with folate repletion, but this is associated with a wide therapeutic window.

One study systematically quantitated the effect of the intracellular THF-cofactor pool size on the activity of a variety of antifolates by growing L1210 cells in different concentrations of 5-CHO-THF (Zhao *et al.*, 2001). The cellular THF-cofactor pool increased nearly in proportion to the ~100-fold rise in extracellular 5-CHO-THF concentration from 0.64 to 62.5 nM – encompassing concentrations below, within, and above the physiological range of plasma 5-CH₃-THF. As the folate level was increased, the intracellular level of DDATHF and pemetrexed polyglutamates decreased. This was associated with a marked increase in the IC₅₀ for these agents (DDATHF > pemetrexed) on continuous exposure to the drugs (Figure 5). The higher the affinity of the monoglutamate for the target enzyme and FPGS, the less the adverse impact of the increase in cellular folate pool (Table 1). There was virtually no effect of folate pool size on the activity of ZD9331, a potent TS inhibitor that does not undergo polyglutamation, consistent with a lack of competition between ZD9331 and 5,10-CH₂-THF at the level of this enzyme (Jackman *et al.*, 1997). For DHFR inhibitors, the more potent the inhibition of DHFR, the less was the modulating effect of the cellular THF-cofactor pool. Hence, when the cellular THF-cofactor pool was high, trimetrexate activity was markedly decreased. This is a relatively weak DHFR inhibitor that does not undergo polyglutamation (Jackson *et al.*, 1984). On the other hand, PT523 & PT632 which also do not undergo polyglutamation but are more potent inhibitors of DHFR than MTX (PT632 > PT523) (Rosowsky *et al.*, 1998; Wright *et al.*, 2000) are minimally affected by the folate pool size. MTX activity was modestly affected by the THF-cofactor pool size. The impact of folate pools on 4-amino DHFR inhibitors is attributed to competition between the drug and DHF at the target enzyme (see above). The higher the level of THF-cofactors, the greater the rise in DHF, as these cofactors interconvert to this product with inhibition of DHFR activity. For all the agents that undergo polyglutamation, alterations in the THF-cofactor pool size have a much more profound impact on activity when the duration of exposure to drugs is brief in contrast to continuous exposure, as the interval over which polyglutamates can build up in the cells is abbreviated (Zhao *et al.*, 2001).

THF-cofactor levels in cells depend not only on the level of folic acid or 5-CHO-THF in the medium *in vitro* or plasma 5-CH₃-THF level *in vivo*, but also on levels of expression of other proteins, such as RFC, γ -GH, and FPGS, since natural folates are also substrates for these transporter/enzymes. Increased expression of RFC and FPGS or decreased expression of γ -GH should augment accumulation of antifolates and the THF-cofactor pool. The opposite changes should depress the folate pool.

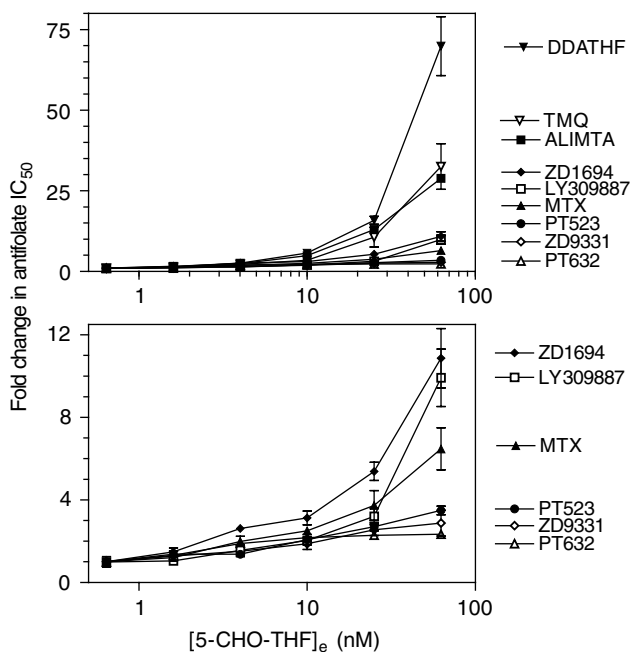


Figure 5 Increase in IC₅₀ for continuous exposure to a variety of antifolates as a function of the level of cellular THF cofactors. Fold increase on the ordinate is the ratio of IC₅₀ at any extracellular 5-CHO-THF level to the IC₅₀ at the lowest extracellular 5-CHO-THF concentration (0.64 nM). The data are from Zhao *et al.* (2001), except for PT632 (5,8-dideazaPT523), a structural analog of PT523 with higher affinity for DHFR (Wright *et al.*, 2000)

Changes in folate pool size will modulate the impact of resistance mechanisms on drug activity. The changes can be an important element that must be considered, along with assessment of transport, FPGS, and target enzymes, in tumor response to antifolates and the crossresistance pattern. The most dramatic example (described above) was a cell line highly resistant to MTX due to a mutation in RFC that was collaterally sensitive to DDATHF when grown in 5-CHO-THF. In this case, impaired transport of 5-CHO-THF, mediated by the defective carrier, depleted cellular THF-cofactors, thereby relaxing the usual feedback inhibition of DDATHF polyglutamation by these endogenous substrates. A similar phenomenon was observed in an earlier study with MTX^{ZR75-1} breast cancer cells, selected for resistance to MTX with markedly impaired influx V_{max} (Dixon *et al.*, 1991). These cells were collaterally sensitive (10-fold) to trimetrexate when grown in 5-CHO-THF, consistent with depletion of cell folates and decreased competition between this agent and DHF at the level of DHFR. In general, when RFC function is impaired and folate transport and accumulation is decreased, there is collateral sensitivity to lipid-soluble antineoplastics.

Another interesting example of this phenomenon was observed in a Chinese hamster ovary cell line (Pyr^R100), selected for resistance to the lipid-soluble DHFR inhibitor pyrimethamine. These cells lost energy-dependent folate export mediated by MRPI (Assaraf and Goldman, 1997; Stark *et al.*, 2003), resulting in marked expansion of cellular THF-cofactor pools (Jansen *et al.*, 1999) and a crossresistance pattern that encompassed antifolates that require polyglutamation for activity (DDATHF, AG2034) and two that do not, AG377 a lipid-soluble TS inhibitor and trimetrexate. The latter is in contrast to the lack of effect of cellular THF-cofactor expansion on the activity of ZD9331 (see above), suggesting competition between 5-10-CH₂-THF and AG337 at the level of TS. Likewise, the cell line resistant to DDATHF due to a mutation in RFC that markedly increased affinity for folic acid expanded the cellular folate cofactor pool only when grown with this substrate (Tse *et al.*, 1998; Tse and Moran, 1998). Under these conditions, there was crossresistance to ZD1694 and to MTX, especially when the duration of exposure to the drug was brief.

Plasma folate levels do not necessarily reflect cellular folate stores or the competence of THF-cofactor-dependent reactions in cells. Hence, normal plasma folate levels could be present despite impaired transport or utilization of folates in tumors or folate-dependent normal tissues. A highly sensitive indicator of cellular folate deficiency or impaired cellular metabolism of folates is plasma homocysteine, which is utilized in the synthesis of 5-CH₃-THF and rises when folates or vitamin B12 are deficient. Homocysteine correlates closely with clinical toxicity to pemetrexed, and supplementation with both folic acid and B12 reduced the incidence of toxicity to this drug (Calvert, 2002; Niyikiza *et al.*, 2002). Likewise, DDATHF toxicity has been substantially reduced by coadministration of low

oral doses of folic acid (Wedge *et al.*, 1995; Roberts *et al.*, 2000).

Alterations in DHFR

A common mechanism of acquired MTX resistance in cultured cells is gene amplification, which occurs in response to sustained exposure to this drug (Schimke, 1988). The process involves an increase in the DHFR gene copy, mRNA, and enzyme. The amplified genes can be found at the DHFR locus as a homogenous staining region on chromosome 21 (Dolnick *et al.*, 1979; Mini *et al.*, 1985) or can be contained in microsatellites (Kaufman *et al.*, 1979; Curt *et al.*, 1983). The former type of amplification is much more stable as the latter is lost during cell division. Low-level gene amplification has been identified in tissue specimens from patients with acute lymphoblastic leukemia (ALL) (Horns *et al.*, 1984) and ovarian cancer (Trent *et al.*, 1984) treated with MTX, indicating that this process plays a role in clinical drug resistance. The low-level amplification detected prior to treatment in human soft-tissue sarcomas suggests that this may be one basis for intrinsic resistance in this disease requiring high-dose therapies (Li *et al.*, 1992b). This mechanism of resistance was subsequently shown to be relevant to resistance to other antifolates and other classes of drugs, most recently STI-571 (Gorre *et al.*, 2001).

Early on, alterations in the affinity of DHFR for MTX among a variety of mouse and human cell lines was shown to correlate with intrinsic drug resistance (Jackson *et al.*, 1976). These changes in the properties of DHFR suggested that MTX resistance was the result of mutations in the protein (Albrecht *et al.*, 1972; Flintoff *et al.*, 1976; Goldie *et al.*, 1980). This was subsequently confirmed. Many tumor cell lines with acquired resistance to 4-amino antifolates have mutations in DHFR that decrease binding of the inhibitor, usually with a lesser decrease in catalytic activity for the DHF substrate. Among the mutated sites identified was leu22Arg in the murine gene located at the MTX-binding site (Simonsen and Levinson, 1983). Mutations were identified in MTX-resistant hamster lung cancer cell lines including a Leu22Phe substitution (Melera *et al.*, 1984; Melera *et al.*, 1988). A variety of human cell lines have been selected for resistance to MTX due to mutations in DHFR. For instance, a Phe31Ser mutation at the ligand-binding site was detected in both MOLT-3 leukemia (Miyachi *et al.*, 1995) and HCT-8R4 (Srimatkandada *et al.*, 1989) human colon cancer cell lines. A mutation at this site, Phe31Trp, was also identified in mouse leukemia cells (McIvor and Simonsen, 1990). Often a DHFR mutation accompanies amplification of the enzyme, possibly a requirement to sustain catalytic activity under conditions in which affinity for the DHF substrate is decreased (Srimatkandada *et al.*, 1989; Dicker *et al.*, 1990; Miyachi *et al.*, 1995). Mutations may not involve binding or catalytic sites. For example, a Gly15Trp mutation in DHFR from L1210 leukemia cells selected for resistance to MTX resulted in a

decrease in affinity for 4-amino-antifolates (with some variability) with a lesser decrease in affinity for DHF, although this highly conserved residue does not appear to interact with substrate, inhibitor, or cofactor, and is located away from the active site of the enzyme (Dicker *et al.*, 1993). The impact of a mutation can be influenced by allelic variation at another site. For instance, the alteration in MTX binding associated with the Leu22Phe mutation is influenced by the residue at position 95 of the CHO enzyme (Yu and Melera, 1993). Despite the frequent occurrence of DHFR mutations in tumor cell lines, mutant forms of DHFR have not been found in tumors from patients treated with MTX (Spencer *et al.*, 1996).

An increase in the DHFR protein occurs rapidly in response to 4-amino antifolates in the absence of amplification or a commensurate increase in message. This has been attributed to a phenomenon in which the DHFR message binds to DHFR, thus regulating its availability for translation. In the presence of an antifolate, the message is released, translation is enhanced, and synthesis of protein is increased (Chu *et al.*, 1993; Ercikan *et al.*, 1993; Ercikan-Abali *et al.*, 1997; Schmitz *et al.*, 2001). Regulation of DHFR expression by this mechanism was absent in the malaria parasite and suggested as a factor in the utility of antifolates in the treatment of this disease (Zhang and Rathod, 2002). A similar mechanism has been proposed for the increase in TS protein after exposure of cells to antifolates or fluoropyrimidines (see below).

Alterations in TS

TS catalyses the conversion of dUMP and 5–10 methylene THF to dTMP and dihydrofolate, and is the sole *de novo* source of deoxythymidylate for DNA replication. Owing to its critical role in DNA biosynthesis, TS has been an important target for chemotherapy, starting with the introduction of fluorouracil (Heidelberg *et al.*, 1957), the second class of antimetabolites to enter the clinic. New-generation antifolates, such as ZD1694, ZD9331, pemetrexed, and AG337, are potent inhibitors of TS. Acquired resistance to antifolates that target TS has been associated with increased expression or, in a few cases, mutations in this enzyme that alter drug binding.

Overexpression of TS is an important mechanism of resistance in cell lines selected in the presence of antifolate inhibitors of this site. As observed with DHFR, this is frequently associated with gene amplification. For example, resistance to ZD1694 in human MCF-7 breast cancer, lymphoblastoid, and ovarian carcinoma cell lines, or to ICI-198584 was associated with TS amplification (O'Connor *et al.*, 1992; Freemantle *et al.*, 1995; Jackman *et al.*, 1995; Drake *et al.*, 1996; Tong *et al.*, 1998a; Kitchens *et al.*, 1999a). The relationship between the increase in expression and the level of resistance can vary. Hence, while 20 000-fold resistance in human lymphoblastoid cells was associated

with a 1000-fold increase in the TS protein level, 14-fold resistance in ovarian carcinoma cells was associated with only a 2.5-fold increase in TS activity (Freemantle *et al.*, 1995). The degree of resistance due to increased expression of TS can vary among antifolates. For instance, in a cell line in which TS expression was increased by transduction of the cDNA with an inducible promoter, a twofold increase in the IC₅₀ for fluorouracil was accompanied by a 9- and 24- fold increase in IC₅₀'s for ZD1994 and ZD9331 with an even greater degree of resistance to pemetrexed (Longley *et al.*, 2001). Amplification of TS in a human lymphoblastic cell line in response to ZD1694 was associated with a lesser but still high level of resistance to other antifolates including pemetrexed (Jackman *et al.*, 1995). On the other hand, a much lesser degree of resistance to pemetrexed was observed in cells selected for resistance to ZD1694 (Schultz *et al.*, 1999). The lower levels of pemetrexed resistance as compared to ZD1694 could be due to pemetrexed's inhibition, albeit to a lesser extent, of GAR transformylase (Shih *et al.*, 1997). In many of these studies, differences in degrees of resistance and patterns of resistance may be attributed to multiple factors that include drug transport and polyglutamation as well as changes in cellular folate pools that must be assessed and considered.

Increases in TS enzyme have also been observed after a brief exposure to the drug based on post-transcriptional changes. For instance, a dose-dependent rise in TS activity after exposure to a variety of TS antifolate inhibitors persisted for only 12 h after drug was removed (Welsh *et al.*, 2000). This was attributed to the same autoregulatory mechanism proposed for antifolate resistance to DHFR described in the previous section. The TS message binds to the enzyme, limiting the transcript available for translation. According to this paradigm, TS inhibitors disrupt the complex, release the message, and translation ensues (Chu *et al.*, 1994; Chu and Allegra, 1996; Schmitz *et al.*, 2001). However, other studies demonstrated that mutation of the protein-message-binding site does not alter translation; rather, increased TS levels could be accounted for by an increase in the stability of the protein (Kitchens *et al.*, 1999a,b). A Pro303Leu mutation in hTS resulted in marked instability of an amplified enzyme, with a reduced half-life resulting in a high level of message relative to enzyme associated with resistance to fluorodeoxyuridine and antifolates (Kitchens *et al.*, 1999a). Interestingly, the induction of TS on exposure to a variety of antifolate inhibitors was greater in nontransformed human fibroblasts and intestinal epithelial cells than in tumor cells, one potential basis for the inherent selectivity of these agents (Welsh *et al.*, 2000).

A very unusual resistance mechanism was observed in a TS-deficient, aminopterin-resistant, human colon cancer cell line subsequently selected for high-level resistance to MTX in the presence of thymidine. The TS activity was absent when grown in low levels of 5-CHO-THF, but present when grown at high folate levels. This was associated with a fivefold increase in the

K_m for 5,10-CH₂-THF due to the presence of two mutations, Asp218Asn and His256Tyr, sites that would affect binding of the folate-cofactor to TS (Houghton *et al.*, 1992). While the selections were likely to be too arcane to be relevant to the clinical setting or even usual laboratory conditions, nonetheless this study demonstrated that mutations could arise that affect binding of the folate-cofactor. Other studies suggested that alterations of the folate-binding site on TS can be the basis for fluorouracil resistance. A naturally occurring Tyr33His mutation in the human enzyme decreased FdUMP binding only in the presence of 5,10-CH₂THF (Barbour *et al.*, 1992; Reilly *et al.*, 1997); when the bacterial enzyme was mutated at the corresponding residue, decreased binding of this folate was demonstrated (Mahdavian *et al.*, 1999). It is possible that changes at this site would also decrease binding of antifolate inhibitors to TS.

Recent studies demonstrate that mutations in TS render cells resistant to antifolate inhibitors of this enzyme. Several mutations in a highly conserved region (Lys47Glu, Asp49Gly, Gly52Ser) were identified in human sarcoma HT1080 cells selected for resistance to AG337, a lipid-soluble inhibitor that does not form polyglutamate derivatives and is transported by passive diffusion (Webber *et al.*, 1996; Tong *et al.*, 1998a). Changes induced by the first two mutations were highly selective. Cells were crossresistant to fluorouracil but not to ZD1694 or GW1843U89, consistent with differences in the binding sites for these antifolates (Tong *et al.*, 1998a). Similarly, site-directed mutagenesis of highly conserved residues confirmed the selective effects of mutations of this enzyme. For instance, Ile108Ala produced marked resistance to ZD1694 and AG337, with lesser resistance to GW1843U89. A Phe225Trp mutation produced 17-fold resistance to the latter compound and about half the level of resistance to fluorouracil, without any increase in the IC₅₀, or the kinetics of enzyme inhibition by ZD1694 and AG337 (Tong *et al.*, 1998b). As observed for DHFR, mutations in TS that arise during selection can be accompanied by amplification of the enzyme, probably required for sufficient catalytic activity for the natural substrate to sustain cell replication (Tong *et al.*, 1998a).

Alterations in GARFT

Although cancer cell lines resistant to the GARFT inhibitor DDATHF have been obtained, significant alterations in the level of this enzyme or mutations in this gene have not been identified. Instead, resistance to DDATHF has been associated with decreased FPGS activity, increased γ -GH activity, decreased drug transport, and/or increased folic acid transport as described in other sections. Hence, at this point it would appear that resistance to GARFT inhibitors is more likely to be associated with changes in sites other than the target enzyme.

Novel gene therapy approaches that exploit mutations in target enzymes that result in drug resistance

The identification of mutations in DHFR and TS that result in drug resistance was the basis for initiatives in which mutant enzymes, identified by selection *in vitro* or by site-directed mutagenesis, were transduced into bone marrow progenitor cells as an approach to protecting these cells from toxicity when infused back into animals or patients who are subsequently treated with antifolates. This approach facilitates enrichment of populations of progenitor cells transfected with mutant enzyme on exposure to antifolate *in vitro*. The strategy has been utilized to achieve resistance to MTX with mutated DHFR in murine progenitor cells (Banerjee *et al.*, 1994; Flasshove *et al.*, 1995a; Takebe *et al.*, 2000) and human CD34+ peripheral human cells *in vitro* and *in vivo* (Flasshove *et al.*, 1995b; Sorrentino *et al.*, 1999). Bicistronic retroviral vectors have been used to expand the spectrum of resistance in progenitors, as with the transduction of both mutated DHFR and Pgp into murine bone marrow progenitors (Galipeau *et al.*, 1997), or with DHFR and cytidine deaminase conferring resistance to both MTX and cytosine arabinoside (Sauerbrey *et al.*, 1999), or with cotransfection of mutated DHFR and wild-type aldehyde dehydrogenase to achieve resistance to both MTX and cyclophosphamide (Takebe *et al.*, 2001). Transplantation of transduced CD34+ cells into irradiated mice in the latter case resulted in substantial protection of the bone marrow from drug toxicity. This approach is also being developed using mutated forms of TS and a TS-DHFR fusion protein (Banerjee *et al.*, 1999; Capioux *et al.*, 2003).

Clinical studies in ALL and osteosarcoma correlating clinical MTX efficacy with cellular pharmacokinetic properties

Over the years, there have been extensive correlative studies within the context of clinical trials in an attempt to understand the determinants of response to methotrexate in acute leukemia. These findings point to the cellular accumulation of polyglutamate derivatives as a critical endpoint of treatment.

Treatment of children with B-lineage ALL is associated with a much better outcome than occurs with T-lineage ALL. This is also associated with a higher level of accumulation of MTX polyglutamates, along with a higher percentage of long-chain-length derivatives, in lymphoblasts obtained from patients after administration of the drug *in vivo* (Synold *et al.*, 1994; Galpin *et al.*, 1997), or when cellular antifolate was measured after exposure of the drug to lymphoblasts *in vitro* (Whitehead *et al.*, 1990). The improved outcome of treatment of children, in contrast to adults, with B- or T-lineage ALL also correlated with the accumulation of higher levels of MTX polyglutamates and a higher ratio of long-chain-length derivatives in lymphoblasts after

treatment (Göker *et al.*, 1993). Treatment of children with high-dose MTX resulted in higher levels of MTX polyglutamates, and higher long-chain derivatives in both B- and T-lineage ALL, although the level in the former was higher than in the latter (Synold *et al.*, 1994). Higher levels of MTX polyglutamates and the ratio of long-chain derivatives correlated with the degree of cytoreduction after high-dose MTX (Masson *et al.*, 1996).

The level of accumulation of MTX polyglutamates in B-lymphoblasts correlates with hyperdiploidy and trisomy 21, and this in turn correlates with RFC expression, a gene that maps to this chromosome. For instance, RFC expression is far greater in hyperdiploid than nonhyperdiploid pre-B- or B-lineage lymphoblasts in ALL (Whitehead *et al.*, 1992, 1998b; Zhang *et al.*, 1998; Belkov *et al.*, 1999); but there was no difference in the expression between nonhyperdiploid B- versus T-lineage lymphoblasts (Belkov *et al.*, 1999). RFC message (Zhang *et al.*, 1998; Belkov *et al.*, 1999) and MTX polyglutamate accumulation (Belkov *et al.*, 1999) correlated with the number of copies of chromosome 21.

MTX transport has been measured in lymphoblasts *in vitro* and correlated with response to treatment. For instance, 13% of patients with untreated ALL, and 70% of patients with relapsed ALL showed impaired transport. In the majority of the latter for whom RNA was available, the RFC1 message was decreased; however, in some cases transport was impaired without a decrease in expression, suggesting the possibility of mutations in the carrier (Gorlick *et al.*, 1997). However, to date, no mutations in RFC have been identified in lymphoblasts obtained from patients. In particular, a search for the Glu45Lys mutation within the first TMD of RFC, detected frequently in a variety of preclinical studies, was not identified in clinical specimens (Whetsline *et al.*, 2001; Gifford *et al.*, 2002).

The level of MTX polyglutamates that accumulate in lymphoblasts is also determined by FPGS activity, and this was higher in B-lineage ALL blasts than ANLL blasts before treatment (Barredo *et al.*, 1994). After treatment, FPGS activity and the percentage of long-chain polyglutamates increased to a greater extent in B- than T-lineage ALL lymphoblasts (Barredo *et al.*, 1994). The level of MTX polyglutamates and the ratio of long-chain derivatives that accumulated in myeloid and

lymphoid leukemia cells *in vitro* was found to correlate best with the ratio of γ -GH:FPGS rather than with either enzyme activity alone (Longo *et al.*, 1997).

Response to treatment does not always correlate with the level of MTX polyglutamate accumulation, indicating that other factors must be at play. For instance, children with B-progenitor ALL with less than 50 chromosomes and translocations of the short arm of chromosome 12 have low-level accumulation of MTX polyglutamates and long-term survival (Whitehead *et al.*, 1998a). In the case of the TEL-AML1 translocation, associated with an excellent clinical outcome, the level of MTX polyglutamates was lower, and hyperdiploidy was rare, in lymphoblasts from patients with, as compared to those without, this translocation (Whitehead *et al.*, 2001). The excellent survival of patients with trisomy of chromosomes 4 and 10 was not associated with augmentation of the accumulation of MTX polyglutamates (Whitehead *et al.*, 1998b). This lack of sole dependence of treatment outcome on polyglutamation of MTX is not unexpected, since all regimens contain antineoplastics in addition to MTX.

Osteosarcoma is another disease in which MTX has been a major element in treatment, particularly high-dose therapies because of the intrinsic resistance of this disease to this drug. In a study involving 42 patients, on the initial biopsy, 65% of the patients had low levels of RFC expression. In all, 36% of the patients with a good response to treatment, and 65% of the patients with a poor response to treatment, had low levels of RFC mRNA, respectively (Guo *et al.*, 1999). Recently, RFC sequence variants were noted in human osteogenic sarcoma tissues involving exons 2 and 3 (Yang *et al.*, 2003).

Abbreviations

MTX, methotrexate; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; TS, thymidylate synthase; γ -GH, gamma glutamyl hydrolase; GARFT, glycinamide ribonucleotide transformylase; AICARFT, phosphoribosylaminoimidazolecarboxamide transformylase; 5-CH₃-THF, 5-methyl tetrahydrofolate; 5-CHO-THF, 5-formyl tetrahydrofolate; 5,10-CH₂-THF, 5,10-methylene tetrahydrofolate; DHF, dihydrofolate; THF, tetrahydrofolate; dTMP, deoxythymidylate; dUMP, deoxyuridylate, SHMT, serinehydroxymethyltransferase; TMD, transmembrane domain.

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