

Pemetrexed: biochemical and cellular pharmacology, mechanisms, and clinical applications

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Abstract

Pemetrexed is a new-generation antifolate, approved for the treatment of mesothelioma and non-small cell lung cancer, currently being evaluated for the treatment of a variety of other solid tumors. This review traces the history of antifolates that led to the development of pemetrexed and describes the unique properties of this agent that distinguish it from other antifolates. These include (a) its very rapid conversion to active polyglutamate derivatives in cells that build to high levels and are retained for long intervals to achieve prolonged and potent inhibition of its major target enzyme thymidylate synthase, (b) its high affinity for three folate transporters, and (c) its marked sensitivity to the level of physiologic folates in cells. The latter results in the unique and paradoxical finding that when transport mediated by the major folate transporter (the reduced folate carrier) is impaired, pemetrexed activity is preserved. This is due to concurrent contraction of competing cellular physiologic folates and utilization of a novel second transport carrier for which pemetrexed has high affinity, recently identified as the proton-coupled folate transporter (PCFT). Laboratory studies are reviewed that raise the possibility of new approaches to the use of folic acid supplementation in clinical regimens with pemetrexed. [Mol Cancer Ther 2007;6(2):404–17]

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Evolution of Antifolates as Chemotherapeutic Agents

The history of the antifolates as anticancer drugs dates back to the 1940s when Lederle Laboratories discovered the structure of the active bacterial growth-promoting principal isolated from beef liver, now known as folic acid (1). In the process, closely related compounds were synthesized; some of which were found to interfere with folic acid-dependent bacterial growth and negated the antianemic and growth-promoting effects of folic acid in chicks (2). One of the folic acid analogues was aminopterin (4-amino-folic acid).

At that time, there was no specific treatment for children with acute leukemia beyond supportive care. Based upon the observation by Lewisohn et al. at the Mt. Sinai Hospital in New York City that “folic acid concentrate” caused regression of breast cancer in mice (3), clinical investigators led by Sidney Farber at the Children’s Hospital in Boston obtained folic acid polyglutamate conjugates from Lederle Laboratories in an attempt to reproduce these findings in children with cancer (4). To their surprise, the administration of folic acid exacerbated the disease in children with leukemia. This suggested that the proliferation of leukemia cells might be limited by the supply of the vitamin, folic acid, or, as learned later, its active tetrahydrofolate (THF) cofactor metabolites. Thus, Sidney Farber obtained folic acid antagonists from Y. SubbaRow et al. at Lederle Laboratories, and in a historic article in the *New England Journal of Medicine* in 1948 (5), Farber et al. reported that one of these agents, aminopterin, produced complete remissions in children with acute leukemia. At the time, a tent city grew on Boston Commons of families who had brought their children with leukemia to be treated with this miracle drug.

A year later, 2-amino-10-methyl-folic acid (amethopterin, methotrexate) was introduced (6), and because of its purported lesser toxicity, it replaced aminopterin in the clinics. Shortly thereafter, the antifolate action of methotrexate was shown to be due to its potent inhibition of dihydrofolate reductase (DHFR; ref. 7). Over the next three decades, there followed an intensive drug synthesis effort to identify a “better” methotrexate. However, methotrexate was simply a remarkable drug, and none of the hundreds of DHFR inhibitors made proved to be superior. Remarkably, methotrexate remains an important component of the treatment regimens for childhood acute lymphoblastic leukemia (8) and continues to be used for the treatment of a variety of neoplasms as well as rheumatoid arthritis (9) and psoriasis (10).

The understanding of the mechanism of methotrexate action and its selectivity was broadened when it was recognized in the mid-1970s that, like the physiologic folates, methotrexate was metabolized to polyglutamate derivatives in cells (11, 12), and that this metabolism permitted high levels of accumulation of active antifolate that markedly prolonged their retention in tumor cells (13–16), resulting in enhanced chemotherapeutic efficacy. Polyglutamation was also shown to be an important element in the selectivity of this agent. These derivatives accumulate to a much greater extent in susceptible tumor cells than in progenitor cells of the bone marrow or the intestinal tract (17–20). Hence, effects of the drug tend to be transient in the latter host cells as the monoglutamate level increases and decreases with the blood level, whereas methotrexate effects in tumor are sustained due to the accumulation and retention of the polyglutamate congeners. This is an inherent selective advantage for any antifolate that forms these derivatives in cells. This metabolic transformation also resulted in an altered spectrum of activity for methotrexate. As a monoglutamate, methotrexate is a highly potent inhibitor of DHFR, but its polyglutamate derivatives are also inhibitory to 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase and thymidylate synthase (21–23). This insight led to an important new perspective on antifolate actions: the realization that the polyglutamate derivatives and not the parent drug could be the active agents (24). There followed a new focus on the identification of antifolates that achieved their major activities in their polyglutamate forms.

The key to the further development of antifolates came when efforts were directed to the development of inhibitors of THF cofactor-dependent biosynthetic enzymes by avoiding the 2,4-diamino-pteridine pharmacophore that inevitably directly drug to the active site of DHFR. The Imperial Cancer Research Laboratories at Sutton, England and their collaborators at the then ICI Pharmaceutical branch, now AstraZeneca Pharmaceuticals, led a successful effort to improve the inhibition of thymidylate synthase shown by the 5,8-dideazafolates. The key intermediate in this development process was the remarkable compound 10-propargyl-5,8-dideazafolate or CB3717 (25). This compound furnished proof-of-principle that antifolates in their polyglutamate forms could be potent inhibitors of THF cofactor-dependent enzymes, in this case, thymidylate synthase, and useful chemotherapeutic agents. This ultimately led to the discovery and development of raltitrexed or Tomudex, which has been in clinical use in Europe and elsewhere but was never approved in the United States (26).

This review will describe the evolution of the development of the third antifolate (after aminopterin and methotrexate) to achieve approval for the treatment of cancer in the United States (pemetrexed) and the several novel properties of this agent.

Origins of Pemetrexed

The synthesis of pemetrexed evolved from a collaboration between E.C. (Ted) Taylor at Princeton and a team of

chemists at Eli Lilly led by Chuan (Joe) Shih as part of an effort to develop antifolate inhibitors of THF cofactor-dependent enzymes. This drug discovery program stemmed from earlier collaborative development at Princeton, Yale, the University of Southern California, and Lilly of the prototypical inhibitor of *de novo* purine synthesis, 5,10-dideazatetrahydrofolate (lometrexol; 27). The structure of lometrexol had been suggested by G. Peter Beardsley to his former mentor Ted Taylor as a potential antifolate, initially thought to be a likely inhibitor of thymidylate synthase; to their surprise, it was not, but it was a potent inhibitor of tumor cell growth (28). Cell culture experiments (28, 29), then direct enzymology on isolated (29, 30) and recombinant (31) proteins in the laboratory of one of the authors, showed that lometrexol was the first potent antifolate inhibitory to purine synthesis due to its direct suppression of glycinamide ribonucleotide transferase (GARFT) activity. Because this was not a nucleotide analogue, it was considered not to pose a mutagenic nor carcinogenic threat. Subsequently, second- and third-generation GARFT inhibitor analogues were synthesized in an attempt to improve on the therapeutic and toxicologic profiles of lometrexol (32–36).

Lometrexol posed a problem; it was made by a complex 23-step process, and the product was a mixture of diastereomers about carbon 6. The decision was made to separate the diastereomers before clinical trials to meet Food and Drug Administration requirements. However, the fractional crystallization used to obtain a pure diastereomer proved to have low yield (29), and, ultimately, another approach was pursued: exploration of related compounds that would eliminate chirality at the 6-position. One strategy replaced the 5-deazapteridine ring of lometrexol with a pyrrolopyrimidine ring, resulting in LY231514 (37), later to be known as pemetrexed. The outcome was unexpected. Cell culture end-product protection experiments and enzymology indicated that this compound was, primarily, a potent inhibitor of thymidylate synthase, although there was weaker inhibition at other enzyme targets (37, 38).

Biochemical Properties of Pemetrexed: Polyglutamation, End-Product Protection, Effect of Cellular Folates

Several factors contribute to the potency of pemetrexed and its unusual properties: rapid transport of drug across the plasma membrane mediated by several transport processes for which pemetrexed has high affinities, remarkable activity as a substrate for folylpolyglutamate synthetase (FPGS), potent inhibition by its polyglutamate derivatives of THF cofactor-dependent reactions, and marked sensitivity to cellular folate pools. These elements will be considered in the following sections.

Polyglutamation

Early studies (37, 39) showed that the kinetics of pemetrexed polyglutamation mediated by mammalian FPGS are matched by only a handful of all the hundreds

of folate analogues that have been studied. This effect is shown with human FPGS *in vitro* in Fig. 1. The magnitude of this kinetic advantage of pemetrexed over methotrexate results in its efficient polyglutamation at low concentrations, either during early phases of drug exposure or during clearance of drug from the plasma, unlike the gradual polyglutamation profile seen with methotrexate. The effect of the high affinity of pemetrexed for FPGS relative to methotrexate is best illustrated by a comparison of the uptake of these agents in leukemia cells (Fig. 2). It can be seen that the initial uptake rates are comparable, reflecting comparable membrane transport. However, whereas net uptake of methotrexate rapidly reaches a steady state, pemetrexed uptake, while slowing from initial rates, continues at a substantial velocity (Fig. 2, top). This difference is due to the rapid formation of pemetrexed polyglutamate derivatives that are retained within the cell, as judged by the absence of this phase in cells in which FPGS was mutated (Fig. 2, bottom). This marked accumulation of active pemetrexed polyglutamate derivatives within cells represents a substantial pharmacologic advantage for this agent in that it produces both prolonged and high-level suppression of its target enzyme(s).

As indicated in Table 1, the polyglutamation of pemetrexed also results in a marked increase in the ability of this agent to inhibit several THF cofactor-dependent enzymes. The potency of the pentaglutamate of pemetrexed is 100 times greater than that of the monoglutamate as an inhibitor of thymidylate synthase (37, 38), and there is a comparable increase in activity against GARFT (38). However, inhibition of thymidylate synthase is 50 times more potent than inhibition of GARFT. Hence, pemetrexed is primarily a thymidylate synthase inhibitor with weaker secondary inhibition of GARFT (Table 1). Although pemetrexed has even weaker inhibitory effects at additional enzyme sites apart from thymidylate synthase and GARFT, the inhibitor constants are so high that, for this and other reasons, these effects are unlikely to be of any pharmacologic importance.

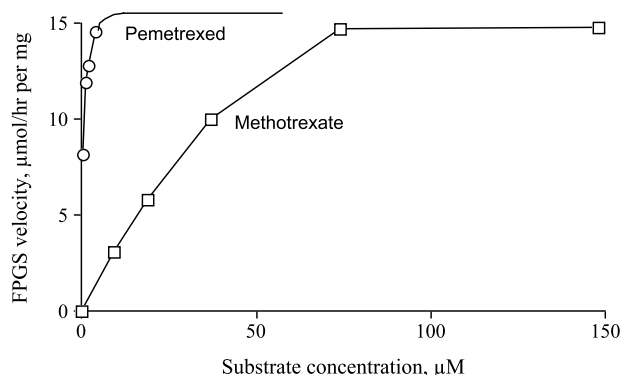


Figure 1. Comparison of pemetrexed and methotrexate polyglutamylation kinetics. Pemetrexed has a far lower Michaelis constant for human recombinant FPGS than does methotrexate. Experimental techniques are detailed in ref. (137).

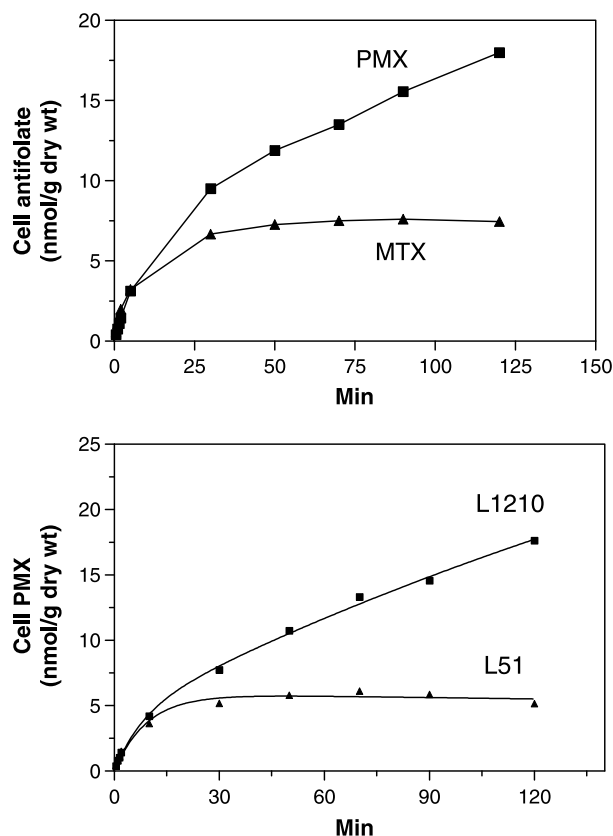


Figure 2. Differences in cellular accumulation of pemetrexed and methotrexate based upon the higher affinity of pemetrexed for FPGS. *Top*, wild-type L1210 murine leukemia cells were exposed to 1 µmol/L tritiated pemetrexed (PMX; ■) or methotrexate (MTX; ▲) at 37°C, and uptake was monitored over 2 h. *Bottom*, L1210 cells (■) or the FPGS-mutated subline-L51 (▲) were exposed to 1 µmol/L tritiated pemetrexed at 37°C, and uptake was monitored over 2 h. Points, average from three experiments; bars, SE. Data are from ref. (81).

End-Production Protection

One powerful approach to identification of the target(s) of enzyme inhibitors, such as the antifolates, has been the protection afforded by the end products of the affected pathways. For instance, because the major effect of methotrexate is suppression of DHFR, this drug depletes cells of reduced folates required for biosynthetic reactions, and protection from the antiproliferative effect of methotrexate requires provision of both a purine and thymidine (40–42). Likewise, lometrexol is a pure inhibitor of *de novo* purine synthesis and has no detectable activity against thymidylate synthase; protection can be achieved with a salvageable purine (such as hypoxanthine or inosine) alone (28, 29). The situation for pemetrexed is more complex. Consistent with the high affinity of pemetrexed polyglutamates for thymidylate synthase, the primary target in cells is this site; at concentrations in the range of the IC₅₀, full protection can be achieved with thymidine alone (37, 38, 43), whereas purine alone (i.e., hypoxanthine) has no protective effect (Fig. 3, top left). However, as the

Table 1. Pemetrexed monoglutamate and pentaglutamate inhibition constants

Enzyme	K_i pemetrexed-monoglutamate (nmol/L)	K_i pemetrexed-pentaglutamate (nmol/L)
rhTS	109 ± 9	1.3 ± 0.3
rmGARFT	9,300 ± 690	65 ± 16
rhDHFR	7.0 ± 1.9	7.2 ± 0.4
rhAICARFT	3,580	265

NOTE: The data, obtained from ref. (38), represents the mean K_i concentrations in nmol/L ± SE in cell-free preparations.

concentration of pemetrexed is increased, protection requires both purine (hypoxanthine) and thymidine, reflecting inhibition of both thymidylate synthase and GARFT (Fig. 3, top left).

Cellular Folate Status

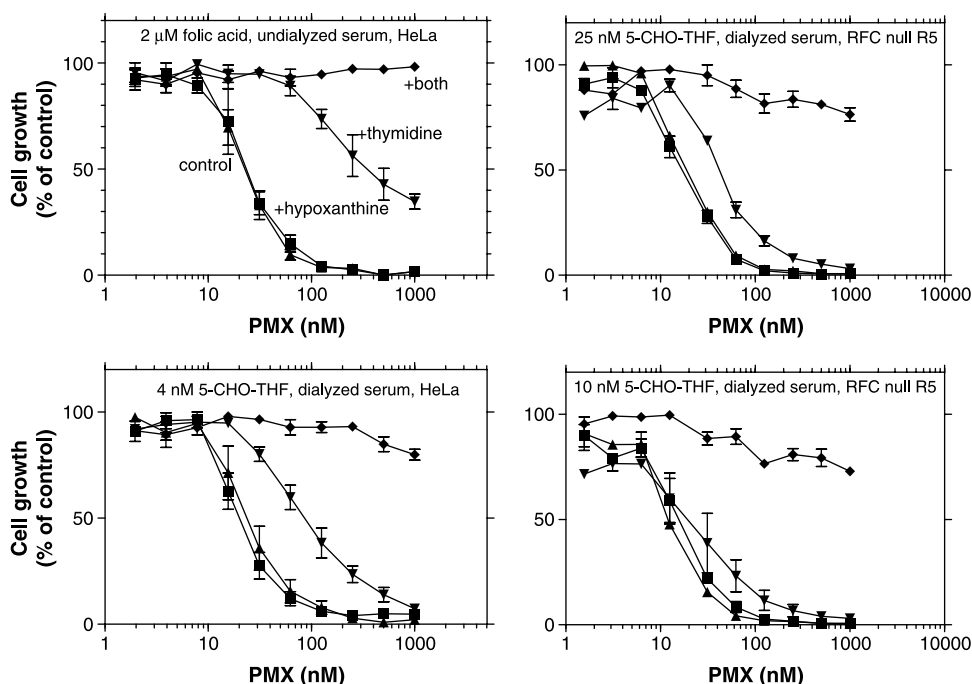
The level of cellular folates is an important determinant of the activity of pemetrexed (37, 44). The basis for this finding is the inhibitory effect of physiologic folates in cells on the polyglutamation of antifolates at the level of FPGS (45) and at the level of their target enzymes. Hence, in leukemia cells, as the extracellular 5-formyltetrahydrofolate level is increased, the formation of the polyglutamate derivatives of pemetrexed and the GARFT inhibitor lomoxetrol are decreased (44). More recently, it has been shown that as extracellular 5-formyltetrahydrofolate is increased over the reference range of blood folates; there is a substantial decrease in pemetrexed activity in human lung cancer, colon cancer, and mesothelioma cell lines (Fig. 4). Different cell lines are affected to different extents by

increasing extracellular folates. These observations are important within the context of the dose of folic acid "protection" used in current clinical regimens with pemetrexed and to the response of tumor cells to decreased transport of this drug, as described below.

The folate status of tumor cells also alters the relative inhibition by pemetrexed of its target enzymes. When extracellular folates are decreased, the protective effect of thymidine is diminished, and the requirement for hypoxanthine is increased at much lower pemetrexed levels, indicating that pemetrexed inhibition of purine synthesis has taken on a larger role in the activity of the drug (Fig. 3, bottom left). Likewise, when the folate level of tumor cells is decreased due to loss of transport function (see below), leading to decreased transport and accumulation of 5-formyltetrahydrofolate, pemetrexed inhibition at the level of GARFT is increased (Fig. 3, right; ref. 43).

Although the targeting of two enzymes essential for a proliferating cell population may seem to be a contributing factor in the efficacy of this agent, it is unclear as to the role that the much weaker inhibition of GARFT actually plays in the action of pemetrexed. However, it is clear that GARFT inhibition can become important under conditions in which thymidylate synthase expression is increased and/or the enzyme is mutated (43, 46). In these settings, inhibition at the primary target may be diminished or eliminated, and inhibition at the secondary target emerges as the concentration of drug is increased. This is confirmed by the observation that growth inhibition by pemetrexed was unaffected by thymidine alone when thymidylate synthase was modestly amplified by ~8-fold, but growth inhibition was fully obviated by a combination of thymidine and purine (43). It was the recognition that pemetrexed has the

Figure 3. Effects of nucleoside products of biosynthetic reactions as indicators of pemetrexed enzyme target. Effects of folate availability on enzyme inhibition by pemetrexed. HeLa cells (left) or the RFC-null subline R5 cells (right) were adapted for at least a week to RPMI containing 2 μ mol/L folic acid and 10% undialyzed serum (top left) or folate-free RPMI containing 10% dialyzed serum supplemented with 5-formyltetrahydrofolate (5-CHO-THF) to a final concentration of 25 nmol/L (top right), 10 nmol/L (bottom right), or 4 nmol/L (bottom left). Cells were then exposed to pemetrexed, and growth inhibition was assessed under these conditions. Protection with 20 μ mol/L hypoxanthine (\blacktriangle), 10 μ mol/L thymidine (\blacktriangledown), or both nucleosides (\blacklozenge) was compared with unprotected cells (\blacksquare). Points, average percentage of cell growth in the absence of drug from three experiments; bars, SE. Data are from ref. (43).



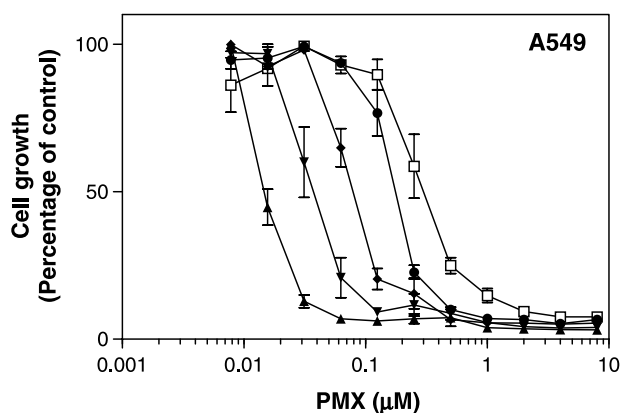


Figure 4. Effect of increasing extracellular 5-formyltetrahydrofolate concentrations on pemetrexed sensitivity in the A549 lung cancer cell line. Cells were adapted for at least a week in folate-free RPMI supplemented with 1.6 nmol/L (▲), 4 nmol/L (▼), 10 nmol/L (◆), 25 nmol/L (●), or 62.5 nmol/L (□) 5-formyltetrahydrofolate. Following this, cells were exposed to a spectrum of pemetrexed concentrations for 6 d, at the end of which cell growth was quantified using the sulforhodamine B reagent. Points, average percentage of cell growth in the absence of drug from three experiments; bars, SE.

potential to inhibit several targets in tumors cells that played an important role in the decision to move this agent forward to the clinics and resulted in its initial name of multitargeted antifol (MTA), that was continued in the trade name Alimta.

Membrane Transport of Pemetrexed

The current understanding of the membrane transport of folates has emerged from studies on the mechanisms of methotrexate transport in tumor cells because of the critical role that transport plays in the activity of this agent (47). Membrane transport of folates and antifolates in mammalian cells is mediated by several distinct processes, each of which has high affinity for pemetrexed. The transport of folates and their analogues was the subject of a recent review (48).

Reduced Folate Carrier

The reduced folate carrier (RFC) was the first folate transporter defined and characterized in mammalian cells in the late 1960s (49–52). This is a carrier-mediated anion exchanger in which the translocation of a folate substrate across the cell membrane is facilitated by the transport of another anion by this carrier in the opposite direction. RFC has a K_t for methotrexate influx of 2 to 5 $\mu\text{mol/L}$ in murine (53) and human tumor cell lines (54); the affinity for pemetrexed is 2-fold higher (53, 54). The affinity for folic acid is very low ($K_i \sim 200 \mu\text{mol/L}$), 1 to 2 orders of magnitude less than the affinity for methotrexate and pemetrexed (53). The affinities of RFC for 5-formyltetrahydrofolate and 5-methyltetrahydrofolate are comparable with and somewhat greater than methotrexate, respectively (53). The rodent and human RFCs were cloned in the mid-1990s, and there have been extensive studies that have confirmed the major elements of its secondary structure,

structure/function, regulation, and the structural and regulatory changes that result in impaired transport and drug resistance in tumor cell lines. Properties of RFC were recently reviewed (48).

Folate Receptors

The folate receptors FR- α and FR- β are high-affinity binding proteins anchored to the cell membranes by a glycosyl-phosphatidylinositol tail that mediates transport by an endocytotic process (48). The former is highly expressed in certain normal tissues, including choroid plexus, lung, female reproductive organs, thyroid, and kidneys, and in a variety of human solid tumors, whereas the latter is found, in particular, in cells derived from hematopoietic tissues and their malignant counterparts and in the placenta (48). Although FR- α has an affinity for its preferred substrates more than 3 orders of magnitude greater than RFC (i.e., the FR- α K_b for folic acid is 1.7 nmol/L), transport into the cells requires a series of complex steps: binding, invagination, vesicle formation and translocation, acidification, and export of substrate from the vesicle into the cytoplasm (55). Because of this, the maximum rate of transport into cells mediated by this mechanism is one hundredth the rate mediated by RFC. Hence, FR-mediated transport contributes little to the uptake of either folates or antifolates unless the receptor is highly overexpressed relative to RFC, and/or transport mediated by RFC and other routes is impaired (56, 57). Such high-level expression has been seen, particularly, in FR-transfected cell lines (57–59), KB cells (60), and ovarian cancers (61–63). FR- α has a low affinity for methotrexate ($K_b \sim 300 \text{ nmol/L}$) but with an affinity for pemetrexed higher than for folic acid ($K_b \sim 1 \text{ nmol/L}$; ref. 53).

A Folate Transport Carrier with a Low-pH Optimum

There is a third folate transport system with a unique and distinct low-pH optimum that is present in the majority of human solid tumor cell lines. This transport activity has properties similar to what has been observed for folate intestinal absorption *in vivo*, in intestinal loops (64, 65), in brush-border intestinal vesicles (66, 67), isolated intestinal cells (68, 69), and in tumor cell lines of intestinal origin (70). This transport activity was shown to be independent of RFC. Hence, low-pH activity persists in (a) HeLa cells in which there has been a genomic deletion of RFC (71), (b) HUH-7 hepatoma cells in which RFC is not expressed (72), and (c) HCT-15 colon cancer cells (73) and rat intestinal epithelial cells (IEC-6) in which the RFC is mutated and not functional (74). This activity was characterized in HeLa cells where influx of methotrexate at pH 5.5 exceeds transport at pH 7.4 by >5-fold (54). At pH 5.5, pemetrexed has an affinity for this transporter ~ 20 -fold greater than methotrexate ($K_t \sim 50 \text{ nmol/L}$ versus 1 $\mu\text{mol/L}$, respectively; ref. 54). Unlike RFC, this transporter has high affinity for folic acid ($K_i \sim 500 \text{ nmol/L}$; refs. 54, 66, 67). It can be seen (Table 2) that the structural specificity and pH sensitivity signatures allow discrimination between transport mediated by the low-pH transporter and transport mediated

by RFC (54). Recent studies in one of our laboratories have established that this low-pH transporter is a member of the superfamily of solute carriers and is a proton-coupled electrogenic process. The transport properties of this carrier, which we have named the "proton-coupled folate transporter (PCFT)," reproduce all the properties of the low-pH transport activity, including a high affinity for pemetrexed, previously observed in human tumors and other cell lines. This transporter was also shown to be required for the intestinal absorption of folates and is mutated in individuals with hereditary folate malabsorption (75).

RFC-Independent Transport at Neutral pH

Beyond the prominent and unchanged low-pH transport activity in RFC-null HeLa cells (71), there is prominent residual influx of methotrexate and pemetrexed at neutral pH in these cells that also has the characteristics of a carrier-mediated process (76). Recent studies indicate that this represents a "tail" of activity of the PCFT with decreased affinities for folate substrates operating at a much less favorable pH (75). Furthermore, as observed for the low-pH process, the affinity of this carrier at neutral pH is greatest for pemetrexed (76). Hence, whereas the influx K_t for pemetrexed via this route at pH 7.4 (12 $\mu\text{mol/L}$; ref. 76) is far higher than its K_t at pH 5.5 (~50 nmol/L; ref. 54), the K_i s for other folates and antifolates are much higher, and the relative order of affinities (inhibition constants) at the two pH values are the same (Table 2). Consistent with a common basis for these transport activities is the observation that when HeLa cells that lack genomic RFC were selected for further resistance to methotrexate at a mildly acid pH, a clonal derivative was obtained (77), in which there was a loss of transport activity at both pH 5.5 and 7.4 and a marked decrease in expression of the "PCFT" (75). As will be discussed below, studies suggest that this RFC-independent transport process, mediated by the PCFT, represents an important route of delivery of pemetrexed into tumor cells when RFC activity is impaired or lost.

Other Carriers and Exporters with Folate/Antifolate Transport Capabilities

Some members of the SLC21 and SLC22 family of organic acid facilitative carriers seem to have folate and antifolate transport function (48, 78). These carriers represent important transport routes for organic anions in epithelial tissues, such as gut, renal tubules, and the biliary system, but their contribution to folate/antifolate absorption, excretion, or uptake into tumor cells is not clear, in general, and for pemetrexed, in particular.

There are several ABC exporters that transport folate and antifolate monoglutamates out of cells. These include members of the multidrug resistance protein family of exporters (79) and the breast cancer resistance protein (80). There is some data to suggest that lower polyglutamate derivatives of folates and antifolates can be exported by some of these transporters, but there is little direct information regarding export of pemetrexed or the extent to which these transporters influence antifolate activity in the clinical setting. One study suggested that the higher concentrative transport of pemetrexed at physiologic pH in murine leukemia cells may be due to a less efficient export relative to methotrexate (81).

Preservation of Pemetrexed Activity with Loss of the Reduced Folate Carrier Function

It has been axiomatic that loss of transport activity for anticancer agents is an important basis for intrinsic and acquired drug resistance. Paradoxically, this does not seem to be the case for pemetrexed.

As discussed above, accumulation of antifolate polyglutamates in cells is determined by a variety of elements. These include (a) the rate of delivery of antifolate substrate into the cells mediated by the various transport systems; (b) the antifolate substrate concentration achieved within the intracellular water, which is determined by the relative influx and efflux activities of the transporters; (c) the level of expression of FPGS and its catalytic activity for the

Table 2. Affinities of RFC and an RFC-independent carrier for folate and antifolate substrates at pH 5.5 and 7.4

Substrate	Transport independent of RFC		RFC-mediated transport	
	K_t or K_i , pH 5.5 (nmol/L)	K_t or K_i , pH 7.4 ($\mu\text{mol/L}$)	K_t or K_i , pH 5.5 (nmol/L)	K_t or K_i , pH 7.4 (nmol/L)
Pemetrexed	0.05*	15 [†]	1.3*	1.1*
6R-5-CHO-THF	0.87*			
6S-5-CHO-THF	0.13*			
6R,S-5-CHO-THF		45 [†]		5 [‡]
ZD1694	0.53*	91 [†]		2 [‡]
Folic acid	0.62*	92 [†]	125*	142*
Methotrexate	1.03*	99 [†]	3.8*	1.7*
PT632	>50,000*	212 [†]	0.19*	0.24*

NOTE: Data represent either direct measurements with radiolabeled substrates (K_t) or the inhibitory effects of substrates on influx of methotrexate (K_i).

*Obtained from ref. (54).

[†]Obtained from ref. (76).

[‡]Obtained from ref. (53).

antifolate substrate; and (d) the level of physiologic folates in cells that feedback inhibit antifolate polyglutamation at the level of FPGS and compete with antifolates at their target enzymes (45). These factors have a profound influence on the pharmacologic activity of pemetrexed. As indicated below, when there are multiple alterations in these elements, there can be unusual consequences that have important clinical ramifications. It is unclear as to whether enhanced activity of γ -glutamyl peptidase (conjugase), which cleaves the polyglutamate side chain, plays an important role as a determinant of antifolate resistance. This is a lysosomal enzyme (82) with limited access to antifolate polyglutamates except perhaps in hepatocytes. Overexpression of conjugase did not alter methotrexate activity in sarcoma 180 cells (83), but an inhibitor of conjugase did cause an extension in the chain length of methotrexate polyglutamates under some conditions, while not affecting total methotrexate levels (84). Acquired resistance to dideazatetrahydrofolate associated with increased expression of this enzyme was accompanied by a marked decrease in transport (85). This issue remains controversial (86).

Commercial cell culture medium is usually formulated with folic acid as the sole source of folates because of its stability. Indeed, folic acid is a "drug-store artifact" originally isolated as a stable breakdown product of the naturally occurring folates; it does not occur in nature. This use of folic acid as a reference folate substrate has been the source of a fair amount of confusion in the experimental literature, with antifolate effects seen in culture that may have little or no relevance to the *in vivo* situation. For instance, when cells are grown with folic acid as the folate source, loss of RFC activity is associated with pemetrexed resistance (76). Folic acid has a low affinity for RFC; it is transported in mammalian cells largely by an RFC-independent process, and when RFC activity is lost, there is only a small contraction of cellular folate pools (71, 76). The major folate form in mammalian serum is 5-methyltetrahydrofolate, but this compound is seldom used *in vitro* because of its instability in tissue culture medium. Rather, the closely related 5-formyltetrahydrofolate is most often used both clinically and in the laboratory because it is a reduced folate that combines stability with transport profiles comparable with that of 5-methyltetrahydrofolate (53).

When RFC activity is lost under conditions that mimic *in vivo* folate availability (i.e., growth on 5-formyltetrahydrofolate), high-level resistance is acquired to many antifolates targeted to a variety of folate-dependent enzymes. However, surprisingly, pemetrexed activity is preserved; indeed, it may be increased (73, 76). In the RFC-deficient HCT-15 human colon cancer PT1 clonal derivative, there is marked resistance to ZD1694, with lesser but still substantial resistance to methotrexate. However, there is collateral sensitivity to pemetrexed. This is illustrated in Fig. 5. This is associated with marked contraction of cellular folate pools, indicated by concurrent collateral sensitivity to trimetrexate, a DHFR inhibitor that enters cells by passive diffusion and is highly sensitive to

the level of cellular folates (refs. 44, 71; Fig. 5). Preservation of pemetrexed activity in HeLa cells can be attributed to several factors. (a) There is residual pemetrexed transport mediated by the RFC-independent PCFT (ref. 75; see above; ref. 76). (b) There is impaired transport of 5-formyltetrahydrofolate resulting in contraction of cellular THF cofactor pools. Consequently, there is decreased competition between cellular THF cofactors and pemetrexed at FPGS resulting in partial preservation of the formation and accumulation of pemetrexed polyglutamate derivatives despite the decrease in transport (73, 76). Furthermore, with contraction of cellular THF cofactor pools, there is enhanced pemetrexed inhibition of GARFT and, to a lesser extent, thymidylate synthase (43, 73). Among the antifolates that undergo polyglutamation, next to lometrexol, pemetrexed is the agent that is most sensitive to the level of physiologic folates in cells (44). It is only when the residual transport mediated by the PCFT is impaired that pemetrexed activity is markedly reduced (77).

Similar to the lack of effect of loss of RFC function on pemetrexed activity, when FR- α expression was suppressed in HeLa cells by the use of small interfering RNA, there was no loss of pemetrexed activity under conditions in which RFC was functional, or in RFC-deficient cells in which residual transport of the drug was mediated by PCFT (87). FR- α expression in HeLa cells was found to be comparable with, or greater than, the level of expression in a variety of human solid tumor cell lines, although far less than has been observed in freshly harvested human ovarian carcinomas and in some ovarian carcinoma cell lines (88).

Contrasting Methotrexate and Pemetrexed

Although pemetrexed has been viewed by some as a "super methotrexate," there are profound differences in the properties of these drugs. Methotrexate, as a monoglutamate, is a potent inhibitor of DHFR, and after a brief exposure to this drug, there is essentially complete suppression of this enzyme, the rapid conversion of THF cofactors to dihydrofolate resulting in THF cofactor depletion and cessation of THF-dependent biosynthetic processes due to this metabolic sequestration of folates as dihydrofolate (47). Methotrexate polyglutamate derivatives slowly build up in cells and have the potential for direct suppression of 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase and thymidylate synthase (21–23), but these effects are minor compared with the inhibition of DHFR and, following inhibition of DHFR by methotrexate, thymidylate synthase and purine synthesis have already ceased due to cofactor depletion. These polyglutamate derivatives become important during "leucovorin rescue" when they selectively suppress utilization of the added reduced folates in tumors in which they accumulate, but not in proliferative cells of the bone marrow and gut in which they accumulate to a far lesser extent (17–20, 89).

The metabolic sequelae of methotrexate inhibition of its primary target enzyme are also quite different from pemetrexed. When methotrexate inhibits the growth of

tumor cells, cytosolic THF cofactors available as one-carbon donors accumulate as dihydrofolate and compete with drug for the target enzyme. Because of this and the marked excess of DHFR in almost all tumor cells over what is needed to optimally drive thymidylate synthesis, methotrexate has no effect on cellular proliferation until in excess of 95% of enzyme is inhibited. As methotrexate blood levels decrease, cell growth resumes to uninhibited rates as $\geq 5\%$ of DHFR becomes available for dihydrofolate reduction. Sustained inhibition of DHFR requires the accumulation of substantial levels of intracellular methotrexate polyglutamates, a slow process for this drug (47).

The situation for pemetrexed is quite different. When pemetrexed inhibits its target enzymes, there is no redistribution of folates within cells; the levels of folate substrates are not changed (90). In addition, inhibition of thymidylate synthase or of GARFT directly decreases the growth rate of tumor cells, as both seem to be limiting to DNA synthesis; that is, inhibition of 50% of either of these enzymes would decrease growth rates of tumors by 50%. Although the rate of pemetrexed transport into cells is controlled by the membrane transport processes, the key event seems to be the very rapid polyglutamation of pemetrexed that begins when the drug is present at very low concentrations in the cytosol due to the superior kinetic characteristics of pemetrexed with FPGS (Fig. 1; refs. 37, 39). The K_m for pemetrexed is one hundredth that of methotrexate for this enzyme (39). Hence, cellular levels of pemetrexed polyglutamates sufficient to completely block thymidylate synthase activity rapidly accumulate. Suppression of GARFT comes later because it requires 50-fold higher concentrations of pemetrexed polyglutamates. Because suppression of thymidylate synthase blocks the formation

of dihydrofolate, the requirement for DHFR is obviated (91); hence, any potential inhibitory effect of pemetrexed at the level of DHFR is superfluous. Neither amplification of DHFR nor loss of RFC function seems to be associated with acquired pemetrexed resistance *in vitro*; more common are mutations or down-regulation of FPGS (92, 93) and increased thymidylate synthase expression (94, 95).

Hence, although both methotrexate and pemetrexed result in suppression of THF-dependent reactions, the effects of methotrexate are achieved primarily by depletion of cellular THF cofactors, whereas pemetrexed acts by a direct block of THF cofactor-dependent biosynthetic enzymes without an effect on the level of cellular THF cofactor pools. Likewise, the mechanisms by which tumor cells develop resistance to these agents are different. This suggests that acquired resistance to methotrexate in the clinical setting is unlikely to be associated with collateral resistance to pemetrexed and vice versa.

Contrasting Pemetrexed and Raltitrexed

Raltitrexed is an antifolate that has much in common with pemetrexed. Both agents have comparable FPGS activity (39), and their higher polyglutamate derivatives have comparable inhibition constants for thymidylate synthase (26, 37). Pemetrexed has an additional target at higher concentrations, and the paradigm has been that this should provide a therapeutic advantage. Yet, the IC_{50} for raltitrexed against *in vitro* tumors is one sixth that of pemetrexed (96), and the maximum tolerated dose for raltitrexed in humans is 2 orders of magnitude lower than that of pemetrexed (97). What can account for this marked difference in activity? Although raltitrexed seems to have only a slightly higher affinity for RFC ($K_i \sim 2 \mu\text{mol/L}$

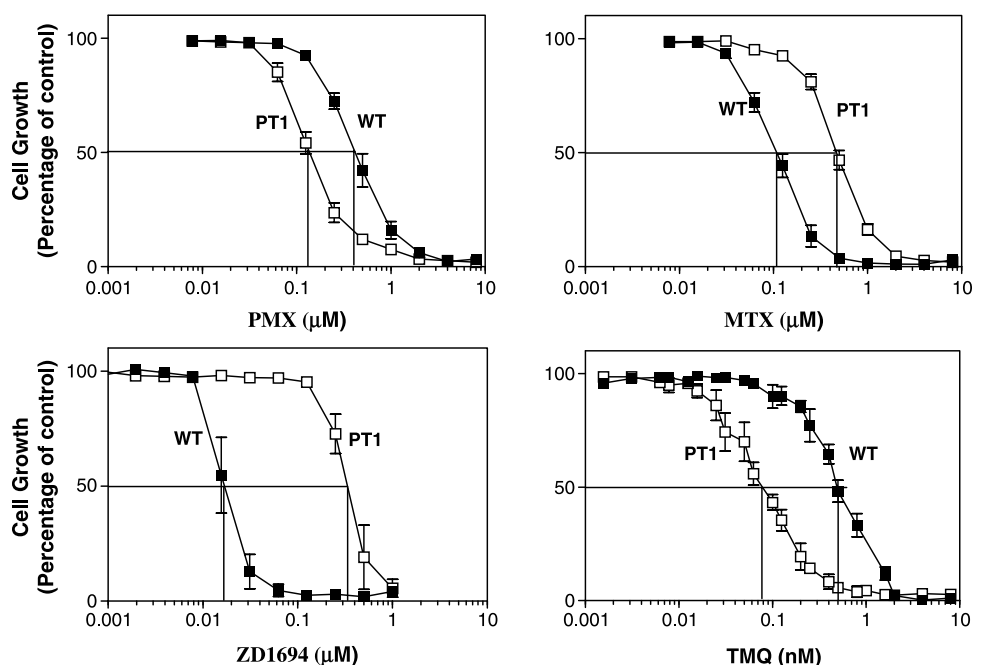


Figure 5. The effect of loss of RFC function on the activity of pemetrexed and other antifolates. Wild-type (WT) HCT-15 colon cancer cells (■) or the RFC-deficient PT1 subline (□) were adapted for at least a week to folate-free RPMI supplemented with 25 nmol/L 5-formyltetrahydrofolate. Cells were then exposed to a spectrum of concentrations of different antifolates, and growth inhibition was assessed after 6 d. Points, average percentage of cell growth in the absence of drug from at least three experiments; bars, SE. Data are from ref. (73).

versus $K_i \sim 5 \mu\text{mol/L}$ for pemetrexed; ref. 53), a direct measurement of raltitrexed transport has not been possible due to the unavailability of radiolabeled drug (53). However, there is evidence for a role for transport in the difference between these drugs. In wild-type HeLa cells growing in 5-formyltetrahydrofolate or folic acid, the IC_{50} for raltitrexed is one sixth to one fifteenth that of pemetrexed (76). However, in HeLa R5 cells that lack genomic RFC, the IC_{50} for raltitrexed is more than 10 times greater than that of pemetrexed in medium containing 5-formyltetrahydrofolate (76) and more than 4 times greater in folic acid medium (76, 77). This may be due to the much lower affinity of raltitrexed relative to pemetrexed for the PCFT that is the predominant pathway for uptake into cells under these conditions (54). In a variant of HeLa R5 cells, in which RFC is absent and the residual low-pH route is markedly diminished, the IC_{50} for these agents is identical during growth in folic acid (the cells grow poorly in 5-formyltetrahydrofolate under these conditions; $1,000 \pm 60$ for both; ref. 77). Hence, the differences in activity of raltitrexed relative to pemetrexed seems to be due, largely, to transport differences that involve RFC and the PCFT.

Contrasting 5-Fluorouracil and Pemetrexed

The mechanisms of action of 5-fluorouracil (5-FU) and its deoxynucleoside 5-fluorodeoxyuridine derivative have been studied for half a century. A tremendous amount of information is available on the disposition of these agents in cells, their biochemical effects, their mechanisms of cytotoxicity, and the spectrum and limitations of their clinical effects (98). 5-FU is converted intracellularly to its active metabolites 5-fluoro-dUMP (FdUMP) and 5-fluoro-UTP (98). These derivatives disrupt DNA synthesis by inhibition of thymidylate synthase (FdUMP) and RNA function by misincorporation of 5-fluoro-UTP into RNA species. Both mechanisms contribute to the effects of 5-FU, although, when 5-FU is used in combination with folinic acid (leucovorin), the mechanism is predominately inhibition of thymidylate synthase.

Both pemetrexed and 5-FU are thymidylate synthase inhibitors. However, their interactions with this enzyme are quite different. Thymidylate synthase has two substrate binding sites: one for a nucleotide (dUMP) and the other for a folate (5,10-methylenetetrahydrofolate polyglutamate). When thymidylate synthase is inhibited after treatment with an inhibitor, the *de novo* synthesis of thymidylate is suppressed, but accumulation of dUMP continues, often to millimolar concentrations (99–101). Although FdUMP can form a stable covalent bond with thymidylate synthase, the initial interaction of thymidylate synthase with FdUMP has an association constant equivalent to that of thymidylate synthase with dUMP (102). Hence, the accumulation of dUMP substantially, and sometimes completely, terminates inhibition of thymidylate synthase by FdUMP (102–104). The same accumulation of dUMP occurs after exposure of tumor cells to pemetrexed (90), but because the

excess dUMP binds to a different site on the enzyme (105), it does not inhibit pemetrexed binding and, in fact, may enhance the binding of pemetrexed polyglutamates to thymidylate synthase.

Tumor cells are often deficient in cellular THF cofactor pools. The covalent complex formed between thymidylate synthase and FdUMP proceeds via chemistry that requires the presence of the THF cofactor (106). If cellular folates are low, the formation of ternary complex either is impeded or does not occur at all. Furthermore, high levels of THF cofactors in cells causes the persistence of cellular thymidylate synthase in the inactive ternary complex due to a kinetic trapping effect (107). Because of these considerations, 5-FU is coadministered with 5-formyltetrahydrofolate in the form of leucovorin (the calcium salt of the mixture of diastereomers about carbon 6). In contrast, low cellular THF cofactor levels enhance pemetrexed polyglutamation and enhance inhibition of its target enzymes (see above). The case could be made, in general, that inhibition of a THF cofactor-dependent enzyme by an analogue of the THF cofactor would be much a more efficient drug than an analogue of the nucleotide substrate because the non-folate substrate will accumulate and block drug action, whereas the folate substrate will not.

Clinical Trials with Pemetrexed

Initial phase I trials with single-agent pemetrexed explored a variety of schedules: (a) weekly $\times 4$ every 6 weeks with a maximum tolerated dose of $40 \text{ mg/m}^2/\text{wk}$ (two minor responses in colorectal cancer; ref. 108), (b) daily for 5 days every 21 days with a maximum tolerated dose of 4 mg/m^2 (three minor responses; ref. 109), and (c) a 10-min infusion every 21 days with a maximum tolerated dose of 600 mg/m^2 (four partial responses in pancreatic and colorectal cancer and six minor responses in a total of 37 patients; ref. 110). None of these trials included folate or B12 supplementation, and neutropenia was the major dose-limiting toxicity. The activity of the latter regimen seemed logical because the high levels of active polyglutamate derivatives generated with the high doses of drug that can be administered on a 3-week schedule should be sustained in tumor cells long after the pemetrexed blood levels have declined and are cleared from intestinal and bone marrow progenitor cells. Subsequent studies used this schedule although the dose was decreased to 500 mg/m^2 . Pharmacokinetic analysis revealed a mean maximum plasma concentration of $137 \mu\text{g/mL}$ ($\sim 290 \mu\text{mol/L}$) and a mean serum half-life of 3.1 h with $>80\%$ of the dose excreted unchanged in the urine during the first 24 h (110). There was no hepatic toxicity, but there was mild reversible renal toxicity with pemetrexed doses of 600 to 700 mg/m^2 (111). Although pemetrexed plasma clearance decreases with decreasing GFR, a pemetrexed dose of 500 mg/m^2 is tolerated in patients with GFR levels of 40 to 79 mL/min (112). Because a reduction in pemetrexed plasma clearance was associated with aspirin (110), and because nonsteroidal anti-inflammatory drugs are known to suppress antifolate

renal tubular secretion (113), cessation of nonsteroidals has been required 2 to 5 days before pemetrexed administration. However, more recently, no alterations in pemetrexed pharmacokinetics were observed with coadministration of aspirin, and only minor changes were noted with ibuprofen (114).

In an early phase I trial designed to evaluate the effects of hydration required with cisplatin coadministration on pemetrexed pharmacokinetics, although there was no change in pemetrexed clearance, nearly 50% of patients with pleural mesothelioma had a partial response, and one third had stable disease (115). In a subsequent phase I study combining pemetrexed with carboplatin in patients with this disease, there was a 32% response rate (116). Mesothelioma is generally considered to be chemoresistant. Although antifolates were known to have good response rates as single agents, ranging from 12% to 37% (117), the results with the pemetrexed-cisplatin combination were sufficiently encouraging to be the basis for the largest-ever trial in mesothelioma, an international phase III study comparing treatment with cisplatin alone or cisplatin in combination with pemetrexed (118). The study began in April 1999, and soon after, drug-related deaths occurred due to bone marrow and mucosal toxicities in the pemetrexed-containing arm. Because blood samples were retained on all the patients, it was possible to go back and attempt to identify factors that might be predictive of toxicity. This analysis revealed that there was a strong positive correlation between increasing toxicity and increasing plasma homocysteine or methylmalonic acid blood levels (119, 120). This relationship held even as the homocysteine blood level fell into the reference range and even when blood folate levels were normal or low normal.

These studies suggested that folate and/or B12 deficiency, even when quite subtle, were important risk factors for pemetrexed toxicity. Based upon these findings, folate/B12 supplementation was added to the regimen, and the number of patients to be accrued was increased within the context of this new study design. This was implemented in December 1999. The trial was completed in the spring of 2003 and showed that in the pemetrexed-cisplatin arm, there was a significant increase in response rate, time to disease progression, and overall survival in the entire cohort of patients (including patients accrued before and after folate supplementation and who were partially supplemented; ref. 118). There was, in addition, impressive objective improvement in vital capacity and in dyspnea, pain, fatigue, cough, anorexia, and global quality of life (assessed by LCSS-Meso; ref. 121) in patients who had received pemetrexed (122). Pemetrexed was approved for the treatment of pleural mesothelioma in the spring of 2004 (123).

The addition of folic acid supplementation substantially diminished toxicity in the pemetrexed/cisplatin arm and allowed an increase in the mean number of cycles of treatment from two in the unsupplemented to six in the supplemented cohorts. This may be the critical determinant of the effect of supplementation. Neutropenia, the predominant toxicity, was reduced from ~41% in suboptimally

and nonsupplemented patients to ~23% in fully supplemented patients. In earlier reports, vitamin supplementation reduced pemetrexed-induced grade 3 or 4 hematologic toxicity from 37% to 6.4% (120). Vitamin supplementation in the phase III study also allowed patients in the cisplatin alone arm to receive more cycles of chemotherapy, with improved response rates and overall survival, a phenomenon that remains unexplained (118).

More recently, another phase III study was completed comparing pemetrexed to docetaxel in previously treated patients with non-small cell lung cancer (124). Pemetrexed and docetaxel had comparable activities (~9% response rate), but there was far less toxicity with pemetrexed (5% versus 40% grade 3 or 4 neutropenia, respectively, along with significantly less febrile neutropenia and alopecia). Although no vitamin supplementation was given to patients in the docetaxel arm in this study, a subsequent analysis revealed that patients in the docetaxel arm who had taken folic acid and vitamin B12 did not have longer survival (125). On the basis of this study, pemetrexed was approved for the second-line treatment of non-small cell lung cancer (126). Pemetrexed with cisplatin is currently being evaluated in a randomized phase III trial as first-line treatment of non-small cell lung cancer in a comparison with the combination of gemcitabine and cisplatin.

Pemetrexed in combination with other chemotherapeutic agents has shown activity in a variety of other solid tumors, including breast, colorectal, gastric, cervical, bladder, and small cell lung cancer (127). Of the various agents with which pemetrexed has been combined, there has been particular interest in its coadministration with gemcitabine, which is synergistic *in vitro* (128). The rationale for this combination is supported by several observations. (a) In pancreatic cancer cell lines, there is increased expression of deoxycytidine kinase, the rate-limiting step in gemcitabine activation, with pemetrexed treatment (129, 130). (b) Pemetrexed increases expression of the hENT1 nucleoside transporter that mediates gemcitabine influx (130). (c) Pemetrexed inhibition of thymidylate synthase in CCRF-CEM cells produces a rapid decrease in intracellular dCTP as well as dTTP and dGTP (90). This should increase the incorporation of gemcitabine, a cytidine analogue, into DNA. Based upon these data, pemetrexed should be administered *before* gemcitabine. Although a phase III trial of the combination of pemetrexed with gemcitabine in pancreatic cancer failed to show improvement over gemcitabine alone (131), pemetrexed was administered *after* gemcitabine. In a recent phase II trial in non-small cell lung cancer comparing a variety of pemetrexed-gemcitabine schedules, the response rate to pemetrexed administered 90 min before gemcitabine was far superior to the response rate with the reverse sequence of administration (132).

Folic Acid Supplementation and Optimization of Pemetrexed Therapeutic Index

The strategy of supplementing all patients with folic acid and vitamin B12 was successful in diminishing pemetrexed

toxicities (118, 133). This approach obviated the need to evaluate the sufficiency of folate and B12 in each patient and was therefore less complicated, less susceptible to error, and less expensive. However, there is clear evidence that increasing the level of folates in tumors diminishes the activity of this agent (see above). A recent meta-analysis that examined the relationship between folic acid dose and reduction of blood homocysteine found that a 90% reduction in homocysteine is achievable with folic acid doses as low as 400 µg/d (134). In another study, serum folate levels increased linearly with oral folic acid supplementation in the range of from 0.1 to 5 mg/d (135). Because early studies that documented pemetrexed toxicity were conducted before food was supplemented with folic acid in the United States, and because many patients currently take over-the-counter vitamin supplements that contain folic acid, it is possible that the level of folate intake at the present time exceeds what is required to achieve acceptable drug toxicity. Hence, pemetrexed activity in clinical trials may be lower than might be achieved if the level of folic acid supplementation was lower. In the current guidelines for folic acid supplementation, the manufacturer recommends 350 to 1,000 µg of folic acid per day with five doses in the preceding week (136). The Food and Drug Administration recommends the same daily dose but with folic acid supplementation beginning 1 to 3 weeks before starting pemetrexed (123). Surely, there is no need to supplement with more folic acid than the lowest recommended dose and schedule (350–400 µg/d for 5 days) before pemetrexed is started. Because 400 µg of folic acid is standard in most multivitamins, it could be administered in this form with patients advised not to take any additional vitamins or food supplements.

Alternatively, all patients might be supplemented with vitamin B12, but with folic acid added only when blood homocysteine is above a certain level. In the phase III mesothelioma study, toxicity decreased with folate/B12 supplementation in all the homocysteine quartiles assessed (<7.4, 7.4–9.2, 9.3–11.5, >11.5). However, the decrease in toxicity below a blood homocysteine level of 9.2 was small (30%) and not statistically significant (119). Hence, only patients with a homocysteine level above 9.2, or some other benchmark, might need to be supplemented with folic acid. Because there are many other variables that must be measured before administration of pemetrexed or other chemotherapeutic agents, the addition of one more required test (plasma homocysteine) would not be unreasonable. Based upon extensive recent information regarding the determinants of the antitumor effects of pemetrexed, perhaps, it is time to consider a clinical trial to address the issue of optimization of folic acid supplementation.

References

1. Stokstad ELR. Some properties of a growth factor for *Lactobacillus casei*. *J Biol Chem* 1943;149:573–4.
2. Hutchings BL, Mowat JH, Oleson JJ, et al. Pteroylaspatic acid, an antagonist for pteroylglutamic acid. *J Biol Chem* 1947;170:323–8.
3. Leuchtenberger R, Leuchtenberger C, Laszlo D, Lewisohn R. The influence of "folic acid" on spontaneous breast cancers in mice. *Science* 1945;101:46.
4. Farber S, Cutler EC, Hawkins JW, et al. The action of pteroylglutamic conjugates on man. *Science* 1947;106:619–21.
5. Farber S, Diamond LK, Mercer RD, Sylvester RF, Wolff VA. Temporary remission in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl glutamic acid (aminopterin). *N Engl J Med* 1948;238:787–93.
6. Farber S. Some observations on the effect of folic acid antagonists on acute leukemia and other forms of incurable cancer. *Blood* 1949;4:160–7.
7. Werkheiser WC. Specific Binding of 4-amino folic acid analogues by folic acid reductase. *J Biol Chem* 1961;236:888–93.
8. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med* 2006;354:166–78.
9. Furst DE. The rational use of methotrexate in rheumatoid arthritis and other rheumatic diseases. *Br J Rheumatol* 1997;36:1196–204.
10. Roenigk HH, Jr., Auerbach R, Maibach H, Weinstein G, Lebwohl M. Methotrexate in psoriasis: consensus conference. *J Am Acad Dermatol* 1998;38:478–85.
11. Baugh CM, Krumdieck CL, Nair MG. Polyglutamyl metabolites of methotrexate. *Biochem Biophys Res Commun* 1973;52:27–34.
12. Whitehead VM. Synthesis of methotrexate polyglutamates in L1210 murine leukemia cells. *Cancer Res* 1977;37:408–12.
13. Rosenblatt DS, Whitehead VM, Dupont MM, Vuchich MJ, Vera N. Synthesis of methotrexate polyglutamates in cultured human cells. *Mol Pharmacol* 1978;14:210–4.
14. Jolivet J, Schilsky RL, Bailey BD, Drake JC, Chabner BA. Synthesis, retention, and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. *J Clin Invest* 1982;70:351–60.
15. Jolivet J, Chabner BA. Intracellular pharmacokinetics of methotrexate polyglutamates in human breast cancer cells. Selective retention and less dissociable binding of 4-NH₂-10-CH₃-4 and 4-NH₂-10-CH₃-5 to dihydrofolate reductase. *J Clin Invest* 1983;72:773–8.
16. Fry DW, Yalowich JC, Goldman ID. Rapid formation of poly-gamma-glutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high pressure liquid chromatography in the Ehrlich ascited tumor cell *in vitro*. *J Biol Chem* 1982;257:1890–6.
17. Poser RG, Sirotnak FM, Chello PL. Differential synthesis of methotrexate polyglutamates in normal proliferative and neoplastic mouse tissues *in vivo*. *Cancer Res* 1981;41:4441–6.
18. Fry DW, Anderson LA, Borst M, Goldman ID. Analysis of the role of membrane transport and polyglutamylation of methotrexate in gut and Ehrlich tumor *in vivo* as factors in drug sensitivity and selectivity. *Cancer Res* 1983;43:1087–92.
19. Fabre I, Fabre G, Goldman ID. Polyglutamylation, an important element in methotrexate cytotoxicity and selectivity in tumor versus murine granulocytic progenitor cells *in vitro*. *Cancer Res* 1984;44:3190–5.
20. Koizumi S, Curt GA, Fine RL, Griffin JD, Chabner BA. Formation of methotrexate polyglutamates in purified myeloid precursor cells from normal human bone marrow. *J Clin Invest* 1985;75:1008–14.
21. Allegra CJ, Drake JC, Jolivet J, Chabner BA. Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. *Proc Natl Acad Sci U S A* 1985;82:4881–5.
22. Allegra CJ, Chabner BA, Drake JC, et al. Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. *J Biol Chem* 1985;260:9720–6.
23. Baggott JE, Vaughn WH, Hudson BB. Inhibition of 5-aminoimidazole-4-carboxamide ribotide transformylase, adenosine deaminase and 5'-adenylate deaminase by polyglutamates of methotrexate and oxidized folates and by 5-aminoimidazole-4-carboxamide riboside and ribotide. *Biochem J* 1986;236:193–200.
24. Chabner BA, Allegra CJ, Curt GA, et al. Polyglutamylation of methotrexate. Is methotrexate a prodrug? *J Clin Invest* 1985;76:907–12.
25. Jones TR, Calvert AH, Jackman AL, et al. A potent antitumor quinazoline inhibitor of thymidylate synthetase: synthesis, biological properties and therapeutic results in mice. *Eur J Cancer* 1981;17:11–9.
26. Jackman AL, Taylor GA, Gibson W, et al. ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth *in vitro* and *in vivo*: a new agent for clinical study. *Cancer Res* 1991;51:5579–86.

27. Taylor EC, Harrington PJ, Fletcher SR, Beardsley GP, Moran RG. Synthesis of the antileukemic agents 5,10-dideazaaminopterin and 5,10-dideaza-5,6,7,8-tetrahydroaminopterin. *J Med Chem* 1985;28:914–21.
28. Beardsley GP, Moroson BA, Taylor EC, Moran RG. A new folate antimetabolite, 5,10-dideaza-5,6,7,8-tetrahydrofolate is a potent inhibitor of *de novo* purine synthesis. *J Biol Chem* 1989;264:328–33.
29. Moran RG, Baldwin SW, Taylor EC, Shih C. The 6*S*- and 6*R*-diastereomers of 5,10-dideaza-5,6,7,8-tetrahydrofolate are equiactive inhibitors of *de novo* purine synthesis. *J Biol Chem* 1989;264:21047–51.
30. Baldwin SW, Tse A, Gossett LS, et al. Structural features of 5,10-dideaza-5,6,7,8-tetrahydrofolate that determine inhibition of mammalian glycinamide ribonucleotide formyltransferase. *Biochemistry* 1991;30:1997–2006.
31. Sanghani SP, Moran RG. Tight binding of folate substrates and inhibitors to recombinant mouse glycinamide ribonucleotide formyltransferase. *Biochemistry* 1997;36:10506–16.
32. Piper JR, McCaleb GS, Montgomery JA, et al. Synthesis and antifolate activity of 5-methyl-5,10-dideaza analogues of aminopterin and folic acid and an alternative synthesis of 5,10-dideazatetrahydrofolic acid, a potent inhibitor of glycinamide ribonucleotide formyltransferase. *J Med Chem* 1988;31:2164–9.
33. Taylor EC, Hamby JM, Shih C, et al. Synthesis and antitumor activity of 5-deaza-5,6,7,8-tetrahydrofolic acid and its N10-substituted analogues. *J Med Chem* 1989;32:1517–22.
34. Habeck LL, Leitner TA, Schackelford KA, et al. A novel class of monoglutamated antifolates exhibits tight-binding inhibition of human glycinamide ribonucleotide formyltransferase and potent activity against solid tumors. *Cancer Res* 1994;54:1021–6.
35. Boritzki TJ, Barlett CA, Zhang C, Howland EF. AG2034: a novel inhibitor of glycinamide ribonucleotide formyltransferase. *Invest New Drugs* 1996;14:295–303.
36. Desharnais J, Hwang I, Zhang Y, et al. Design, synthesis and biological evaluation of 10-CF3CO-DDACTH analogues and derivatives as inhibitors of GAR Tfase and the *de novo* purine biosynthetic pathway. *Bioorg Med Chem* 2003;11:4511–21.
37. Taylor EC, Kuhnt D, Shih C, et al. A dideazatetrahydrofolate analogue lacking a chiral center at C-6, *N*-[4-(2-(2-amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl)benzoyl]-L-glutamic acid, is an inhibitor of thymidylate synthase. *J Med Chem* 1992;35:4450–4.
38. Shih C, Chen VJ, Gossett LS, et al. LY231514, a pyrrolo[2,3-*d*]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. *Cancer Res* 1997;57:1116–23.
39. Habeck LL, Mendelsohn LG, Shih C, et al. Substrate specificity of mammalian folylpolyglutamate synthetase for 5,10-dideazatetrahydrofolate analogs. *Mol Pharmacol* 1995;48:326–33.
40. Straw JA, Talbot DC, Taylor GA, Harrap KR. Some observations on the reversibility of methotrexate toxicity in normal proliferating tissues. *J Natl Cancer Inst* 1977;58:91–7.
41. Pinedo HM, Zaharko DS, Bull JM, Chabner BA. The reversal of methotrexate cytotoxicity to mouse bone marrow cells by leucovorin and nucleosides. *Cancer Res* 1976;36:4418–24.
42. Hakala MT, Taylor E. The ability of purine and thymine derivatives and of glycine to support the growth of mammalian cells in culture. *J Biol Chem* 1959;234:126–8.
43. Zhao R, Zhang S, Hanscom M, Chattopadhyay S, Goldman ID. Loss of reduced folate carrier function and folate depletion result in enhanced pemetrexed inhibition of purine synthesis. *Clin Cancer Res* 2005;11:1294–301.
44. Zhao R, Gao F, Goldman ID. Marked suppression of the activity of some, but not all, antifolate compounds by augmentation of folate cofactor pools within tumor cells. *Biochem Pharmacol* 2001;61:857–65.
45. Andreassi JL, Moran RG. Mouse folylpoly-gamma-glutamate synthetase isoforms respond differently to feedback inhibition by folylpolyglutamate cofactors. *Biochemistry* 2002;41:226–35.
46. Schultz RM, Patel VF, Worzalla JF, Shih C. Role of thymidylate synthase in the antitumor activity of the multitargeted antifolate, LY231514. *Anticancer Res* 1999;19:437–43.
47. Zhao R, Goldman ID. Resistance to antifolates. *Oncogene* 2003;22:7431–57.
48. Matherly LH, Goldman DI. Membrane transport of folates. *Vitam Horm* 2003;66:403–56.
49. Sirotnak FM, Kurita S, Hutchison DJ. On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukemia. *Cancer Res* 1968;28:75–80.
50. Goldman ID, Lichtenstein NS, Oliverio VT. Carrier-mediated transport of the folic acid analogue methotrexate, in the L1210 leukemia cell. *J Biol Chem* 1968;243:5007–17.
51. Goldman ID. Transport energetics of the folic acid analogue, methotrexate, in L1210 cells: enhanced accumulation by metabolic inhibitors. *J Biol Chem* 1969;244:3779–85.
52. Goldman ID. The characteristics of the membrane transport of amethopterin and the naturally occurring folates. *Ann N Y Acad Sci* 1971;186:400–22.
53. Westerhof GR, Schornagel JH, Kathmann I, et al. Carrier- and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: correlates of molecular-structure and biological activity. *Mol Pharmacol* 1995;48:459–71.
54. Wang Y, Zhao R, Goldman ID. Characterization of a folate transporter in HeLa cells with a low pH optimum and high affinity for pemetrexed distinct from the reduced folate carrier. *Clin Cancer Res* 2004;10:6256–64.
55. Mineo C, Anderson RG. Potocytosis. Robert Feulgen Lecture. *Histochem Cell Biol* 2001;116:109–18.
56. Sierra EE, Brigle KE, Spinella MJ, Goldman ID. Comparison of transport properties of the reduced folate carrier and folate receptor in murine L1210 leukemia cells. *Biochem Pharmacol* 1995;50:1287–94.
57. Spinella MJ, Brigle KE, Sierra EE, Goldman ID. Distinguishing between folate receptor- α -mediated transport and reduced folate carrier-mediated transport in L1210 leukemia cells. *J Biol Chem* 1995;270:7842–9.
58. Dixon KH, Mulligan T, Chung K-N, Elwood PC, Cowan KH. Effects of folate receptor expression following stable transfection into wild type and methotrexate transport-deficient ZR-75-1 human breast cancer cells. *J Biol Chem* 1992;267:24140–7.
59. Henderson GB, Tsuji JM, Kumar HP. Mediated uptake of folate by a high-affinity binding protein in sublines of L1210 cells adapted to nanomolar concentrations of folate. *J Membr Biol* 1988;101:247–58.
60. Theti DS, Bavetsias V, Skelton LA, et al. Selective delivery of CB300638, a cyclopenta[*g*]quinazoline-based thymidylate synthase inhibitor into human tumor cell lines overexpressing the alpha-isoform of the folate receptor. *Cancer Res* 2003;63:3612–8.
61. Wu M, Gunning W, Ratnam M. Expression of folate receptor type alpha in relation to cell type, malignancy, and differentiation in ovary, uterus, and cervix. *Cancer Epidemiol Biomarkers Prev* 1999;8:775–82.
62. Mantovani LT, Miotti S, Ménard S, et al. Folate binding protein distribution in normal tissues and biological fluids from ovarian carcinoma patients as detected by the monoclonal antibodies MOv18 and MOv19. *Eur J Cancer [A]* 1994;30A:363–9.
63. Toffoli G, Cernigoi C, Russo A, et al. Overexpression of folate binding protein in ovarian cancers. *Int J Cancer* 1997;74:193–8.
64. Strum WB. Enzymatic reduction and methylation of folate following pH-dependent, carrier-mediated transport in rat jejunum. *Biochim Biophys Acta* 1979;554:249–57.
65. Said HM, Strum WB. A pH-dependent, carrier-mediated system for transport of 5-methyltetrahydrofolate in rat jejunum. *J Pharmacol Exp Ther* 1983;226:95–9.
66. Selhub J, Rosenberg IH. Folate transport in isolated brush border membrane vesicles from rat intestine. *J Biol Chem* 1981;256:4489–93.
67. Schron CM, Washington C, Jr., Blitzer BL. The transmembrane pH gradient drives uphill folate transport in rabbit jejunum. Direct evidence for folate/hydroxyl exchange in brush border membrane vesicles. *J Clin Invest* 1985;76:2030–3.
68. Said HM, Ma TY, Ortiz A, Tapia A, Valerio CK. Intracellular regulation of intestinal folate uptake: studies with cultured IEC-6 epithelial cells. *Am J Physiol Cell Physiol* 1997;272:C729–36.
69. Rajgopal A, Sierra EE, Zhao R, Goldman ID. Expression of the reduced folate carrier SLC19A1 in IEC-6 cells results in two distinct transport activities. *Am J Physiol Cell Physiol* 2001;281:C1579–86.
70. Vincent ML, Russell RM, Sasak V. Folic acid uptake characteristics of a human colon carcinoma cell line, Caco-2. A newly-described

- cellular model for small intestinal epithelium. *Hum Nutr Clin Nutr* 1985; 39:355–60.
71. Zhao R, Gao F, Hanscom M, Goldman ID. A prominent low-pH methotrexate transport activity in human solid tumor cells: contribution to the preservation of methotrexate pharmacological activity in HeLa cells lacking the reduced folate carrier. *Clin Cancer Res* 2004; 10:718–27.
 72. Zhao R, Hanscom M, Goldman ID. The relationship between folate transport activity at low pH and reduced folate carrier function in human Huh7 hepatoma cells. *Biochim Biophys Acta* 2005;1715: 57–64.
 73. Chattopadhyay S, Zhao R, Krupenko SA, Krupenko N, Goldman ID. The inverse relationship between reduced folate carrier function and pemetrexed activity in a human colon cancer cell line. *Mol Cancer Ther* 2006;5:438–49.
 74. Wang Y, Rajgopal A, Goldman ID, Zhao R. Preservation of folate transport activity with a low-pH optimum in rat IEC-6 intestinal epithelial cell lines that lack reduced folate carrier function. *Am J Physiol Cell Physiol* 2005;288:C65–71.
 75. Qiu A, Jansen M, Sakaris A, et al. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell* 2006;127:917–28.
 76. Zhao R, Hanscom M, Chattopadhyay S, Goldman ID. Selective preservation of pemetrexed pharmacological activity in HeLa cells lacking the reduced folate carrier; association with the presence of a secondary transport pathway. *Cancer Res* 2004;64:3313–9.
 77. Zhao R, Chattopadhyay S, Hanscom M, Goldman ID. Antifolate resistance in a HeLa cell line associated with impaired transport independent of the reduced folate carrier. *Clin Cancer Res* 2005;10: 8735–42.
 78. Hagenbuch B, Meier PJ. The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta* 2003;1609:1–18.
 79. Kruh GD, Belinsky MG. The MRP family of drug efflux pumps. *Oncogene* 2003;22:7537–52.
 80. Volk EL, Schneider E. Wild-type breast cancer resistance protein (BCRP/ABCG2) is a methotrexate polyglutamate transporter. *Cancer Res* 2003;63:5538–43.
 81. Zhao R, Babani S, Gao F, Liu L, Goldman ID. The mechanism of transport of the multitargeted antifolate, MTA-LY231514, and its cross resistance pattern in cell with impaired transport of methotrexate. *Clin Cancer Res* 2000;6:3687–95.
 82. Halsted CH, Ling EH, Luthi-Carter R, et al. Folylpoly-gamma-glutamate carboxypeptidase from pig jejunum. Molecular characterization and relation to glutamate carboxypeptidase II. *J Biol Chem* 1998;273: 20417–24.
 83. Cole PD, Kamen BA, Gorlick R, et al. Effects of overexpression of gamma-Glutamyl hydrolase on methotrexate metabolism and resistance. *Cancer Res* 2001;61:4599–604.
 84. Whitehead VM, Kalman TI, Vuchich MJ. Inhibition of gamma-glutamyl hydrolases in human cells by 2-mercaptomethylglutaric acid. *Biochem Biophys Res Commun* 1987;144:292–7.
 85. Rhee MS, Wang Y, Nair MG, Galivan J. Acquisition of resistance to antifolates caused by enhanced gamma-glutamyl hydrolase activity. *Cancer Res* 1993;53:2227–30.
 86. Schneider E, Ryan TJ. Gamma-glutamyl hydrolase and drug resistance. *Clin Chim Acta*. Epub 2006.
 87. Chattopadhyay S, Wang Y, Zhao R, Goldman ID. Lack of impact of the loss of constitutive folate receptor alpha expression, achieved by RNA interference, on the activity of the new generation antifolate pemetrexed in HeLa cells. *Clin Cancer Res* 2004;10:7986–93.
 88. Parker N, Turk MJ, Westrick E, et al. Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. *Anal Biochem* 2005;338:284–93.
 89. Matherly LH, Barlowe CK, Phillips VM, Goldman ID. The effects on 4-aminoantifolates on 5-formyltetrahydrofolate metabolism in L1210 cells. A biochemical basis of the selectivity of leucovorin rescue. *J Biol Chem* 1987;262:710–7.
 90. Chen VJ, Bewley JR, Andis SL, et al. Preclinical cellular pharmacology of LY231514 (MTA): a comparison with methotrexate, LY309887 and raltitrexed for their effects on intracellular folate and nucleoside triphosphate pools in CCRF-CEM cells. *Br J Cancer* 1998;78 Suppl 3:27–34.
 91. Moran RG, Mulkins M, Heidelberger C. Role of thymidylate synthetase activity in development of methotrexate cytotoxicity. *Proc Natl Acad Sci U S A* 1979;76:5924–8.
 92. Zhao R, Titus S, Gao F, Moran RG, Goldman ID. Molecular analysis of murine leukemia cell lines resistant to 5,10-ideazetetrahydrofolate identifies several amino acids critical to the function of folypolyglutamate synthetase. *J Biol Chem* 2000;275:26599–606.
 93. Liani E, Rothen L, Bunni MA, et al. Loss of folypoly-gamma-glutamate synthetase activity is a dominant mechanism of resistance to polyglutamylation-dependent novel antifolates in multiple human leukemia sublines. *Int J Cancer* 2003;103:587–99.
 94. Schultz RM, Chen VJ, Bewley JR, et al. Biological activity of the multitargeted antifolate, MTA (LY231514), in human cell lines with different resistance mechanisms to antifolate drugs. *Semin Oncol* 1999; 26 Suppl 6:68–73.
 95. Jackman AL, Kelland LR, Kimbell R, et al. Mechanisms of acquired resistance to the quinazoline thymidylate synthase inhibitor ZD1694 (Tomudex) in one mouse and three human cell lines. *Br J Cancer* 1995;71: 914–24.
 96. Backus HH, Pinedo HM, Wouters D, et al. Folate depletion increases sensitivity of solid tumor cell lines to 5-fluorouracil and antifolates. *Int J Cancer* 2000;87:771–8.
 97. Clarke SJ, Hanwell J, de Boer M, et al. Phase I trial of ZD1694, a new folate-based thymidylate synthase inhibitor, in patients with solid tumors. *J Clin Oncol* 1996;14:1495–503.
 98. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 2003;3:330–8.
 99. Jackson RC, Jackman AL, Calvert AH. Biochemical effects of a quinazoline inhibitor of thymidylate synthetase, *N*-(4-((2-amino-4-hydroxy-6-quinazolyl)methyl)prop-2-ynylamino) benzoyl)-L-glutamic acid (CB3717), on human lymphoblastoid cells. *Biochem Pharmacol* 1983;32: 3783–90.
 100. Pfall EM, Curtin NJ, Aherne GW, Harris AL, Marks V. The quantitation by radioimmunoassay of 2'-deoxyuridine 5'-triphosphate in extracts of thymidylate synthase-inhibited cells. *Anal Biochem* 1989;177: 347–52.
 101. Mitrovski B, Pressacco J, Mandelbaum S, Erlichman C. Biochemical effects of folate-based inhibitors of thymidylate synthase in MGH-U1 cells. *Cancer Chemother Pharmacol* 1994;35:109–14.
 102. Myers CE, Young RC, Chabner BA. Biochemical determinants of 5-fluorouracil response *in vivo*. The role of deoxyuridylate pool expansion. *J Clin Invest* 1975;56:1231–8.
 103. Myers CE, Young RC, Johns DG, Chabner BA. Assay of 5-fluorodeoxyuridine 5'-monophosphate deoxyuridine 5'-monophosphate pools following 5-fluorouracil. *Cancer Res* 1974;34:2682–8.
 104. Moran RG, Spears CP, Heidelberger C. Biochemical determinants of tumor sensitivity to 5-fluorouracil: ultrasensitive methods for the determination of 5-fluoro-2'-deoxyuridylate, 2'-deoxyuridylate, and thymidylate synthetase. *Proc Natl Acad Sci U S A* 1979;76:1456–60.
 105. Galivan JH, Maley GF, Maley F. Factors affecting substrate binding in *Lactobacillus casei* thymidylate synthase as studied by equilibrium dialysis. *Biochemistry* 1976;15:356–62.
 106. Carreras CW, Santi DV. The catalytic mechanism and structure of thymidylate synthase. *Annu Rev Biochem* 1995;64:721–62.
 107. Keyomarsi K, Moran RG. Mechanism of the cytotoxic synergism of fluoropyrimidines and folic acid in mouse leukemic cells. *J Biol Chem* 1988;263:14402–9.
 108. Rinaldi DA, Burris HA, Dorr FA, et al. Initial phase I evaluation of the novel thymidylate synthase inhibitor, LY231514, using the modified continual reassessment method for dose escalation. *J Clin Oncol* 1995; 13:2842–50.
 109. McDonald AC, Vasey PA, Adams L, et al. A phase I and pharmacokinetic study of LY231514, the multitargeted antifolate. *Clin Cancer Res* 1998;4:605–10.
 110. Rinaldi DA, Kuhn JG, Burris HA, et al. A phase I evaluation of multitargeted antifolate (MTA, LY231514), administered every 21 days, utilizing the modified continual reassessment method for dose escalation. *Cancer Chemother Pharmacol* 1999;44:372–80.
 111. Rinaldi DA. Overview of phase I trials of multitargeted antifolate (MTA, LY231514). *Semin Oncol* 1999;26 suppl 6:82–8.
 112. Mita AC, Sweeney CJ, Baker SD, et al. Phase I and pharmacokinetic

- study of pemetrexed administered every 3 weeks to advanced cancer patients with normal and impaired renal function. *J Clin Oncol* 2006;24:552–62.
113. Frenia ML, Long KS. Methotrexate and nonsteroidal antiinflammatory drug interactions. *Ann Pharmacother* 1992;26:234–7.
114. Sweeney CJ, Takimoto CH, Latz JE, et al. Two drug interaction studies evaluating the pharmacokinetics and toxicity of pemetrexed when coadministered with aspirin or ibuprofen in patients with advanced cancer. *Clin Cancer Res* 2006;12:536–42.
115. Thodtmann R, Depenbrock H, Dumez H, et al. Clinical and pharmacokinetic phase I study of multitargeted antifolate (LY231514) in combination with cisplatin. *J Clin Oncol* 1999;17:3009–16.
116. Hughes A, Calvert P, Azzabi A, et al. Phase I clinical and pharmacokinetic study of pemetrexed and carboplatin in patients with malignant pleural mesothelioma. *J Clin Oncol* 2002;20:3533–44.
117. Fizazi K, John WJ, Vogelzang NJ. The emerging role of antifolates in the treatment of malignant pleural mesothelioma. *Semin Oncol* 2002;29:77–81.
118. Vogelzang NJ, Rusthoven JJ, Symanowski J, et al. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *J Clin Oncol* 2003;21:2636–44.
119. Niyikiza C, Baker SD, Seitz DE, et al. Homocysteine and methylmalonic acid: markers to predict and avoid toxicity from pemetrexed therapy. *Mol Cancer Ther* 2002;1:545–52.
120. Bunn P, Paoletti P, Niyikiza C, et al. Vitamin B12 and folate reduce toxicity of ALIMTA (pemetrexed disodium, LY231514, MTA), a novel antifolate/antimetabolite [abstr 300]. *Proc Am Soc Clin Oncol* 2001;20:76a.
121. Gralla RJ, Hollen PJ, Liepa AM, et al. Improving quality of life in patients with malignant pleural mesothelioma: results of the randomized pemetrexed + cisplatin vs. cisplatin trial using the LCSS-meso instrument [abstract 2946]. *Proc Am Soc Clin Oncol* 2003;22:621.
122. Paoletti P, Pistolesi M, Rusthoven JJ, et al. Correlation of pulmonary function tests with best tumor response status: results from the phase III study of pemetrexed + cisplatin vs. cisplatin in malignant pleural mesothelioma [abstract 2651]. *Proc Am Soc Clin Oncol* 2003;22:659.
123. Hazarika M, White RM, Johnson JR, Pazdur R. FDA drug approval summaries: pemetrexed (Alimta). *Oncologist* 2004;9:482–8.
124. Hanna N, Shepherd FA, Fossella FV, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. *J Clin Oncol* 2004;22:1589–97.
125. Fossella F, Obasaju C, Paul S. Pemetrexed vs docetaxel for non-small cell lung cancer (NSCLC): could vitamin supplementation affect docetaxel survival? [abstract 7131]. An exploratory analysis. *J Clin Oncol* 2004;22 (July 15 Suppl):649S.
126. Cohen MH, Johnson JR, Wang YC, Sridhara R, Pazdur R. FDA drug approval summary: pemetrexed for injection (Alimta) for the treatment of non-small cell lung cancer. *Oncologist* 2005;10:363–8.
127. Adjei AA. Pemetrexed (ALIMTA), a novel multitargeted antineoplastic agent. *Clin Cancer Res* 2004;10:4276–80S.
128. Tonkinson JL, Worzalla JF, Teng CH, Mendelsohn LG. Cell cycle modulation by a multitargeted antifolate, LY231514, increases the cytotoxicity and antitumor activity of gemcitabine in HT29 colon carcinoma. *Cancer Res* 1999;59:3671–6.
129. Giovannetti E, Mey V, Danesi R, Mosca I, Del Tacca M. Synergistic cytotoxicity and pharmacogenetics of gemcitabine and pemetrexed combination in pancreatic cancer cell lines. *Clin Cancer Res* 2004;10:2936–43.
130. Giovannetti E, Mey V, Nannizzi S, et al. Cellular and pharmacogenetics foundation of synergistic interaction of pemetrexed and gemcitabine in human non-small-cell lung cancer cells. *Mol Pharmacol* 2005;68:110–8.
131. Oettle H, Richards D, Ramanathan RK, et al. A phase III trial of pemetrexed plus gemcitabine versus gemcitabine in patients with unresectable or metastatic pancreatic cancer. *Ann Oncol* 2005;16:1639–45.
132. Ma CX, Nair S, Thomas S, et al. Randomized phase II trial of three schedules of pemetrexed and gemcitabine as front-line therapy for advanced non-small-cell lung cancer. *J Clin Oncol* 2005;23:5929–37.
133. Scagliotti GV, Shin DM, Kindler HL, et al. Phase II study of pemetrexed with and without folic acid and vitamin B12 as front-line therapy in malignant pleural mesothelioma. *J Clin Oncol* 2003;21:1556–61.
134. Homocysteine Lowering Trialists' Collaboration. Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. *Am J Clin Nutr* 2005;82:806–12.
135. Wald NJ, Law MR, Morris JK, Wald DS. Quantifying the effect of folic acid. *Lancet* 2001;358:2069–73.
136. Eli Lilly and Company. Alimta product information. Physicians' desk reference. 59th ed. Montvale (NJ): Thomson PDR; 2005. p. 1824–8.
137. Sanghani PC, Moran RG. Purification and characteristics of recombinant human folylpoly-gamma-glutamate synthetase expressed at high levels in insect cells. *Protein Expr Purif* 2000;18:36–45.