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Advances in Brief

A Novel Folate Transport Activity in Human Mesothelioma Cell Lines with High Affinity and Specificity for the New-Generation Antifolate, Pemetrexed[1]

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Abstract

Pemetrexed is a novel antifolate effective in the treatment of mesothelioma. Studies were undertaken to characterize the transport of this antifolate in this tumor. We report the presence of a novel, concentrative high-affinity transport activity in three human mesothelioma cell lines, characterized in detail in the NCI-H28 line, with a pemetrexed influx K_t of 30 nM and V_{max} of 10 nmol/g protein/min. This route is highly specific for pemetrexed, with a substrate specificity pattern quite different from that of the reduced folate carrier and folate receptors. In particular, there is an apparent relatively low affinity for other antifolate inhibitors of dihydrofolate-reductase (MTX, aminopterin, PT523) and thymidylate synthase (ZD1694, ZD9331). Besides its impact on the transport of pemetrexed, this high-affinity route may represent another pathway by which physiological folates are transported into human cells.

Introduction

Mesothelioma is a rapidly fatal malignancy of the pleura, heretofore refractory to chemotherapy. In initial Phase I studies, a new generation antifolate pemetrexed, ALIMTA, LY-231514, (*N*-{4-[2-(2-amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-D]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic acid) showed activity in a variety of neoplastic diseases and, in combination with cisplatin, was active in mesothelioma (1, 2). Pemetrexed activity is dependent on its metabolism to polyglutamate derivatives; it is among the most potent substrates for folylpolyglutamate synthetase, the enzyme that mediates this reaction (3). The pentaglutamate is a potent inhibitor of thymidylate synthase (K_i = 1.3 nM) and a lesser, but still potent, inhibitor of GAR transformylase[3] (K_i = 65 nM; Refs. 4, 5). Pemetrexed is transported into murine and human leukemia cells by the RFC, with transport kinetics similar to MTX, and binds to FR-α with an affinity similar to that of folic acid (6, 7). Pemetrexed also appears to be a substrate for MRP exporters (8). To further define the pharmacological properties of pemetrexed that might underlie its activity, studies were undertaken to explore the mechanism of membrane transport of this agent in human mesothelioma cell lines. These studies revealed the presence of a high-affinity transport system with properties distinct from any other folate/antifolate transporter previously described in human cells. This transport system exhibits a high degree of structural specificity that favors pemetrexed over many other antifolates and results in its very rapid transport and accumulation in mesothelioma cells.

Materials and Methods

Chemicals. [3′,5′,7-^{3}H]MTX and [3′,5′,7′,9-^{3}H] folic acid was from Amersham Corp., Arlington Heights, IL. Pemetrexed bearing 4-[ethyl-[1,2-$^3H_4$]]-benzoyl modification (4.1 Ci/mmol), and unlabeled pemetrexed, DDATHF (Lomotrexol), and LY309887 (6*R*-2′,5′-thienyl-5,10-dideazatetrahydrofolate) were provided by the Eli Lilly Company, Indianapolis, IN. PT523 [N^{α}–(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine] was provided by Dr. Andre Rosowsky, Dana-Farber Cancer Institute. ZD1694 (Tomudex, Raltitrexed)[4] and ZD9331[5] were from Dr. Ann Jackman, Institute for Cancer Research, Sutton, England. Purity of the tritiated agents was maintained by high-performance liquid chromatography (9).

Cells and Media. Human mesothelioma cell lines were obtained from the American Type Tissue Culture Collection, Manassas, VA (NCI-H28) and from the National Cancer Institute (NCI-2373, NCI-2052). Cells were grown as monolayers in liquid scintillation glass vials in RPMI 1640 with 10% FBS (Gemini Bioproducts), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2.

Transport Studies and Folate Binding Assay. Transport measurements were performed in 25-ml scintillation vials with HBS buffer by methods described previously that allow very rapid uptake determinations on cells growing in monolayer cultures (10). To assess folate binding, cells were washed with ice-cold acid buffer [10 mM sodium acetate, 150 mM NaCl (pH 3.5)], followed by a wash with ice-cold PBS at pH 7.4, then incubated with 1 ml PBS containing 5 pmol of [^{3}H]folic acid with and without a 1000-fold excess of nonlabeled folic acid (to inhibit specific binding to receptor) at 4°C for 30 min. The cells were then washed twice with ice-cold PBS buffer. Bound [^{3}H] folic acid was extracted with the acid buffer (0.5 ml) and radioactivity in the extract was measured. The data reported is the average ± SE based on three replicate experiments performed on different days.

Sequence Analysis of RFC and FR cDNAs: Northern Blot Analysis of FRs. Polyadenylated RNA was purified from cell lines using a Dynabeads mRNA DIRECT kit (Dynal). cDNAs were prepared by Superscript Reverse Transcriptase (Invitrogen) and amplified with Taq polymerase (Stratagene) using oligonucleotide primers that flank the coding region. For RFC the primers were: 5′-TGTCACCTTCGTCCCCTCCG-3′ and 5′-TAGCAGGATAAGCGGAGGCC-3′. Primers for FR-α were: 5′- TCTCCCAGGGACAGACATGGCTCA-3′ and 5′ GCGGAGGTCAGCTGAGCAGCCACAGCA-3′. Primers for FR-β were: 5′-GGACAGAAAGACATGGTCTGGAAATGGATG-3′ and 5′-GCCTGAACTCAGCCAAGGAGCCAGAGTT-3′. The RFC PCR amplifications were performed for 35 cycles of 45 s at 95°C, 45 s at 60°C, and 110 s at 72°C. Amplifications for FRs were 39 cycles of 30 s at 95°C, 30 s at 55°C, 80 s at 72°C. The predicted PCR products were purified on an agarose gel (Qiagen) and cloned into a pCR4-TOPO vector (Invitrogen). For RFC and FR-α, five randomly picked cDNA clones were sequenced using primers that covered the whole coding region. The sequence analysis was performed on an ABI-3700 capillary electrophoresis system in the Albert Einstein Cancer Center's DNA Sequencing Facility. Northern analyses for FR-α and FR-β were performed with total RNA isolated using TRIzol (Life Sciences, Inc.) as described previously (11). Full-length FR-α and FR-β cDNAs along with positive RNA controls were generously provided by Dr. M. Ratnam (Medical College of Ohio, Toledo, OH).

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[3] The abbreviations used are: GAR transformylase, glycinamide ribonucleotide formyltransferase (EC 2.1.2.2); DDATHF, (6*R*)-5,10-dideazatetrahydrofolate; MTX, methotrexate; RFC, reduced folate carrier; FR, folate receptor; 5-Formyl-THF, 5-formyltetrahydrofolate; 5-Methyl-THF, 5-methyltetrahydrofolate.

[4] ZD1694 is the common name for (*N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl-methyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid).

[5] ZD9331 is the common name for (2*S*)-2-{*O*-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydro-quinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]benzamido}-4-(tetrazol-5-yl)-butyric acid.

Results

The Characteristics of Pemetrexed and MTX Influx in the NCI-H28 Human Mesothelioma Cell Line at an Extracellular Concentration of 1 μM. Pemetrexed influx and net accumulation were evaluated in the NCI-H28 human mesothelioma cell line and compared with that of MTX. The drug concentration initially chosen (1 μM) was similar to the level usually used to assess transport mediated by RFC. Fig. 1, *top panel*, illustrates a time course of uptake of these drugs. It can be seen that the initial rate and subsequent net uptake of pemetrexed far exceeded that of MTX. After this, net pemetrexed accumulation fell to a much slower, constant velocity, whereas net uptake of MTX did not increase further. The late, linear uptake component is consistent with the accumulation of pemetrexed polyglutamates that are retained in cells (7). The inset focuses on the initial uptake component captured by measurements every 3–4 s over a 20-s interval. On the basis of the average of three experiments, pemetrexed influx (13.56 ± 4.23 nmol/g protein/min) exceeded that for MTX (1.19 ± 0.17 nmol/g protein/min) by a factor of 11.

Additional studies revealed unique aspects of transport of these antifolates in this cell line. In contrast to an expected substantial inhibition of pemetrexed influx if the transport process was mediated by RFC, 25 μM MTX had only a negligible inhibitory effect on the influx of 1 μM [^{3}H]pemetrexed (Fig. 1, *bottom panel*). On the other hand, 10 μM pemetrexed inhibited the influx of 1 μM [^{3}H]pemetrexed by >90%. Because the affinity of RFC for pemetrexed is only slightly greater than for MTX (7, 8), these data suggested the presence of another transport mechanism in this mesothelioma cell line with a high affinity for pemetrexed and a relatively low affinity for MTX.

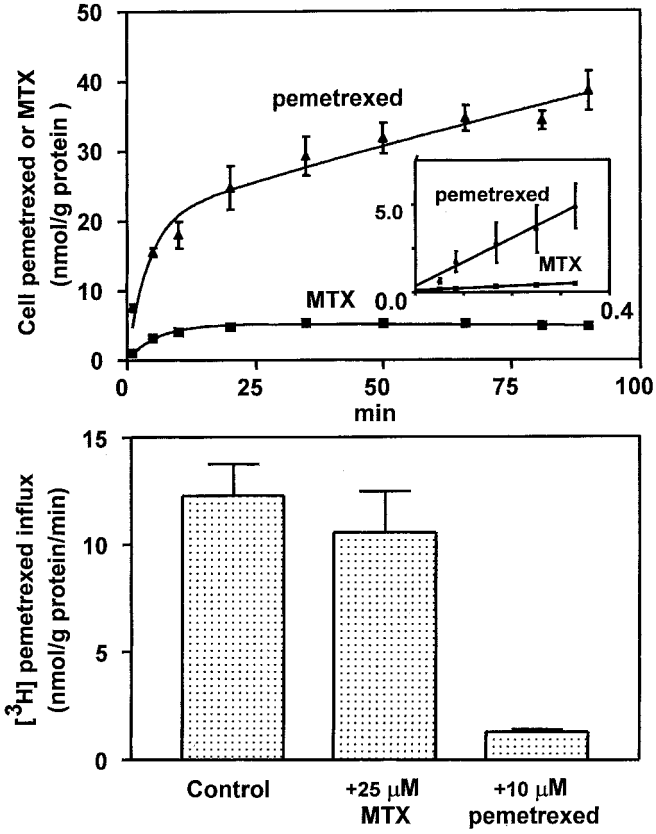

Fig. 1. *Top panel*, A comparison of the time course of [^{3}H]pemetrexed and [^{3}H]MTX uptake in the NCI-H28 mesothelioma cell line at an extracellular concentration of 1 μM. The inset is a comparison of initial rates of uptake (influx) over a 20-s interval at the same extracellular level. The data are the mean of three experiments. *Bottom panel*, the effects of 25 μM nonlabeled MTX or 10 μM nonlabeled pemetrexed on the influx of 1 μM [^{3}H]pemetrexed. The data reflect the average of three experiments.

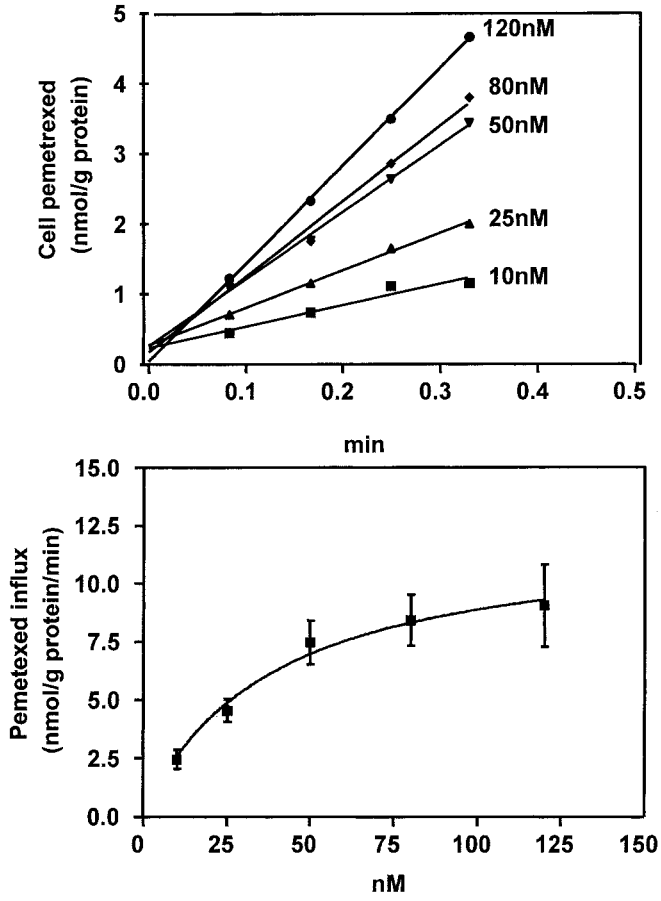

Fig. 2. The kinetics of [^{3}H]pemetrexed influx in NCI-H28 cells. *Top panel*, initial uptake over the concentration range studied, on which rates were computed as indicated in the *bottom panel*. *Bottom panel*, pemetrexed influx as a function of the extracellular concentration. The data are the average of three experiments.

Sequence Analysis of RFC cDNA. The possibility that the NCI-H28 mesothelioma line might carry a mutated carrier with altered structural specificity, as has been reported for antifolate-resistant cell lines (12), was excluded by direct sequencing of the RFC cDNA. The open reading frame from five cDNA clones from the NCI-H28 line was sequenced. No mutations were detected. RFC does have a polymorphism (Arg/His) at amino acid 27, and this cell line was homozygous for Arg at this site. However, functional differences among these polymorphisms are small (13), the NCI-2373 mesothelioma cell line is homozygous for His at this site and as indicated below (Fig. 3) influx of pemetrexed in this and the NCI-H28 line is comparable.

FR Expression and cDNA Sequence Analysis. Three separate folic acid membrane binding measurements were done to assess the level of expression of FRs in the NCI-H28 cell line as compared with L1210 cells, which have minimal FR expression, and the L1210 LL1 variant with high FR-α expression (14). Specific folic acid binding in L1210 cells and the LL1 line was 0.017 ± 0.004 and 1.006 ± 0.077 nmol/g protein, respectively. There was no specific binding, at all, detected in the NCI-H28 line. Consistent with the binding studies, Northern blot analysis for FR-α mRNA showed negligible expression; a FR-β message could not be detected. Attempts to amplify FR-β did not yield any PCR product. A FR-α PCR product was obtained, and the open reading frames from five clones were sequenced; no mutations were detected.

The Kinetics of Pemetrexed Influx: Demonstration of a Novel High-Affinity Transport Route. Attempts to determine pemetrexed influx kinetics in the range of the RFC K_t did not demonstrate a

typical pattern of saturability in NCI-H28 cells. Accordingly, the concentrations were progressively decreased until clear Michaelis-Menten kinetics were observed over a pemetrexed range of 10–120 nM (Fig. 2, *bottom panel*). On the basis of three such experiments, the influx K_t was 29.9 ± 3.1 nM with an influx V_{max} of 10.1 ± 1.4 nmol/g protein/min. This influx K_t is nearly two orders of magnitude lower than that of the RFC K_t for pemetrexed and more than 30-fold higher than the pemetrexed FR binding constant. The high V_{max} is the basis for the high activity of this transporter that dominates uptake of this drug at concentrations far in excess of the K_t (see Fig. 1). Fig. 2, *top panel*, demonstrates the linearity of uptake over the spectrum of concentrations studied, which assures that accurate unidirectional rates were measured.

Influx of Pemetrexed, as Compared with MTX, in Other Mesothelioma Cell Lines. To assess the presence of the high-affinity transport activity in other mesothelioma cell lines, influx of pemetrexed was compared with influx of MTX, each at a concentration of 50 nM. A high ratio is indicative of transport by the high-affinity route because, as indicated above, both antifolates have comparable RFC-mediated influx kinetics. As shown in Fig. 3, high rates of pemetrexed influx in comparison with barely detectable rates of MTX influx were observed in the three human mesothelioma cells lines studied. Influx in the NCI-2373 line was slightly less than that observed in NCI-H28 cells. Pemetrexed influx in the NCI-2052 line was even less but still far greater than the rate of MTX influx. Also seen in this figure is the presence of a low level of pemetrexate transport activity in HeLa cells relative to MTX. No significant difference between MTX and pemetrexed influx was observed in L1210 murine leukemia cells under these conditions (data not shown).

The Specificity of the High-Affinity Influx Mechanism for Pemetrexed. This novel transport route exhibits a high degree of structural specificity as assessed in the NCI-H28 line (Fig. 4, *top panel*). Tritiated pemetrexed (50 nM) influx was abolished by 1 μM nonlabeled drug. However, a significant inhibition of 50 nM pemetrexed influx could not be demonstrated by a 1-μM concentration of the dihydrofolate reductase inhibitors (MTX, aminopterin, PT523) and antifolate inhibitors of thymidylate synthase (D1694, ZD9331). Inhibition was observed for natural folates (5-Formyl-THF > 5-Methyl-THF > folic acid). Inhibition was observed for the GAR transformylase inhibitors DDATHF and, in particular, LY309887. This high-affinity transport activity may represent another route by which mesothelioma cells take up 5-Methyl-THF, the predominant folate in the plasma of man and rodents. However, additional studies will be necessary to quantitate influx K_t and K_is for these folates and antifolates and to confirm utilization, and not just inhibition, of this transport mechanism. The possibility that other transport routes for reduced folates might be present in mice, in addition to RFC and FRs, was suggested by the observation that folate-supplemented animals of RFC-deleted mice ultimately succumb after birth because of failure of hematopoietic tissues, whereas virtually all other tissues are histologically intact including some that do not express FRs (15).

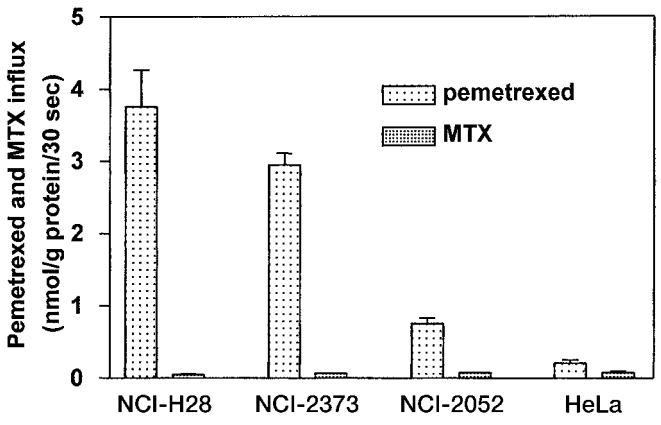

Fig. 3. A comparison of the initial rates of MTX and pemetrexed uptake (over a period of 30 s) in three mesothelioma cell lines and HeLa cells. The extracellular concentration of drugs was 50 nM. The data are the average of three experiments.

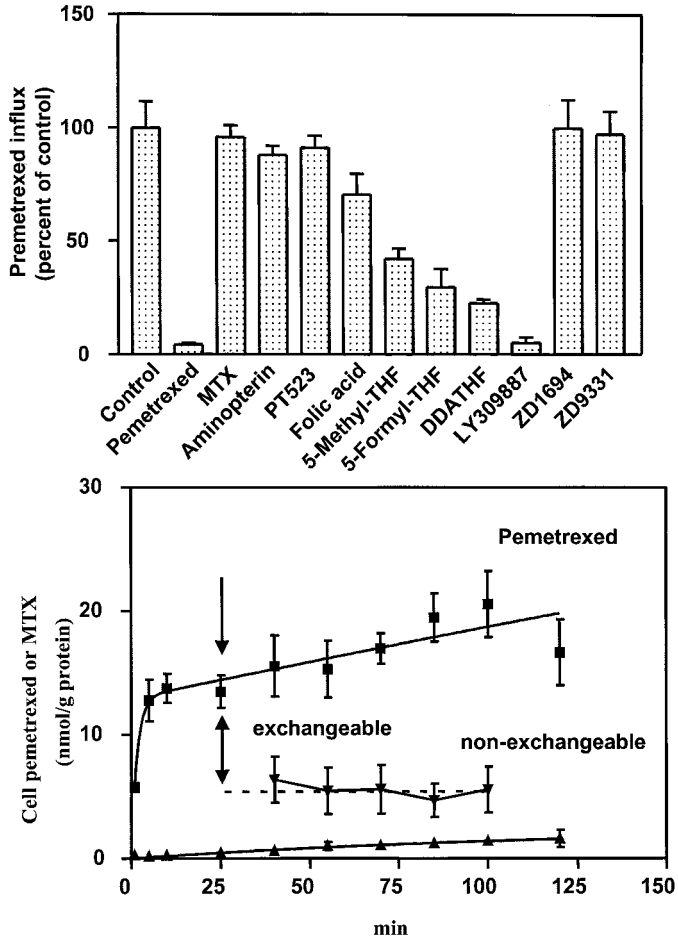

Fig. 4. *Top panel*, the inhibitory effects of folates and antifolates on the influx of [^{3}H]pemetrexed over a peiod of 30 s mediated by the high-affinity transporter. The extracellular [^{3}H]pemetrexed level was 50 nM; all of the inhibitors were present at a 20-fold higher concentration of 1 μM. The data are the average of three experiments. *Bottom panel*, a comparison of net [^{3}H]pemetrexed and [^{3}H]MTX transport at an extracellular level of 50 nM. After 25 min of incubation (*arrow*), some of the vials containing pemetrexed were aspirated, washed with 0°C drug-free buffer, and then incubated with fresh buffer at 37°C to discriminate the fraction retained within the cell (▼) from exchangeable intracellular drug (↕); --------, the average nonexchangeable level extrapolated to the time of resuspension. The data are the average of three experiments.

Net Pemetrexed Transport in NCI-H28 Cells When the Extracellular Concentration Was in the Range of the Influx K_t for the High-Affinity Transport Route: Comparison with MTX. The Fig. 4, *bottom panel*, compares the time course of [^{3}H]pemetrexed and [^{3}H]MTX uptake over 90 min when the extracellular levels were 50 nM. MTX uptake was trivial compared with that of pemetrexed. After incubation with [^{3}H]pemetrexed for 25 min, cells in some vials were washed and incubated in drug-free buffer to assess the exchangeable and nonexchangeable components. It can be seen that the major fraction of intracellular pemetrexed exited the cells within 15 min, which suggests that this component of drug (*vertical arrow*) was free within the intracellular water. If this was the case, then the concentration of exchangeable pemetrexed achieved, expressed as micromoles per liter of intracellular water, was ~1 μM. This represents a gradient of 20:1 over the extracellular drug level of 50 nM.

Discussion

These data indicate the presence in mesothelioma cell lines of a unique high-affinity folate transport mechanism, never before described in human cells, with very novel properties that favor pemetrexed over many other antifolates including other thymidylate synthase inhibitors. The characteristics of this transporter are distinct from those of RFC. The latter has K_t's in the 5–7-μM range and is minimally concentrative (6, 11). RFC has comparable affinities for MTX and pemetrexed but a much higher affinity for ZD9331. RFC affinities for ZD1694 and PT523 are greater than for MTX (6, 16). This high-affinity transporter also has properties distinct from the FRs that have binding constants for pemetrexed and folic acid of ~1 nM and have relatively high affinities for ZD1694 and ZD9331 (6). FR expression based on a binding assay was not detected in the NCI-H28 mesothelioma line studied. FR-β message was not detectable nor was a PCR product. FR-α message was negligible, and no mutations were detected in the open reading frame ruling out the possibility of expression of a mutated form with altered binding characteristics (17). FR-α message has also been detected in mesothelioma tissues in other studies, and the level was almost always far less than that of RFC, and there was little contribution to MTX uptake by this route (18, 19).

These studies demonstrate a very striking transport advantage for pemetrexed in mesothelioma cells lines over several other antifolate thymidylate synthase and dihydrofolate reductase inhibitors. It would appear, intuitively at least, that this may play a role in the efficacy of pemetrexed in this disease. High rates of drug transport and enhanced concentration within cells mediated by this high-affinity process should augment the rate, extent, and duration of polyglutamation and suppression of thymidylate synthase and GAR transformylase. However, it is not clear as to which of these elements are limiting in the interactions among pemetrexed, its active metabolites, and cellular components, and the extent to which subsequent downstream events are key determinants of the activity of this drug. Although the three mesothelioma cell lines evaluated all showed this transport activity, to different degrees, additional studies are required to assess the functional expression of this transporter in other mesothelioma cell lines and in human tumor tissue, and whether this parameter correlates with clinical response to pemetrexed. Also of interest is the extent to which tumors of other tissue origin express this transport activity. Finally, the physiological function of this transporter remains to be explored along with its expression and role in normal tissues.

Because RFC is expressed and active in mesothelioma cells (19), the delivery of pemetrexed will be the sum of this process and this high-affinity route. When the high-affinity route is present at the levels detected in the current study, it will be the dominant process and may overwhelm RFC over a broad range of extracellular drug levels especially in terms of concentrative transport, as seen in Fig. 1. On the other hand, when expression of this transporter is absent or low, delivery of drug will depend on the activity of RFC. In any event, it would appear that these pathways offer alternative routes for pemetrexed uptake in mesothelioma cells. The high-affinity transporter functions most efficiently at very low (nanomolar) concentrations. It should be especially effective in maintaining high intracellular pemetrexed levels in mesothelioma cells for long intervals (days) beyond 24 h after i.v. administration of a 500-mg/m^2 dose, when extracellular drug concentrations are in a range in which this transporter is the major, or sole, route of transport (20). This should sustain pemetrexed polyglutamation during this period and thereby prolong the duration over which the drug inhibits its target sites.

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