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Decreased expression of the reduced folate carrier and folypolyglutamate synthetase is the basis for acquired resistance to the pemetrexed antifolate (LY231514) in an L1210 murine leukemia cell line

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Abstract

Pemetrexed (LY231514) is a new-generation antifolate that, in its polyglutamyl forms, is a potent inhibitor of thymidylate synthase and glycinamide ribonucleotide formyltransferase (GAR transformylase). This study explored the mechanisms of resistance to pemetrexed in L1210 murine leukemia cells using chemical mutagenesis with 5-formyltetrahydrofolate (5-formylTHF) as the growth substrate. A cell line, MTA-13, was identified that was 8.5-fold resistant to pemetrexed with comparable cross-resistance to ZD1694 (Tomudex) and lesser cross-resistance (5-fold) to ZD9331 [(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], DDATHF (dideazatetrahydrofolate) (3.5-fold), and methotrexate (MTX) (2.7-fold) but comparable sensitivity to trimetrexate. Influx of pemetrexed, MTX, and 5-formylTHF into MTA-13 cells was decreased by 56, 47, and 38% compared to wild-type cells. Folate receptor expression was negligible in both cell lines. Net drug uptake declined within 15 min to a slower, constant rate over the next 45 min, reflecting the rate of accumulation of pemetrexed polyglutamate derivatives. This rate in the MTA-13 line was half that of the wild-type cells. Accumulation of 50 nM [^{3}H]pemetrexed, 25 nM [^{3}H]5-formylTHF, or 50 nM [^{3}H]DDATHF after 3 days was decreased to 35, 46, and 56% the level of L1210 cells. The reduced folate carrier (RFC) message and protein were decreased by 50%, and folypolyglutamate synthetase (FPGS) message was decreased by 65% in MTA-13 cells. No mutations were detected in either protein by DNA sequence analysis. There was a slight decrease (~25%) in thymidylate synthase mRNA, without mutations in the protein, and there was no change in GAR transformylase message. The data indicate that resistance to pemetrexed in the MTA-13 cell line was due to changes in both RFC and FPGS expression, two proteins that act in tandem to regulate polyglutamation of folates and antifolates in cells, resulting in cellular depletion of these active pemetrexed congeners.

Keywords: Pemetrexed; Alimta; Multi-targeted antifolate; Antifolate resistance; Antifolates

1. Introduction

Pemetrexed is a new-generation antifolate that in its polyglutamyl forms is a direct inhibitor of tetrahydrofolate cofactor-requiring enzymes in thymidylate (thymidylate synthase) and purine (GAR transformylase) biosynthesis [1,2]. This is unlike the classical antifolates, MTX and aminopterin, that block tetrahydrofolate regeneration from dihydrofolate by inhibition of dihydrofolate reductase, thus depleting cells of the folate cofactors required for these reactions [3,4]. The mono- and polyglutamates of pemetrexed are also inhibitors of dihydrofolate reductase, but with an affinity for this enzyme of about three orders of magnitude less that of MTX [2]. Its multiple sites of enzyme inhibition are the basis for the characterization

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Abbreviations: Pemetrexed (™Alimta, LY231514), *N*-{4-[2-(2-amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic acid; DDATHF (Lomotrexol), (6*R*)-5,10-dideazatetrahydrofolate; ZD1694 (Tomudex, Raltitrexed), *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid; ZD9331, (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; GAR transformylase, glycinamide ribonucleotide formyltransferase (EC 2.1.2.2); AICAR transformylase, aminoimidazole carboxamine ribonucleotide formyltransferase (EC 2.1.2.3); GAT, 200 μM glycine, 100 μM adenosine, 10 μM thymidine; MTX, methotrexate; RFC, reduced folate carrier; FPGS, folylpolyglutamate synthetase; 5-formylTHF, 5-formyltetrahydrofolate; and 5-methylTHF, 5-methyltetrahydrofolate.

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of pemetrexed as a "multi-targeted" antifolate [2]. Pemetrexed has substantial activity in model tumor systems and has shown activity in the clinics, as well, particularly in combination with cisplatin in the treatment of pleural mesothelioma, a disease heretofore refractory to chemotherapeutics [5,6]. Because of its clinical utility and novel pharmacological actions, mechanisms by which tumor cells develop resistance to this agent are of particular interest.

Resistance to antifolates has been attributed to: (i) impaired transport, (ii) increased expression, amplification, or mutation of target enzymes, and (iii) impaired polyglutamation due to alterations in the expression or function of FPGS [7–11]. A major route for MTX transport in tumor cells is RFC [12], and resistance on the basis of impaired transport by this mechanism in the clinical setting has been shown to be due largely to decreased expression [13–15]. However, tumors have been identified with impaired transport with unchanged expression likely due to mutations [14] in RFC, and recently mutations in RFC have been documented in a human osteogenic sarcoma [16]. In murine and human cell lines, mutations in RFC have been associated with resistance to MTX by virtue of alterations in the binding properties of the carrier and/or the mobility of the carrier loaded with its substrates [17,18]. These mutant carriers can be highly selective with relative preservation of natural folate transport or the transport of other antifolates [17–19].

Intracellular folate pools influence the efficacy of antifolates that require polyglutamation for activity [20,21]. High folate pools compete at the level of FPGS, suppressing formation of active antifolate polyglutamates. Low folate pools decrease competition at the level of FPGS, thereby augmenting antifolate polyglutamation [21]. Cell lines selected for resistance to MTX in the presence of physiological levels of 5-formylTHF (leucovorin) with impaired transport have decreased cellular folate cofactor pools and may have lesser cross-resistance to pemetrexed despite the fact that pemetrexed transport is also reduced [22].

Most studies on acquired antifolate resistance use selection approaches in which the folate growth substrate is folic acid. Since folic acid is transported largely by a mechanism independent of RFC [23,24], marked changes in the properties of this carrier that restrict entry of the drug can be achieved without depriving cells of this essential nutrient [7]. On the other hand, the natural folate in blood is 5-methylTHF, transported in many cells by RFC alone. Hence, acquired resistance to an antifolate that utilizes this system must allow sufficient reduced folate transport to sustain growth and replication—a much more restrictive, yet physiological, condition. There is limited information on the mechanisms of resistance to pemetrexed, particularly low-level, clinically relevant resistance. In this paper, we evaluated and report on the basis for acquired low-level pemetrexed resistance in an L1210 leukemia cell line selected with 5-formylTHF as the folate substrate.

2. Materials and methods

2.1. Chemicals

[3′,5′,7-^{3}H]-(6*S*)-5-formylTHF was obtained from Moravek Biochemicals; [3′,5′,7-^{3}H]MTX and [3′,5′,7,9-^{3}H]-folic acid were from the Amersham Corp. Pemetrexed bearing a 4-[ethyl-[1,2-$^3H_4$]]benzoyl modification (4.1 Ci/mmol) and unlabeled pemetrexed were provided by the Eli Lilly Co. Trimetrexate (TMQ) was a gift from Dr. David Fry (Pfizer, Inc.). Tritiated agents as well as unlabeled MTX and 5-formylTHF (Lederle) and folic acid (Sigma) were purified by high performance liquid chromatography prior to use [25].

2.2. Cell culture conditions, cell growth, and growth inhibition studies

Cells were grown in folate-free RPMI 1640 medium containing 25 nM 5-formylTHF supplemented with 5% dialyzed bovine calf serum (HyClone), 2 mM glutamate, 20 μM 2-mercaptoethanol, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37° in a humidified atmosphere of 5% CO_2. Cells were selected for pemetrexed resistance in this medium. Folate growth requirement (EC_{50}) and antifolate growth inhibition (IC_{50}) were assessed as reported previously [18].

2.3. Isolation of the pemetrexed-resistant MTA-13 cell line

L1210 cells were maintained in GAT-containing medium for 1 week to deplete endogenous folate pools prior to treatment with 1.6 mM ethylmethanesulfonate for 12 hr, a concentration that killed 90% of the cells. After cells were washed to remove the mutagen, they were grown in GAT medium for an additional 2 days, transferred to folate-free medium supplemented with dialyzed calf serum, 25 nM 5-formylTHF, and 130 nM pemetrexed, and plated on 24-well clusters. This concentration of pemetrexed was 10 times greater than the IC_{50} values under these conditions and completely suppressed growth of wild-type L1210 cells. Three resistant clones were isolated, all of which had diminished pemetrexed and MTX transport. One with the greatest transport defect, MTA-13, was studied in detail. This cell line has been maintained in drug-free, RPMI 1640 medium and has displayed a stable phenotype for more than 2 years.

2.4. Transport and net accumulation of cellular folates

Folate and antifolate influx was assessed by methods described previously [26]. Net antifolate accumulation was measured in cells (3×10^6/mL) grown in folate-free RPMI 1640 medium with dialyzed bovine calf serum, 25 nM 5-formylTHF, GAT, and either 50 nM radiolabeled

pemetrexed or DDATHF. For 5-formylTHF accumulation, 25 nM [^{3}H]5-formylTHF was utilized. After 3 days of exponential growth, cells were harvested, washed twice with ice-cold HEPES-buffered saline (HBS; 20 mM HEPES, 140 mM sodium chloride; 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4), and processed for measurement of intracellular tritium as reported previously [26]. Exchangeable intracellular pemetrexed was assessed after cells loaded with drug were separated by centrifugation and resuspended into a large volume of drug-free buffer. Prior studies with L1210 cells from this laboratory have demonstrated that (i) after an initial phase of rapid uptake (~30 min) the rate of accumulation of radiolabel is constant over at least 60 min and represents the rate of formation and retention of polyglutamate derivatives, (ii) intracellular radiolabel after 3 days represents virtually entirely radiolabeled polyglutamate derivatives of these substrates, and (iii) nonexchangeable drug represents pemetrexed polyglutamates, a component that is eliminated in cells with mutated FPGS that do not synthesize these derivatives [22].

2.5. Northern analyses

Total RNA was isolated using the TRIzol reagent (Life Technologies, Inc.). RNA (40 μg) was fractionated by electrophoresis on 1.0% formaldehyde–agarose gels. Transfer and hybridization were performed as described previously [26]. All northern blot transcripts were quantitated by PhosphorImager analysis of the hybridization signals and normalized to β-actin. The data presented were based upon two separate experiments in which RNA and protein were prepared and analyzed.

2.6. Sequence analysis of RFC, FPGS, and thymidylate synthase cDNA

Poly(A)$^+$ RNA was purified from MTA-13 and L1210 cells using the Dynabeads mRNA DIRECT kit (Dynal). First strand synthesis was carried out with Superscript Reverse Transcriptase (Life Technologies) for RFC and FPGS, and according to the 3′ RLM-RACE protocol (Ambion) for thymidylate synthase. The cDNAs were amplified with *pfu* Turbo polymerase (Stratagene) utilizing the following oligonucleotide primers which flank the coding regions: for RFC, 5′-GCGGATCCTGGAGTGTCATCTTGG-3′ and 5′-GCCTCGAGCTGGTTCAGGTGGAGT-3′; for FPGS, 5′-GGAGCCGGGCATGGAGTATC-3′ and 5′-TGTGGAAAGGCAGACCGATG-3′; and for thymidylate synthase, 5′-TGCTCCGTTATGCTGGTGGTTGGCTCCGA-3′ and 5′-GCGAGCACAGAATTAATACGACT-3′ (the 3′ RACE Outer Primer).

The PCR amplifications were performed for 35 cycles of 45 sec at 95°, 45 sec at 60°, and 4 min at 72°. The predicted PCR products were purified on an agarose gel (Qiagen) and cloned into a pCR-blunt vector (Invitrogen). For each protein, five randomly picked cDNA clones were sequenced using primers that covered the entire coding region. The sequence analysis was performed on an Applied Biosystems model 3700 capillary electrophoresis system in the Albert Einstein Cancer Center's DNA Sequencing Facility.

2.7. Preparation of cell lysate for western blot analysis

This laboratory has developed a polyclonal antibody (AE390) targeted to the distal C-terminus (Met^{499} through Ala^{512}) of RFC [27]. To assess RFC protein, total cell lysate (2×10^7 cells) was sonicated on ice for 20 sec with proteinase inhibitor. Protein concentration in the lysate was determined with the BCA Protein Assay Kit (Pierce). Fifteen micrograms of protein from the MTA-13 lysate was loaded for SDS–PAGE along with different amounts of protein from wild-type L1210 cell lysate. The loading buffer consisted of 62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.00125% bromophenol blue. Dithiothreitol (DTT) was omitted as was heat denaturation. After electrophoresis, protein was transferred to PVDF filters. The blots were probed with the affinity-purified rabbit anti-mouse RFC antibody followed by the addition of peroxidase-conjugated goat anti-rabbit IgG (Promega). Enhanced chemiluminescence (ECC) detection was performed according to the manufacturer's instructions (Amersham). This procedure has been described in detail previously [27].

2.8. Folate binding assay

Cells were harvested and 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. The cells were incubated with 1 mL PBS containing 5 pmol of [^{3}H]folic acid with and without an excess of nonlabeled folic acid, at 4° for 30 min, to quantitate specific folate binding, and then were 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 on a liquid scintillation spectrometer.

3. Results

3.1. Development of pemetrexed-resistant cell lines—cross-resistance patterns and folate growth requirements

L1210 cells were subjected to chemical mutagenesis with ethylmethanesulfonate followed by selection in 130 nM pemetrexed with 25 nM 5-formylTHF as the sole folate growth source in medium containing dialyzed serum. Three pemetrexed-resistant clones were identified with impaired transport; the one with the greatest defect, MTA-13, was chosen for further characterization. As indicated in Table 1, this cell line was 8.5-fold resistant to pemetrexed with comparable cross-resistance to ZD1694,

Table 1
Growth inhibition by a series of antifolates, and growth requirement for folates

	L1210 cells	MTA-13 cells	Fold-change
IC_{50} (nM)			
Pemetrexed	13.6 ± 3.0	116.0 ± 9.4	8.5
MTX	20.2 ± 3.8	54.5 ± 9.0	2.7
ZD1694	3.3 ± 0.6	28.0 ± 2.9	8.5
ZD9331	12.5 ± 1.8	63.5 ± 12.7	5.0
DDATHF	59.1 ± 9.0	205.1 ± 9.6	3.5
Trimetreaxate	12.8 ± 1.9	10.6 ± 1.8	0.8
EC_{50} (nM)			
Folic acid	88.3 ± 7.2	121.7 ± 1.7	1.4
5-formylTHF	0.77 ± 0.15	1.0 ± 0.1	1.3

Cells were maintained under usual growth conditions as described in "Section 2". Cell numbers were determined after 72 hr. Data are the average ± SEM of three experiments.

lesser cross-resistance to ZD9331 (5-fold) and DDATHF (3.5-fold), and comparable sensitivity to trimetrexate. MTA-13 had a small increase (~30–40%) in growth requirement for both folic acid and 5-formylTHF.

3.2. Influx and net uptake rates in the pemetrexed-resistant MTA-13 cell line

Pemetrexed resistance was related, in part, to a decline in transport (Fig. 1). The initial rate of pemetrexed uptake in the MTA-13 cell line was decreased by 56 ± 3.4% as compared to wild-type cells. There was a 47 ± 1.7% decline in MTX influx with a somewhat lesser 38.0 ± 6.0% decrease in 5-formylTHF influx. This change in pemetrexed influx could not be related to changes in folate receptor expression that were trivial and equal in both wild-type and MTA-13 cells (13 ± 1.8 and 12 ± 2.4 pmol/g dry weight of cells, respectively). Beyond the decrease in initial uptake rates, there was a decline in the overall rate and extent of pemetrexed uptake (Fig. 2) over 15–60 min. Previous studies from this laboratory demonstrated that this late uptake component represents the rate of formation of pemetrexed polyglutamate derivatives synthesized within the cells [22]. This latter uptake component in MTA-13 cells was 50% the rate in wild-type L1210 cells, consistent with a 50% fall in the rate of synthesis of pemetrexed polyglutamates. At 20 min, portions of the cell suspensions were separated, washed, and resuspended into drug-free buffer to permit efflux of exchangeable drug. In both cell lines, more than 90% of the radiolabel was nonexchangeable, consistent with the rapid rate of formation of pemetrexed polyglutamates, shown previously to be retained in L1210 cells [22]. This nonexchangeable polyglutamate component was so large, accounting for the major portion of intracellular drug, that it was not possible to accurately quantitate and compare the much smaller free monoglutamate levels.

To assess total folate accumulation under growth conditions and in the presence of lower, more relevant drug concentrations, cells were grown for 3 days with tritiated

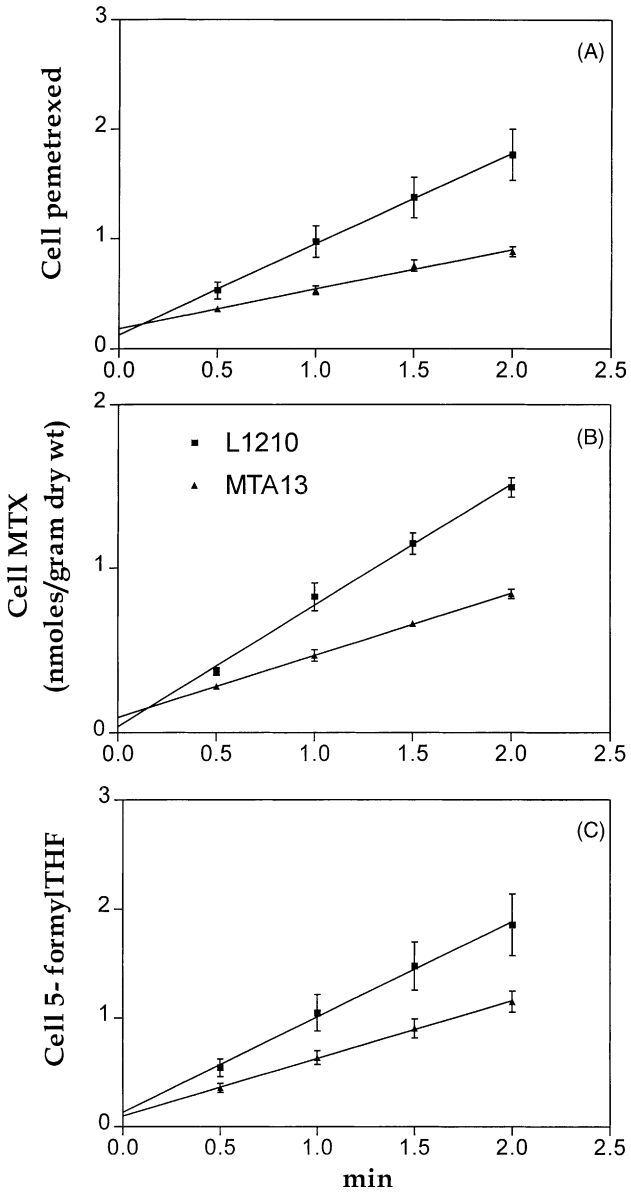

Fig. 1. Initial uptake of 5-formylTHF and antifolates. Wild-type L1210 and MTA-13 cells were harvested, washed twice with HBS buffer, and then incubated in 4.5 mL of HBS buffer for 25 min at 37°. This was followed by the addition of 1 μM tritiated pemetrexed, MTX, or 5-formylTHF. Data are the average ± SEM of three separate experiments. The three panels indicate (A) initial uptake rates for pemetrexed, (B) MTX and (C) 5-formylTHF in L1210 (■) and MTA-13 cells (▲).

25 nM 5-formylTHF, 50 nM pemetrexed, or 50 nM DDATHF. Under the latter two conditions, the folate growth source was 25 nM 5-formylTHF. As indicated in Table 2, pemetrexed accumulation in MTA-13 cells was 35% that of wild-type L1210 cells. The level of DDATHF was 46% that of wild-type L1210 cells, while the level of natural folates was 56% of L1210 cells. Under these conditions, virtually all intracellular radiolabel represents polyglutamate derivatives of natural folates and these drugs [22]. Hence, antifolate resistance correlated with diminished accumulation of active polyglutamate derivatives and contraction of the cellular folate pool.

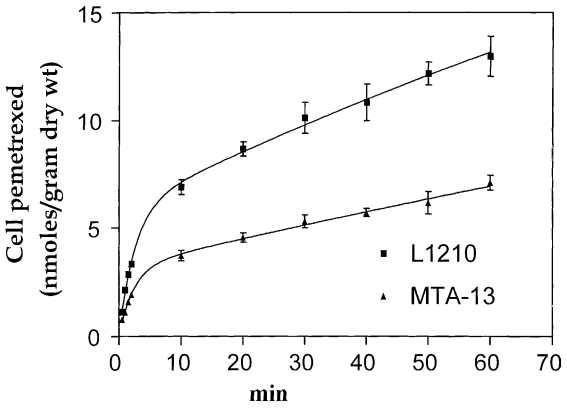

Fig. 2. Net uptake of pemetrexed. Cells were prepared as described in the legend to Fig. 1, and then incubated with 1 μM tritiated pemetrexed. Data represent the average ± SEM of three separate experiments.

Table 2
Accumulation of tritiated pemetrexed, DDATHF, and 5-formylTHF after 3 days under growth conditions

Cell line	Pemetrexed	DDATHF (nmol/g dry weight)	5-formylTHF
L1210	15.7 ± 2.4	7.68 ± 2.83	11.7 ± 0.6
MTA-13	5.52 ± 1.33	3.58 ± 1.47	6.64 ± 0.48
% Decrease	65	54	44

MTA-13 and wild-type L1210 cells were incubated with 25 nM [^{3}H]5-formylTHF, 50 nM [^{3}H]DDATHF, or 50 nM [^{3}H]pemetrexed in RPMI 1640 medium with 5% dialyzed bovine calf serum containing GAT. In the latter two conditions, 25 nM 5-formylTHF was added to the medium. After 3 days cells were harvested, and total folate/antifolate was measured as described in "Section 2". Data are the mean ± SEM of three separate experiment.

3.3. Expression of RFC, FPGS, and GAR transformylase

A variety of proteins are important determinants of pemetrexed activity, and alterations in their expression or function can result in drug resistance. Figure 3 shows northern and western blots for several key enzymes with changes quantitated by PhosphorImager analysis. There was a 50% decrease in RFC mRNA expression and protein in MTA-13 cells. The entire RFC open reading frame was sequenced in five clones; no mutations were detected. The level of FPGS mRNA was decreased by 65%. The open reading frame of FPGS from five separate clones was sequenced; no mutations were detected. GAR transformylase message was unchanged in MTA-13 cells. There was a small ~25% decrease in thymidylate synthase message; no mutations were detected in the protein based upon sequence analysis of five clones. Hence, the major changes detected were a decrease in expression of both RFC and FPGS without mutations in the coding regions.

4. Discussion

In the mid-1980s it was recognized that polyglutamation of MTX produced congeners with high affinity for thymidylate synthase and AICAR transformylase [28,29]. This insight led to a new focus on antifolates that, in their polyglutamyl forms, might be even more potent inhibitors of tetrahydrofolate-cofactor-dependent enzymes. Since then, several agents that inhibit GAR transformylase have been developed and brought into clinical trial [30,31]. Likewise, ZD1694 was developed as a highly potent inhibitor of thymidylate synthase [32,33]. These drugs are generally very good substrates for FPGS with affinities for this enzyme about two orders of magnitude better than

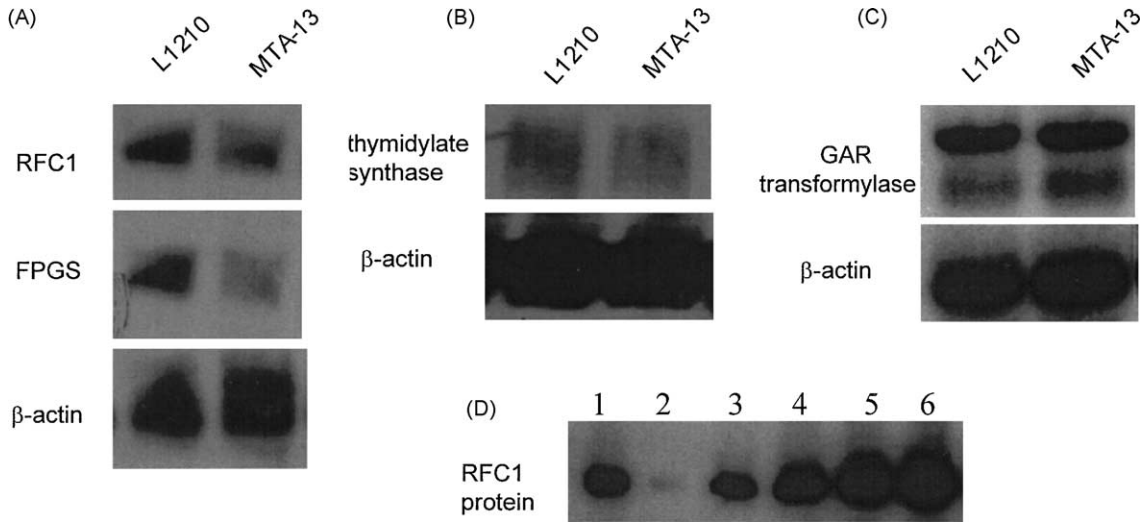

Fig. 3. Northern blot analyses of RFC and FPGS (A), thymidylate synthase (B), and GAR transformylase (C) along with β-actin standards. Panel D is a western blot using antibody to an epitope in the distal C-terminus of RFC. The amount of protein loaded on the gel was 15 μg for MTA-13 in lane 1, and 1, 5, 10, 15, and 20 μg for wild-type L1210 cells in lanes 2–6. The signals were quantitated on the PhosphorImager with KODAK ID Image Analysis Software, as indicated in the text. Data are representative of two separate experiments.

MTX. In their polyglutamyl forms, all are very potent inhibitors of their target enzymes (~1 nM or less), and all are good substrates for RFC with K_m values comparable to that of MTX [34].

One of the recent additions to the antifolate armamentarium is pemetrexed. This agent is among the most potent substrates for FPGS with a K_m of 0.8 μM, and its mechanism of action is unique [35]. In its monoglutamyl form, pemetrexed is a weak inhibitor of thymidylate synthase (K_i =~ 100 nM) and GAR transformylase (K_m =~ 9 μM); but as the pentaglutamate these K_i values decrease to 1.3 and 65 nM, respectively [1,2]. While pemetrexed polyglutamates are more potent inhibitors of AICAR transformylase than the monoglutamate (260 nM vs 3.6 μM, respectively), they are much weaker inhibitors than MTX tetraglutamate (56 nM) [2,5,28]. At concentrations in the range of the IC_{50}, pemetrexed activity appears to be due to inhibition of thymidylate synthase since cells can be fully protected with thymidine alone. However, at higher pemetrexed concentrations, protection requires both thymidine and a purine source, such as hypoxanthine [1,2]. Indeed, thymidine alone does not protect ZD1694-resistant cells with high thymidylate synthase activity from pemetrexed cytotoxicity, but hypoxanthanine alone does, suggesting a shift in the target site to GAR transformylase [36]. The mono- and polyglutamates of pemetrexed are comparable weak inhibitors of dihydrofolate reductase (DHFR) (K_i = 7 nM [2] as compared to an MTX K_i of 1–10 pM), and suppression at this site is not likely to contribute to the activity of this agent.

Intrinsic to pemetrexed activity is the formation of polyglutamate derivatives. The extent to which this occurs will depend upon the rate of pemetrexed entry into cells, the concentration achieved in the intracellular water, and the activity of FPGS. Polyglutamation of antifolates is also influenced by the level of natural folates within cells that compete for FPGS. As the natural tetrahydrofolate cofactor pool increases, the level of polyglutamate derivatives and the activities of these agents decrease, although the magnitude of change varies among the different antifolates [21]. The order of loss of activity as the folate cofactor pool was increased in L1210 cells was DDATHF > pemetrexed > ZD1694 and LY309887. For pemetrexed, an increase in the intracellular folate pool that accompanied an increase in extracellular 5-formylTHF of from 10 to 62.5 nM in L1210 cells resulted in a 20-fold increase in IC_{50} upon continuous exposure to the drug. With transient exposure the change was much greater [21]. An interesting DDATHF-resistant phenotype was detected in cells selected with folic acid as a growth source [37,38]. In this case, two mutations in RFC each contributed to enhanced affinity of carrier for folic acid without a change in DDATHF transport. This resulted in augmented folate pools and impaired polyglutamation of the drug. Likewise, loss of folate exporter function in a pyrimethamine-resistant CHO cell line with markedly augmented cellular folate pools resulted in cross-resistance to a variety of antifolates, including DDATHF [39,40].

A critical element in acquired antifolate resistance *in vitro* is the folate growth source utilized in the selection procedure. Folic acid is a very poor substrate for RFC, with a K_m about two orders of magnitude above that of the physiological blood folate, 5-methylTHF [41], and enters cells largely by a mechanism distinct from RFC. However, 5-methylTHF and 5-formylTHF enter hematopoietic and many other cells by RFC alone (when folate binding proteins are not expressed). Hence, when drug resistance associated with impaired antifolate transport arises in the clinical setting, sufficient transport of 5-methylTHF must be preserved to sustain tumor cell growth and replication.

Studies from this laboratory indicate that the frequency of emergence of MTX-resistant clones in the presence of a chemical mutagen is far lower in the presence of 5-formylTHF than when folic acid is the growth source [7]. However, cell lines resistant to MTX have been identified with sufficient preservation of 5-formylTHF and 5-methylTHF transport to allow growth and replication despite marked contraction of the tetrahydrofolate cofactor pool [7]. This depletion of natural cellular folates can result in the preservation of activity of other antifolates that require polyglutamation. For instance, a V104M mutation in RFC resulting in marked resistance to MTX was associated with collateral sensitivity to DDATHF despite a substantial, although lesser, fall in DDATHF transport [19]. Likewise, failure of MTX transport due to an S46N [18] or A130P [42] mutation in RFC that increased the MTX IC_{50} by factors of 10 and 11, respectively, when cells were grown in the presence of 5-formylTHF, increased the pemetrexed IC_{50} by factors of only 3.2 and 2.4, respectively [22].

There is limited information on mechanisms of resistance to pemetrexed. Of interest have been cell lines identified with primary resistance to an inhibitor of thymidylate synthase, such as ZD1694 or 5-fluorouracil (5-FU), which have a much lesser level of cross-resistance to pemetrexed [5]. For instance, an MCF-7 cell line 6400-fold resistant to ZD1694 due to an increase in thymidylate synthase was only 5-fold resistant to pemetrexed [36]. At levels of resistance to ZD1694 more relevant to the clinical setting, there might be no cross-resistance to pemetrexed. This preservation of pemetrexed activity is presumably due to inhibition of GAR transformylase when suppression of TS is negated [1,2]. A series of cell lines selected for primary resistance to DDATHF with mutations in both alleles of FPGS showed comparable, or somewhat greater, cross-resistance to pemetrexed [43]. In cell lines selected for resistance to ZD1694 with depressed uptake due to impaired polyglutamation and/or transport, there was also much lesser, or comparable, cross-resistance to pemetrexed [5,44]. In cell lines selected for resistance to pemetrexed in the presence of folic acid, cross-resistance to ZD1694 was generally higher, sometimes markedly so,

when resistance was due to either increased thymidylate synthase activity or impaired accumulation of the drug [5,36]. Overexpression of glutamyl hydrolase *in vitro* resulted in resistance to pemetrexed that was comparable to that of MTX and DDATHF [44].

In this study, few cell lines resistant to pemetrexed were obtained, possibly due to the stringency of the selection procedure. However, additional studies will be required to determine whether the frequency of emergence of clones resistant to pemetrexed might be different from other antifolates. The clone chosen for study had a modest, though clinically relevant, degree of resistance that was found to be associated with two complementary changes—impaired transport and polyglutamation—due to decreased expression of both proteins. Despite the use of a chemical mutagen, no mutations were detected in the open reading frames of RFC or FPGS, although it is possible that mutations did arise in the regulatory regions of these genes. No changes were noted in expression of GAR transformylase. There was a small, unexplained decrease in expression of thymidylate synthase that would tend to increase, rather than decrease, sensitivity to pemetrexed, and there were no mutations in this enzyme.

The declines in RFC and FPGS mRNA were small, but both proteins are required for the formation of pemetrexed polyglutamates. The degree to which the expression of each was suppressed was similar and comparable to the decrease in accumulation of pemetrexed polyglutamates. It might have been expected that these separate effects would be at least additive. However, there are a number of factors that might influence the impact of these changes. First, the level of FPGS message has not been a reliable indicator of the depression of FPGS protein or activity in antifolate-resistant lines. Hence, low levels of protein and activity have been associated with normal levels of FPGS message, indicative of changes at the level of translation [8,45,46]. On the other hand, there have been no reports in which message declined to a greater degree than enzyme activity. It is also possible that there was a shift in the distribution of pemetrexed polyglutamates to higher chain lengths that could modulate the impact of a decline in FPGS expression and total polyglutamates in the resistant line.

Also complicating interpretation of the impact of changes in RFC and FPGS expression on the level of resistance was the inability to measure accurately free intracellular pemetrexed levels, the substrate for FPGS, because of the rapid rate and extent of polyglutamation [22]. In other studies, it was noted that free levels of DDATHF were relatively preserved when influx was impaired in a resistant line, and this may be the case for pemetrexed [19]. Further, as transport of pemetrexed via RFC was reduced, transport of 5-formylTHF was reduced as well, depleting cellular tetrahydrofolate cofactors tending to preserve polyglutamation of the drug. The importance of the change in FPGS activity is indicated by the lesser degree of cross-resistance to ZD9331 in the MTA-13 line, a very potent thymidylate synthase inhibitor that does not undergo polyglutamation but utilizes RFC [47].

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