The inverse relationship between reduced folate carrier function and Pemetrexed activity in a human colon cancer cell line

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Abstract

Pemetrexed, a new generation antifolate recently approved for the treatment of mesothelioma and non–small cell lung cancer, is an excellent substrate for the reduced folate carrier (RFC). To explore the carrier's effect on pemetrexed activity, RFC was inactivated in HCT-15 colon cancer cells by mutagenesis and PT632 selective pressure. A clone (PT1) was obtained with a glycine to arginine substitution at amino acid 401, resulting in the loss of RFC function. PT1 cells were resistant to PT632 (178-fold), methotrexate (4-fold), and ZD1694 (Tomudex, raltitrexed; 20-fold), but were 3-fold collaterally sensitive to pemetrexed when grown in 25 nmol/L of 5-formyltetrahydrofolate. PT1 cells transfected with wild-type RFC had antifolate sensitivities comparable to that of wild-type HCT-15 cells, indicating that the RFC mutation was the sole basis for resistance. Folate pools were contracted in PT1 cells by 32% or 60%, as measured by radiolabeling intracellular folates or by an enzyme binding assay, respectively. This was reflected in marked (6.5-fold) collateral sensitivity to trimetrexate. The initial uptake of pemetrexed in PT1 cells was markedly reduced (~85%) but intracellular pemetrexed levels increased to ~60% and ~70% to that of wild-type cells after 2 hours and 6 days, respectively. There was increased pemetrexed inhibition of glycinamide ribonucleotide transformylase and, to a lesser extent, thymidylate synthase in PT1 cells growing in 5-formyltetrahydrofolate based on nucleoside protection analyses. Hence, loss of RFC function leads to collateral sensitivity to pemetrexed in HCT-15 cells, likely due to cellular folate pool contraction resulting in partial preservation of pemetrexed polyglutamylation and increased target enzyme inhibition. [Mol Cancer Ther 2006;5(2):438–49]

Introduction

Pemetrexed is a new generation antifolate active in the treatment of malignant pleural mesothelioma (1) and non–small cell lung cancer (2). This is the first antifolate to be approved by the Food and Drug Administration for cancer treatment in the U.S. (3, 4) since the introduction of aminopterin and methotrexate more than 50 years ago. Pemetrexed has unique properties as compared with methotrexate and other antifolates. Methotrexate is an inhibitor of dihydrofolate reductase (DHFR) and results in the cessation of purine and thymidylate synthesis by depleting cells of tetrahydrofolate-cofactors available for these reactions (5). Methotrexate forms polyglutamate derivatives slowly in cells; it is a poor substrate for folylpoly-γ-glutamate synthetase. These methotrexate derivatives are direct inhibitors of thymidylate synthase (TS) and aminoimidazole carboxamide ribonucleotide formyltransferase, but this inhibition is a late event, occurring only after suppression of DHFR by the monoglutamate (5). The pharmacologic effect of these polyglutamylated derivatives is due to their retention in cells, which prolongs the duration of DHFR suppression, and the subsequent inhibition of the utilization of exogenous reduced folates in tumor cells, especially during leucovorin rescue (5, 6). Pemetrexed, on the other hand, is an excellent substrate for folylpoly-γ-glutamate synthetase (7); polyglutamate derivatives accumulate rapidly in cells (8) resulting in marked suppression of TS, the primary target (9). There is lesser, but still potent, suppression of glycinamide ribonucleotide transformylase (GARFT; ref. 9, 10). Pemetrexed affinity for DHFR is 1/1,000th that of methotrexate (9, 10), and because of this, and its rapid and potent block at TS that results in the cessation of the generation of dihydrofolate, inhibition at DHFR is not of pharmacologic importance.

Pemetrexed is an excellent substrate for the known transporters of folates and antifolates. Its affinity for the reduced folate carrier (RFC) is twice that of methotrexate (11, 12) and its affinity for folate receptor-α (FR-α) is 2 orders greater than that of methotrexate and comparable to that of folic acid (11). Recent studies in this laboratory suggest that transport of pemetrexed via FR-α makes little

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contribution to the activity of this agent in human solid tumor cell lines, even those that express increased levels of the receptor (13). One property of pemetrexed that distinguishes this agent from most other antifolates is the observation, counterintuitively, that when RFC activity is lost in HeLa cells, the growth-inhibitory activity of this agent is preserved when the cells are grown in 5-formyltetrahydrofolate (5-CHO-THF; ref. 14). Under these conditions, there is marked resistance to ZD1694 (Tomudex, raltitrexed), PT523, and methotrexate. This retention of pemetrexed activity was attributed to the contraction of cellular tetrahydrofolate-cofactor pools due to diminished transport of 5-CHO-THF when RFC activity is lost, along with the presence of substantial residual RFC-independent carrier-mediated transport activity with high affinity for pemetrexed relative to other antifolates (12, 14, 15).

This report describes studies undertaken to determine whether the relationship between RFC activity and growth inhibition by pemetrexed could be extended to a human solid tumor cell line of different tissue origin and, in particular, whether this occurs when the residual RFC-independent transport (observed in HeLa cells) is much lower. The HCT-15 colon cancer cell line was chosen for this study. The approach taken to isolate cells with impaired RFC function was selective pressure with PT632 (5,8-dideaza-PT523) following chemical mutagenesis. PT632, like its predecessor PT523, is a potent inhibitor of DHFR, does not form polyglutamate derivatives, and is highly specific for RFC (16). This agent also has a very low affinity for FR-α and RFC-independent transport route(s) at physiologic and low pH (12, 14). Therefore, transport-mediated resistance would be expected to be restricted to loss of RFC function. This strategy has been used previously for developing RFC-null IEC-6 intestinal cells (17). The properties of an HCT-15–derived clone (PT1) are described in which RFC was mutated, transport activity was lost, and there was only a very low level of residual pemetrexed influx activity.

Materials and Methods

Chemicals

Unlabeled and [^{3}H]pemetrexed (1 Ci/mmol) was provided by the Eli Lilly Company (Indianapolis, IN). [3′,5′,7-^{3}H]-(6S)-5-formyltetrahydrofolate (5-CHO-THF) and [3′,5′,7-^{3}H]methotrexate were purchased from Moravek Biochemicals (Brea, CA). [3′,5′,7,9-^{3}H]Folic acid was obtained from Amersham Corp. (Arlington Heights, IL). Unlabeled (6 R,S) 5-CHO-THF and folic acid were obtained from Sigma (St. Louis, MO). G418 sulfate was obtained from Cellgro (Herndon, VA). ZD1694 was obtained from AstraZeneca, trimetrexate from Parke-Davis (Ann Arbor, MI), and AG331 from Agouron Pharmaceuticals, Inc. 5,8-Dideaza-PT523 (PT632) was provided by Dr. Andre Rosowsky, Dana-Farber Cancer Institute (Boston, MA). All radiochemicals were purified by high-pressure liquid chromatography (HPLC) before use (8). Stability was regularly checked by HPLC and compounds were repurified as necessary.

Cell Culture Conditions

HCT-15 cells were obtained from the National Cancer Institute Developmental Therapeutics Program and were maintained in RPMI 1640 containing ~2 μmol/L folic acid supplemented with 10% fetal bovine serum (undialyzed unless indicated otherwise; Gemini Bio-Products, Woodland, CA), 2 mmol/L glutamine, 20 μmol/L 2-mercaptoethanol, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 5% CO_2. Most experiments were carried out in cells growing under these conditions. For other experiments—growth inhibition, net accumulation of pemetrexed, HPLC analysis of pemetrexed polyglutamates, folic acid surface binding, and folate pool studies—cells were adapted for at least 1 week to folate-free RPMI to which 25 nmol/L of 5-CHO-THF was added in addition to the supplements noted above. Cells were monitored regularly for *Mycoplasma* contamination with the MycoSensor PCR assay kit (Stratagene, La Jolla, CA) and maintained free of this microorganism.

Growth Inhibition Studies

Growth inhibition studies were carried out in cells seeded in 96-well plates at a density of 1,000 cells/well with continuous exposure to antifolates, at 11 different concentrations, for 6 days, and quantified by the sulforhodamine B assay (18). The serum used in these experiments was undialyzed. For growth inhibition studies to examine the protective effect of nucleosides, the media (RPMI or folate-free RPMI supplemented with 25 nmol/L 5-CHO-THF) were prepared as above except that *dialyzed* fetal bovine serum was used. Cells, adapted for at least a week to these media, were seeded in 96-well plates at 1,000 cells/well and exposed to 11 different pemetrexed concentrations for 6 days either in the absence of nucleosides or the presence of 100 μmol/L hypoxanthine, 10 μmol/L thymidine or both. Cell growth was quantified by the sulforhodamine B assay.

Development of a PT632 Resistant Clone

HCT-15 cells growing in RPMI were mutagenized by a 16-hour exposure to ethylmethanesulfonate at 4.8 mmol/L, a concentration sufficient to kill >90% cells. Cells were then passaged into fresh plates and 24 hours later exposed to 30 nmol/L PT632. Cells that survived under these conditions were grown into clonal colonies and 10 to 15 days later were picked up and expanded in 12 wells, and later grown in 100 mm, plates. The clone which grew best under these conditions was named PT1, and chosen for further investigation. PT1 cells were grown under the same conditions as wild-type HCT-15 cells, except that 30 nmol/L PT632 was added to the media to maintain selective pressure.

Drug Uptake Studies

Radiolabeled drug influx and net uptake studies followed a protocol designed for rapid uptake determinations with adherent cells (19). Cells were seeded in 20 mL glass scintillation vials (Research Products International, Corp., Prospect, IL) at a density of 3 to 4 × 10^5 cells/vial to reach confluence 3 days later. For uptake experiments at pH 7.4, cells were washed twice with HEPES-buffered saline (HBS;

20 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $MgCl_2$, and 5 mmol/L glucose; pH 7.4) and incubated in the same buffer for 20 minutes at 37°C. The buffer was then aspirated, and cells exposed to 0.5 mL of HBS containing 0.5 μmol/L of [^{3}H]methotrexate or [^{3}H]pemetrexed. For uptake at pH 5.5, cells were washed with, and incubated in MBS buffer (20 mmol/L 2-(4-morpholino)-ethane sulfonic acid, 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $MgCl_2$, and 5 mmol/L glucose; pH 5.5) then exposed to 0.1 μmol/L [^{3}H]pemetrexed in the same buffer. At designated intervals, uptake was stopped by the addition of 5 mL of ice-cold HBS. After three washes with ice-cold HBS, cells were lysed with 0.5 mL of 0.2 N NaOH at 70°C for 40 minutes, 400 μL of lysate was transferred to glass vials, and radioactivity was measured. Ten microliters of lysate were used for protein determination using the bicinchoninic acid assay (Pierce, Rockford IL). Radiolabeled drug uptake is expressed as pmol/mg protein.

Northern Blot Analysis

Total RNA was isolated from HCT-15 and PT1 cells using the TRIzol method (Invitrogen, Carlsbad, CA). Thirty micrograms of RNA were run on a 1% formaldehyde denaturing gel and blotted onto a Nytran N-membrane (Schleicher&Schuell, Keene, NH). The membrane was probed with human RFC cDNA containing the open reading frame. Two bands were visible with the RFC probe in the HCT and PT1 lanes with low-stringency washes. One band was considerably less prominent when the membrane was washed under conditions of high stringency (i.e., 65°C washes with 1× SSC, 0.1% SDS twice for 15 minutes each followed by two washes with 0.1× SSC, 0.1% SDS for 20 minutes). The membrane was then stripped and reprobed with β-actin cDNA.

Detection of an hRFC Mutation

Total RNA (5 μg) isolated as described above was used for cDNA synthesis with the SuperScript II reverse transcriptase kit (Invitrogen). The coding region of hRFC was then PCR amplified from cDNA using primers designed from the hRFC sequence (GenBank accession No. U19720). The sense primer was 5′ TGTCACCTTCGTCCCCTCCG 3′ and the antisense primer 5′ TAGCAGGATAAGCGGAGGCC 3′. The PCR product was cloned into the pCR-4-TOPO vector (Invitrogen) following the manufacturer's protocol. The cloned product was sequenced using the primers supplied with the vector as well as two additional sense primers 5′ AACTACATCTCGCTGGCCTT 3′ and 5′ TACCAGTTCCTCGTGCCCAT 3′ and an antisense primer 5′ GAACACGCCCAGCAGCACCG 3′. A mutation was detected in RFC cDNA derived from PT1 cells, and to determine whether it was homozygous, the PCR product was sequenced directly using a sense primer specific for that site, 5′ TGGTCTTCCTTCTGGCGCAC 3′. Sequencing was carried out in the DNA Sequencing Shared Resource of the Albert Einstein College of Medicine Cancer Center.

Construction of Green Fluorescent Protein – Tagged Wild-type and Mutated hRFC

Wild-type hRFC was PCR-amplified from cDNA using the primers 5′ GGCTCGAGGGATGGTGCCCTCCAGCCCA 3′ (sense) and 5′ GCGGATCCTCACTGGTTCACATTCTGAA 3′ (antisense) which contain *Xho*I and *Bam*HI restriction sites, respectively. The PCR product and the phr-green fluorescent protein (GFP)-N1 vector (Stratagene) were digested with the above enzymes and ligated together. The 1296 G to A mutant RFC was created from this GFP-tagged wild-type RFC by QuikChange mutagenesis (Stratagene) using the primers 5′ GTGCCCTGGTCTTC**A**GGGTCAACACGTTCTTT 3′ (sense) and 5′ AAAGAACGTGTTGACCC**T**GAAAGACCAGGGCAC 3′ (antisense). The wild-type and mutant RFC sequences were confirmed by automated sequencing.

Transient Transfection

To study the disposition and function of the mutated carrier, the GFP-tagged wild-type RFC, mutant RFC, and vector control plasmids were transiently transfected in duplicate into RFC-null PT1 cells (seeded 1 day prior in six-well plates at 3×10^5 cells/well to reach 30–40% confluence on the day of transfection), using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. Cells were examined under a fluorescent microscope 2 days later. These cells were also used to assess the function of the transfected RFC proteins by measuring the influx of 0.5 μmol/L tritiated methotrexate or pemetrexed over 2 minutes (pH 7.4). Because transfection efficiency in PT1 cells is low, to confirm carrier localization to the cell membrane, the same constructs were transfected under the same conditions into RFC-null HeLa R5 cells (15) which have high transfection efficiency.

Stable Transfection of RFC into PT1 Cells

PT1 cells were seeded in six-well plates and transfected (as described in transient transfection) either with pcDNA3.1+ plasmid into which the full-coding length of hRFC was cloned, or with the plasmid alone. Two days later, cells were passaged into fresh 10 cm plates at 1:10, 1:30, and 1:90 dilutions and G418 added to a final concentration of 500 μg/mL. Stably transfected clones were picked 15 days later, amplified into six-well plates and then screened for RFC activity by measuring influx of 0.5 μmol/L tritiated methotrexate over 2 minutes (pH 7.4; as described above). An RFC-transfected clone, which exhibited a methotrexate initial uptake rate similar to wild-type HCT-15 cells, and a random vector-transfected clone, were picked for growth inhibition studies.

Net Accumulation of Pemetrexed under Growth Inhibition Conditions

HCT-15 or PT1 cells were seeded in six-well plates in folate-free RPMI containing 25 nmol/L 5-CHO-THF and GAT (200 μmol/L glycine, 100 μmol/L adenosine, and 10 μmol/L thymidine) at a density of 3×10^5 cells/well. [^{3}H]Pemetrexed was added to achieve a concentration of 50 nmol/L. Three days later, when cells were confluent, they were passaged into other six-well plates with fresh medium containing 50 nmol/L [^{3}H]pemetrexed. After three additional days (total exposure to [^{3}H]pemetrexed of 6 days), cells were washed with ice-cold HBS, lysed, and radioactivity was measured and normalized to protein content as in the drug uptake studies. Cellular uptake is expressed in units of pmol/mg protein.

Assessment of Total Radiolabeled Intracellular Folates

To estimate total intracellular folate pools, cells were seeded in six-well plates at a density of 3×10^5 cells/well in folate-free RPMI supplemented with 25 nmol/L [^{3}H]5-CHO-THF. Cells were grown under these conditions for a total of 7 days (with one passage at day 3—a total of approximately seven divisions) at the end of which all intracellular folates were radiolabeled. Thereafter, cells were washed and processed as in the pemetrexed accumulation studies and total intracellular radioactivity was measured.

Folate Pool Analysis

HCT-15 wild-type cells and the PT1 subline, growing for at least a week in 25 nmol/L 5-CHO-THF medium, were seeded in triplicate in 100 mm plates and grown to 70% to 80% confluence to ensure that cells were in the log phase of growth. Cells were then trypsinized, resuspended in ice-cold folate-free RPMI, and centrifuged. The cell pellets were washed thrice with ice-cold PBS then immediately frozen at −80°C. For folate pool analyses, the cell pellet was resuspended in 50 mmol/L Tris-HCl buffer (pH 7.4) containing 50 mmol/L sodium ascorbate. Cells were lysed by heating for 3 minutes in a boiling water bath, then the lysates were chilled on ice and centrifuged for 5 minutes at 17,000 × *g* at 4°C. Folate pools were measured in cell lysates by a ternary complex TS assay as described previously (20). Folate levels were calculated per milligram of cellular protein measured by the Bradford assay.

HPLC Analysis of Pemetrexed Polyglutamates

Wild-type and PT1 cells, adapted to 25 nmol/L 5-CHO-THF, were grown in 100 mm plates to reach confluence in 3 days. Cells were then washed twice with HBS, preincubated with this buffer for 20 minutes, and then exposed to 0.5 μmol/L [^{3}H]pemetrexed for 2 hours. Cells were then washed thrice with ice-cold HBS, mechanically dissociated from the plate using a rubber policeman, and resuspended in 1 mL of 50 mmol/L phosphate buffer (pH 6.0) containing 100 mmol/L 2-mercaptoethanol. Fifty microliters was processed for the measurement of total [^{3}H]pemetrexed as above with the exception that protein content was estimated using the Bio-Rad assay (Hercules, CA). The remainder was boiled for 10 minutes, centrifuged, and the supernatant was injected onto a reversed-phase HPLC column (Waters Spherisorb; 5 μmol/L ODS2; 4.6 × 250 mm) after the addition of pemetrexed-monoglutamate, -triglutamate, and -pentaglutamate nonlabeled standards, as reported previously (8).

Folate Binding Capacity

Cells near confluence were washed with ice-cold acid buffer (10 mmol/L NaAC, 150 mmol/L NaCl, pH 3.5) followed by a wash with ice-cold HBS (20 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $MgCl_2$, 5 mmol/L glucose, pH 7.4). Cells were exposed to 5 nmol/L [^{3}H]folic acid for 15 minutes in ice-cold HBS, then washed thrice with fresh folic acid–free buffer. [^{3}H]folic acid bound to the cell surface was then released with acid buffer (0.5 mL) and measured on a liquid scintillation spectrometer. The adherent cells were subsequently lysed with 0.2 mol/L NaOH (0.5 mL) and 10 μL of the lysate was used for protein determination (as in drug uptake studies). Folate binding capacity is expressed in pmol/g protein.

Statistical Analyses

An unpaired Student's *t* test was employed for analyses of differences in IC_{50}'s in growth inhibition studies, low pH transport, folate pool measurements, and polyglutamate derivatives of pemetrexed. Two-tailed *P* values were calculated based on 95% confidence intervals.

Results

Loss of RFC Function in PT632-Resistant Cells

HCT-15 cells were mutagenized then selected for resistant clones in the presence of 30 nmol/L PT632. The PT1 clone which grew best under these conditions was used for further studies. Transport function was examined by assessing influx of 0.5 μmol/L [^{3}H]methotrexate (pH 7.4). As depicted in Fig. 1, methotrexate influx in PT1 cells was <10% that of wild-type HCT-15 cells. Northern blot analysis (Fig. 2) revealed that RFC mRNA expression in PT1 cells was equivalent to that of wild-type cells (PT1 RNA loading was greater than wild-type HCT-15 RNA). Although two bands are visible with the RFC probe, in both lines the upper band may not be specific for RFC because it can be markedly reduced by more stringent washes. However, an alternative splice form cannot be ruled out. To examine whether RFC from PT1 cells was mutated, PCR-amplified RFC cDNA cloned into the pCR-4-ToPo vector was sequenced using two different primer sets as described in Materials and Methods. PT1 RFC revealed a G to A mutation at position 1296 (based on GenBank accession No. U19720), which resulted in a glycine to arginine substitution at amino acid 401 within the 11th transmembrane domain. Sequencing the PT1 RFC PCR product yielded only the mutated carrier, indicating either that this mutation was homozygous or that the wild-type allele was not present.

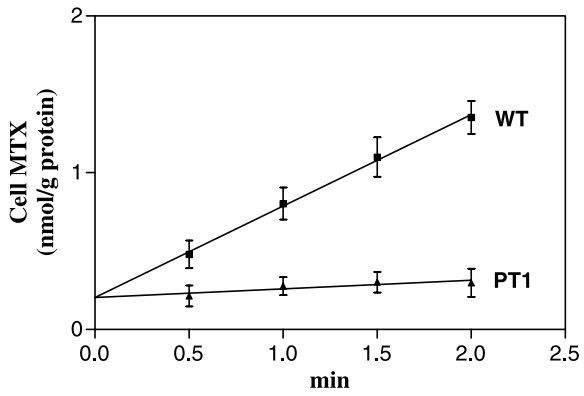

Figure 1. Initial uptake of methotrexate. Influx of 0.5 μmol/L [^{3}H]methotrexate (*MTX*) was assessed in wild-type (*WT*) and PT1 cells. *Points,* average of three independent experiments; *bars,* ±SE.

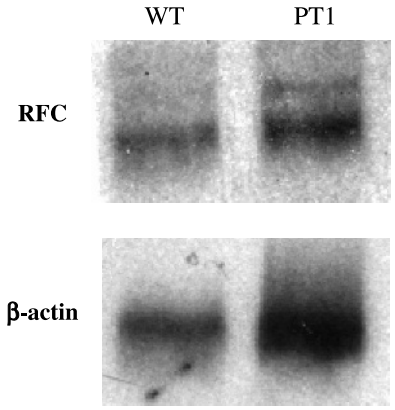

Figure 2. Northern blot analysis of RFC expression. Total RNA from PT1 and wild-type HCT-15 cells was run on a formaldehyde denaturing RNA gel and blotted onto a Nytran-N membrane as described in Materials and Methods. The membrane was probed with RFC cDNA followed by high-stringency washes as described in Materials and Methods (*top lanes*) then stripped and reprobed with β-actin cDNA (*bottom lanes*).

To assess the localization and function of the mutated protein, wild-type and mutated RFC were tagged with GFP and, along with the GFP vector-control, were transfected into PT1 cells. When examined for fluorescence, both wild-type and mutant RFC proteins were expressed equally, and detected at sites within the cell and at the plasma membrane, indicating that the mutant carrier localized to the cell membrane (data not shown). To assess function, the influx of tritiated methotrexate and pemetrexed was evaluated in the same transfectants. As indicated in Fig. 3, although uptake of methotrexate and pemetrexed was increased by a factor of 2.5 and 2.4, respectively, in PT1 cells transfected with wild-type RFC, as compared with vector control, there was no change in influx of either antifolate in cells transfected with the mutated carrier. The same constructs were also transfected into RFC-null HeLa-R5 cells. This produced similar results (data not shown), indicating that the loss of function of the mutated RFC was not cell-specific.

Effect of Loss of RFC Function on Antifolate Sensitivities

Growth inhibition studies with various antifolates were carried out in cells growing in folate-free RPMI supplemented with 25 nmol/L 5-CHO-THF as the folate source. As indicated in Fig. 4 and Table 1, PT1 cells were ~178-fold resistant to PT632 and >4-fold resistant to methotrexate compared with wild-type cells. However, the loss of RFC function resulted in ~3-fold collateral sensitivity to pemetrexed relative to parental cells, despite the fact that pemetrexed and methotrexate have comparable affinities for RFC (8, 11, 12). These cells were also highly resistant to ZD1694 (>20-fold), another antifolate with high affinity for RFC that, like pemetrexed, requires polyglutamylation for activity (5). On the other hand, there was no cross-resistance to the lipophilic TS antagonist AG331 excluding changes in PT1 at the level of this enzyme.

Pemetrexed activity is highly dependent on intracellular folate pools (21) due to the inhibitory effect of physiologic folates on the polyglutamylation of this drug and potential competition at the level of its target enzymes. Trimetrexate, a lipophilic DHFR inhibitor is even more sensitive to cellular folate pools; its activity is markedly increased if intracellular folates are decreased (21). As indicated in Fig. 4, PT1 cells were ~6.5-fold more sensitive to this agent than wild-type cells, consistent with marked contraction of cellular folate pools.

PT1 cells were developed by chemical mutagenesis and to ensure that the mutation in RFC was the only determinant of resistance in these cells, wild-type RFC was transfected into PT1 cells to determine if the wild-type phenotype could be restored. As indicated in Table 1, both resistance to methotrexate and PT632 and the collateral sensitivity to pemetrexed were reversed in the RFC transfectants. Although there were small variations in the IC_{50} values between untransfected and vector-transfected PT1 cells and between wild-type HCT-15 and RFC-transfected PT1 cells (possibly due to clonal variability), PT1 cells transfected with RFC or the empty vector had a pattern of antifolate sensitivities similar to wild-type HCT-15 or PT1 cells, respectively. Apart from AG331, all differences between HCT-15 and PT1 cells or between RFC- and vector-transfected PT1 cells were significant at $P < 0.01$.

To examine the effect of the folate growth source on antifolate sensitivities, cells were grown in the usual RPMI which contains 2 μmol/L folic acid. Because folic acid is transported largely by a mechanism distinct from RFC, only small changes in cellular folate pools are expected and observed when RFC function is lost (22). Under these

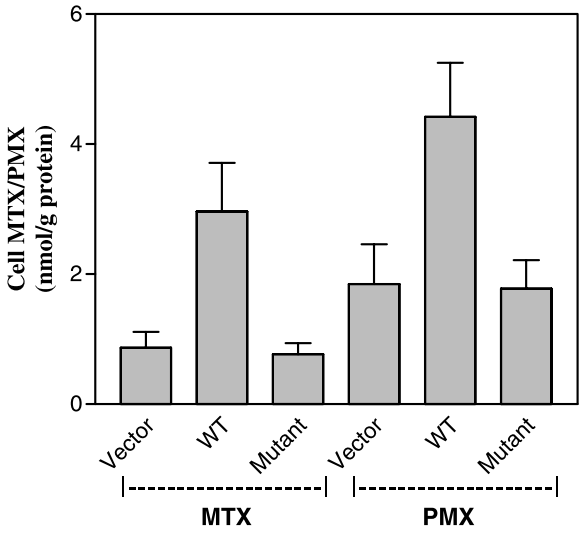

Figure 3. Effect of the G1296A mutation on RFC function. GFP-tagged wild-type and mutant RFC along with the GFP vector alone were transiently transfected, in duplicate, into PT1 cells growing in six-well plates using the LipofectAMINE Plus reagent. Forty-eight hours later, the influx of either 0.5 μmol/L [^{3}H]methotrexate (*MTX*) or [^{3}H]pemetrexed (*PMX*) was assessed. *Columns,* nmol/g of protein from three independent experiments; *bars,* ±SE.

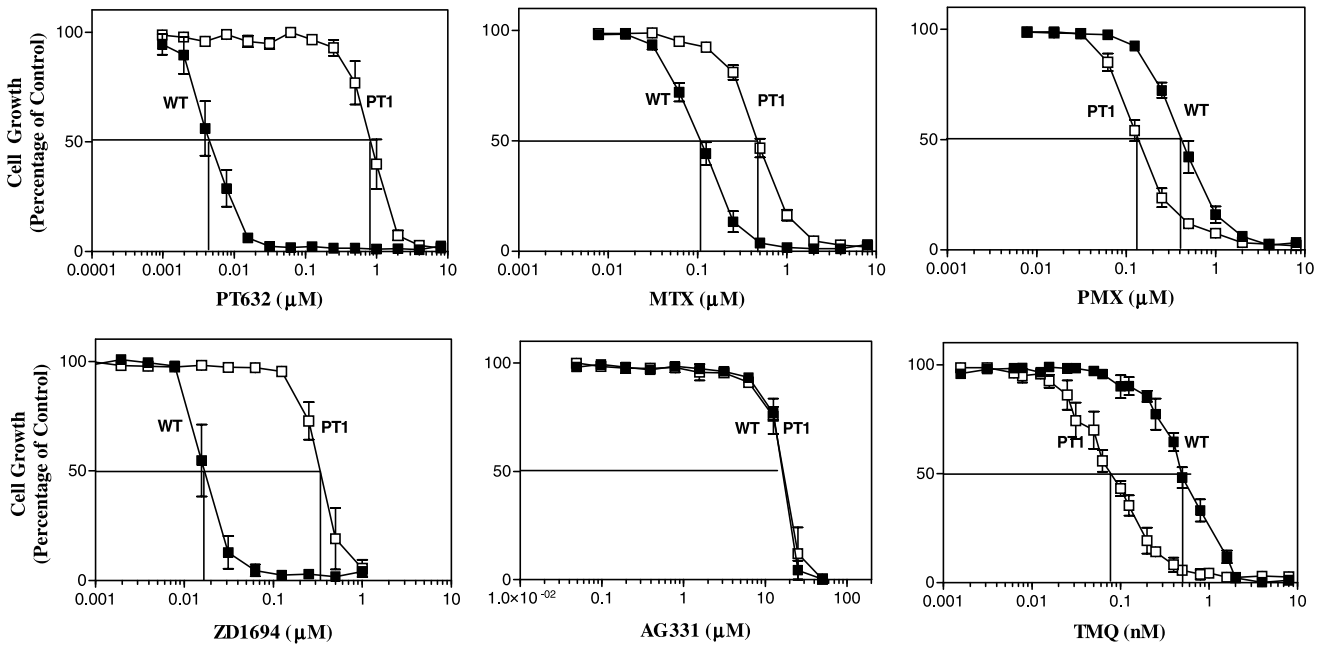

Figure 4. Inhibitory effects of antifolates in cells grown with 5-CHO-THF. PT1 or wild-type HCT-15 cells, growing for at least a week in folate-free RPMI supplemented with 25 nmol/L 5-CHO-THF, were seeded in 96-well plates at a density of 1,000 cells/well and exposed to a variety of antifolates, over a broad range of concentrations. After 6 d, cell protein was quantitated with the sulforhodamine B assay. *Points,* average percentages of cell growth relative to untreated cells from seven different experiments conducted over an extended period, with the exception of ZD1694 and AG331 groups, which are the averages of three and two experiments, respectively; *bars,* ±SE.

conditions, wild-type and PT1 cells had similar sensitivities to trimetrexate consistent with similar cellular folate pools; the apparent, small decrease in IC_{50} in PT1 cells was not statistically significant ($P = 0.19$). As indicated in Fig. 5, cells growing in folic acid were now ~4.5-fold resistant to pemetrexed, and resistance to both methotrexate and PT632 was increased to ~15-fold and ~320-fold, respectively, levels much greater than observed in cells grown in 5-CHO-THF. RFC- and vector-transfected PT1 cells followed a similar pattern of sensitivities as wild-type HCT-15 and nontransfected PT1 cells, respectively (Table 1). The small decrease in trimetrexate IC_{50} in PT1-RFC cells was not statistically significant ($P = 0.18$).

Assessment of Pemetrexed and 5-CHO-THF Accumulation under Growth Conditions

To assess the effect of the RFC mutation in PT1 cells on pemetrexed and folate accumulation, intracellular levels were measured after cells were grown for 6 days with

Table 1. Growth inhibition by antifolates

Cell line	HCT-15 (wild-type)	PT1 (nontransfected)	PT1-RFC	PT1-Vector
Cells grown in 5-CHO-THF				
Methotrexate	110 ± 11 (7)	474 ± 33 (7)	70 ± 7 (4)	350 ± 20 (4)
Pemetrexed	410 ± 61 (7)	128 ± 13 (7)	382 ± 36 (4)	155 ± 23 (4)
PT632	4.9 ± 1.0 (7)	871 ± 122 (7)	2.5 ± 0.2 (4)	1,125 ± 25 (4)
Trimetrexate	510 ± 59 (7)	79 ± 9 (7)	305 ± 39 (4)	31 ± 7 (4)
ZD1694	17 ± 3 (3)	360 ± 61 (3)		
AG331 (μmol/L)	19 ± 1 (2)*	19 ± 1 (2)*		
Cells grown in folic acid				
Methotrexate	44 ± 7 (6)	670 ± 45 (6)	19 ± 1 (3)	550 ± 29 (3)
Pemetrexed	250 ± 54 (6)	1,133 ± 185 (6)	77 ± 15 (3)	1,200 ± 58 (3)
PT632	2.7 ± 0.7 (6)	860 ± 162 (6)	0.9 ± 0.1 (3)	783 ± 109 (3)
Trimetrexate	112 ± 19 (6)*	81 ± 10 (6)*	48 ± 7 (3)*	97 ± 29 (3)*

NOTE: HCT-15 wild-type cells, PT1 cells, and PT1 cells transfected with either wild-type RFC or empty vector were grown for at least a week with the folate growth sources indicated, then exposed to a spectrum of concentrations of different antifolates for a period of 6 days. The IC_{50}s are the average ± SE and are expressed in nmol/L, except for AG331. The number of experiments is indicated in parentheses. All differences between HCT-15 and PT1 cells and between PT1-RFC and PT1-Vector cells are statistically significant ($P < 0.01$) except for the values marked with an asterisk (*, $P > 0.1$).

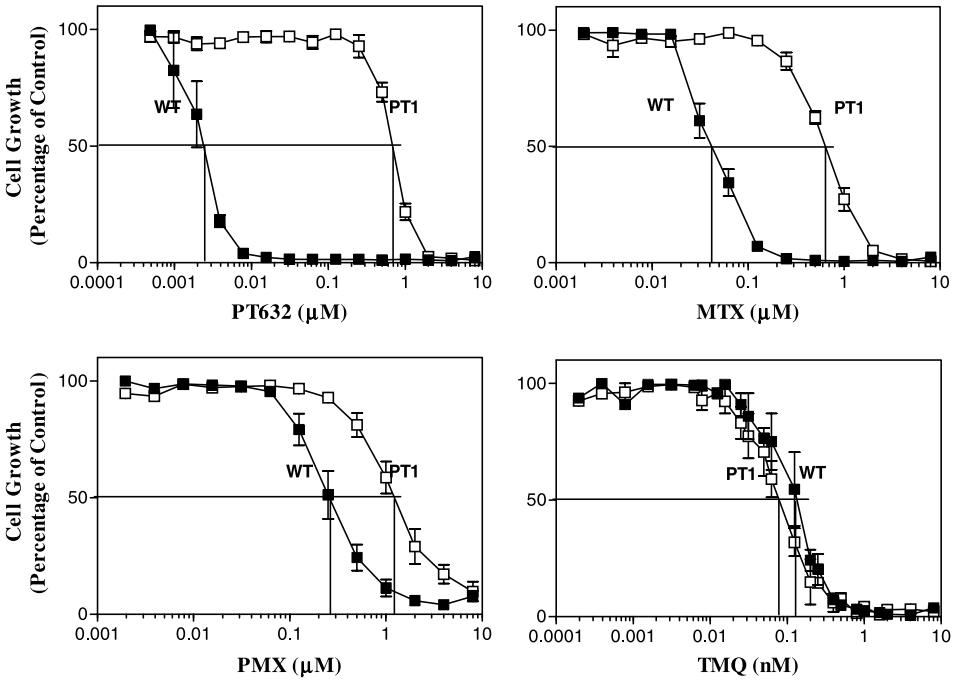

Figure 5. Inhibitory effects of antifolates in cells grown with folic acid. Cells growing in RPMI containing ~2 μmol/L folic acid were seeded in 96-well plates at a density of 1,000 cells/well and exposed to a variety of antifolates. Cell growth was estimated at the end of 6 d with the sulforhodamine B assay. *Points,* average percentage of cell growth relative to control cell growth from six different experiments conducted over an extended period with the exception of the assay with PT632, which is the average from five experiments; *bars,* ±SE.

50 nmol/L [^{3}H]pemetrexed (the duration of growth inhibition studies) and for 7 days with [^{3}H]5-CHO-THF (an interval over which all cellular folates become radiolabeled). Figure 6 indicates that cell pemetrexed (*left*) and total cell folate (*right*) levels were decreased by only ~30% and ~32%, respectively, in PT1 cells compared with wild-type cells despite the loss of RFC-mediated influx activity.

The distribution of oxidized and reduced cellular folates between different pools in wild-type and PT1 cells grown in 25 nmol/L 5-CHO-THF was assessed by a ternary complex TS assay (20). The pools measured included tetrahydrofolate + 5,10-methylenetetrahydrofolate, 5-methyltetrahydrofolate, 10-formyltetrahydrofolate, and dihydrofolate + folic acid. These folate coenzymes represent the major intracellular reduced folates and therefore the sum of their concentrations was represented as the total folate pool. The results of these measurements are shown in Table 2. Most notably, the total folate pool was decreased by nearly 60% in PT1 cells compared with the wild-type cells (7.1 ± 0.4 versus 16.8 ± 0.5 nmol/g protein, respectively). This was due to a decrease in 5,10-methylenetetrahydrofolate/tetrahydrofolate and 5-methyltetrahydrofolate pools by 60% and 66%, respectively. The dihydrofolate/folic acid pool was a trivial component of total folates and the 2-fold decrease in PT1 cells was not significant. The 10-formyltetrahydrofolate pool, also very low, was unchanged in PT1 cells. Intracellular 5-CHO-THF is not measured by this method.

Initial and Net Rates of Pemetrexed Uptake, Levels of FR-α Expression, Pemetrexed Polyglutamylation, and Uptake at Low pH

As observed for methotrexate, the pemetrexed initial uptake rate is decreased at pH 7.4 in PT1 cells by >85% (Fig. 7, *top*). However, after a period of 2 hours, there was only a 40% decrease in pemetrexed uptake as compared with wild-type cells (Fig. 7, *bottom*). This difference in net uptake was not associated with a change in distribution of pemetrexed polyglutamate derivatives as assessed by HPLC. Although total pemetrexed accumulation in PT1 cells was 60% that of wild-type cells, the relative proportion of higher pemetrexed polyglutamates (triglutamate and higher) was similar (Table 3). The FR-α expression levels in HCT and PT1 cells were measured by surface binding capacity, and although PT1 cells had higher surface binding than wild-type cells, the actual

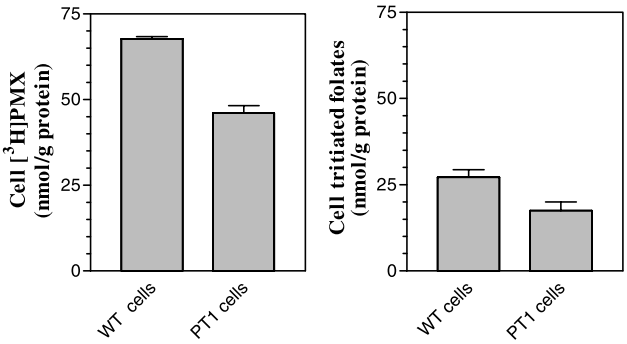

Figure 6. Net accumulation of [^{3}H]pemetrexed and [^{3}H]5-CHO-THF. Cells growing for at least a week in 25 nmol/L 5-CHO-THF were seeded in six-well plates at a density of 3×10^5 cells/well. *Left,* cells were grown with 50 nmol/L [^{3}H]pemetrexed, 200 μmol/L glycine, 100 μmol/L adenosine, and 10 μmol/L thymidine for 6 d and intracellular radioactivity was measured as described in Materials and Methods. *Right,* cells were grown in folate-free RPMI supplemented with 25 nmol/L [^{3}H]5-CHO-THF for 7 d at the end of which intracellular tritium was measured. *Columns,* average of three separate experiments (nmol/g protein); *bars,* ±SE.

Table 2. Analysis of cellular folate pools

	5,10-Methylenetetrahydrofolate + tetrahydrofolate ($P < 0.001$)	5-Methyltetrahydrofolate ($P < 0.001$)	10-Formyltetrahydrofolate ($P = 0.51$)	Dihydrofolate + folic acid ($P = 0.51$)	Total folates ($P < 0.001$)
Wild-type	6.7 ± 0.7	8.5 ± 0.6	0.9 ± 0.2	0.8 ± 0.2	16.8 ± 0.5
PT1	2.7 ± 0.2	2.9 ± 0.3	1.1 ± 0.2	0.4 ± 0.1	7.1 ± 0.4

NOTE: Wild-type and PT1 cells growing for at least a week in 25 nmol/L 5-CHO-THF were trypsinized, resuspended in ice-cold folate-free RPMI, then washed thrice with ice-cold PBS. The final pellet was analyzed for folate pool distribution as indicated in Materials and Methods. The results are the average + SE of four independent experiments, each conducted in triplicate.

binding capacities were barely detectable (0.041 ± 0.020 pmol/mg protein in PT1 cells versus 0.022 ± 0.018 pmol/mg protein in wild-type cells, from two experiments), a level 1/100th that of HeLa cells (13). Uptake of pemetrexed at low pH was also assessed (data not shown). Transport at pH 5.5 was negligibly decreased (15% $P = 0.053$) consistent with other observations from this laboratory that this transport activity is almost entirely independent of RFC (15, 17).

Protection of Pemetrexed Cytotoxicity by Purine and Pyrimidine Nucleosides

Pemetrexed is primarily an inhibitor of TS, depleting cells of thymidylate but, at higher concentrations, it also suppresses *de novo* purine synthesis due to the inhibition of GARFT. Previous studies in HeLa cells have shown that pemetrexed inhibition of GARFT increases as cellular folate pools are decreased (23). Studies were undertaken to determine the relative suppression of these enzymes in wild-type and PT1 cells and how this might relate to the preservation of pemetrexed activity in the latter cells with the loss of RFC function. Cells were adapted to medium containing 10% dialyzed serum with either 2 μmol/L folic acid or 25 nmol/L 5-CHO-THF following which pemetrexed growth inhibition was assessed in the presence or absence of nucleoside protection. As depicted in Fig. 8, the pemetrexed IC_{50} for wild-type cells was the same whether it was grown on 25 nmol/L 5-CHO-THF (*top left*) or 2 μmol/L folic acid (*bottom left*). In both cases, the addition of thymidine increased the IC_{50} by 1 order of magnitude (from 120 nmol/L to ~2 μmol/L) in 5-CHO-THF or folic acid. Full protection to ~30 μmol/L pemetrexed, however, required the addition of both hypoxanthine and thymidine, which is now consistent with a block at the level of GARFT. Alone, hypoxanthine had essentially no protective effect. When PT1 cells were grown on folic acid (*bottom right*), the pemetrexed IC_{50} was ~8-fold greater than that of wild-type HCT-15 cells (*bottom left*) and there was a 1 order of magnitude (~0.9 to ~8 μmol/L) increase in IC_{50} with the addition of thymidine. Full protection beyond a pemetrexed concentration of 8 μmol/L was achieved when hypoxanthine was added. PT1 cells grown on 5-CHO-THF (*top right*) had an IC_{50} 2-fold less than wild-type cells (*top left*) grown under the same conditions. Because hypoxanthine alone had no protective effect, this was attributed to a 2-fold greater inhibition at the level of TS in PT1 cells. Thymidine alone added to these cells resulted in much less protection (IC_{50} increased from ~60 to ~110 nmol/L), suggesting that GARFT inhibition occurred in PT1 cells at an 18-fold lower pemetrexed concentration than in wild-type cells. The combination of hypoxanthine with thymidine afforded full protection beyond a pemetrexed concentration of 110 nmol/L. Hence, there seemed to be enhanced target enzyme inhibition, which was prominent at the level of GARFT but was also seen at the level of TS in PT1 cells grown on 5-CHO-THF. Of note, the enhanced 2-fold pemetrexed sensitivity of PT1 (*top right*) relative to wild-type cells (*top left*) growing in 5-CHO-THF was less than

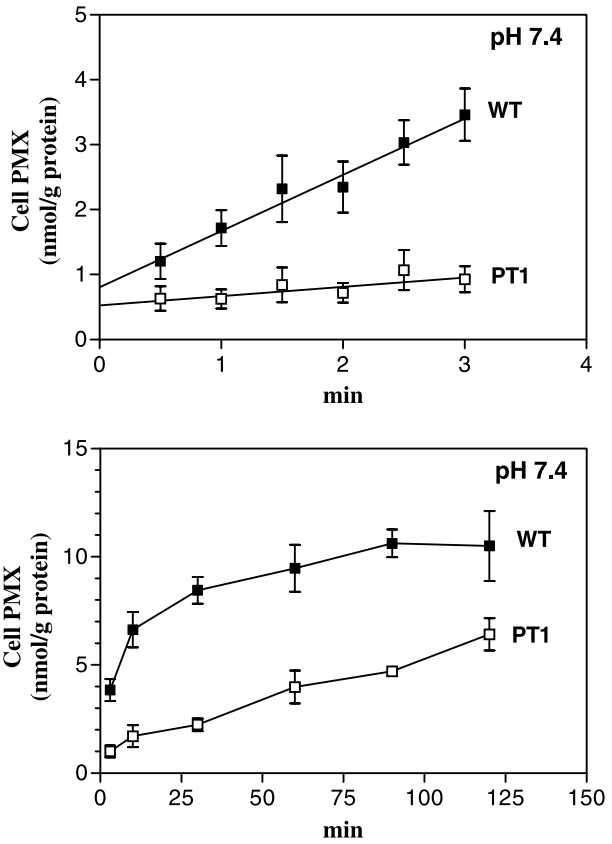

Figure 7. Initial and net uptake of pemetrexed. Initial uptake of 0.5 μmol/L [^{3}H]pemetrexed (*top*) was assessed in wild-type (*WT*) and PT1 cells growing in RPMI (pH 7.4) in HBS buffer. Net uptake of 0.5 μmol/L [^{3}H]pemetrexed (*bottom*) was assessed in cells grown for at least a week in folate-free medium supplemented with 25 nmol/L 5-CHO-THF. *Points,* average of at least three different experiments (nmol/g protein); *bars,* ±SE.

Table 3. HPLC analysis of intracellular pemetrexed

	HCT-15 wild-type	PT-1
≥Triglutamate (%)	68.0 ± 3.3	62.5 ± 5.0
Diglutamate (%)	10.5 ± 1.0	12.3 ± 2.3
Monoglutamate (%)	21.8 ± 3.9	25.2 ± 4.6
Total pemetrexed (nmol/g protein)	23.2 ± 3.3	12.6 ± 1.8

NOTE: [^{3}H]Pemetrexed polyglutamate derivatives were analyzed after a 2-hour interval of uptake with 0.5 μmol/L of radiolabeled drug as described in Materials and Methods. The results are the average ± SE of three independent experiments. The *P* value between wild-type and PT1 cells for each category of cell pemetrexed was >0.1.

the 3-fold difference observed in cells growing in non-dialyzed serum (Fig. 4). The basis for this difference is not clear but the former condition is more representative of *in vivo* conditions.

Discussion

Previous studies from this laboratory showed that in HeLa cells, loss of the *RFC* gene results in the preservation of pemetrexed growth inhibition in medium in which 5-CHO-THF is the source of intracellular folates (14). This was attributed to (*a*) a substantial (~50%) level of residual pemetrexed transport mediated by an RFC-independent process (14) and (*b*) a substantial depletion of cellular tetrahydrofolate-cofactors when RFC function is lost due to impaired transport of 5-CHO-THF, resulting in (*c*) partial preservation of pemetrexed polyglutamylation, compensating in part for the loss of transport, and (*d*) increased inhibition at the level of GARFT (23). The current study was undertaken to determine whether this phenomenon is dependent on a high level of residual RFC-independent transport and whether this would be observed in another human solid tumor cell line of different tissue origin. The data shows that, in the HCT-15 colon carcinoma cell line, loss of RFC function results in a marked reduction in pemetrexed transport with only a low level (~15%) of residual pemetrexed influx. This was associated with marked contraction of cellular pools of reduced folates when cells were grown in 5-CHO-THF and 3-fold collateral sensitivity to pemetrexed. This was in contrast to the high level of resistance to ZD1694, PT632 and, to a lesser extent, methotrexate—all antifolates that share a high affinity for RFC and, in the case of ZD1694, a high affinity for folylpoly-γ-glutamate synthetase. Transfection of RFC back into PT1 cells increased sensitivity to methotrexate and PT632 but decreased sensitivity to pemetrexed to levels observed in wild-type cells, confirming (*a*) the inverse relationship between RFC function and pemetrexed activity in these cells, regardless of the low level of RFC-independent transport activity and (*b*) that the PT1 phenotype was due entirely to the loss of RFC function. This is a unique property of pemetrexed, suggesting that loss of RFC function is unlikely to be an important primary mechanism of drug resistance and that this agent will be active under conditions in which there is loss of RFC function following treatment with methotrexate or another antifolate. The mechanisms that underlie this retention of pemetrexed activity were further clarified in the current study.

Pemetrexed activity and polyglutamylation is modulated by the level of physiologic folates in cells due to feedback inhibition of folylpoly-γ-glutamate synthetase by these compounds (24). When cellular folate pools are contracted, polyglutamylation of pemetrexed is enhanced

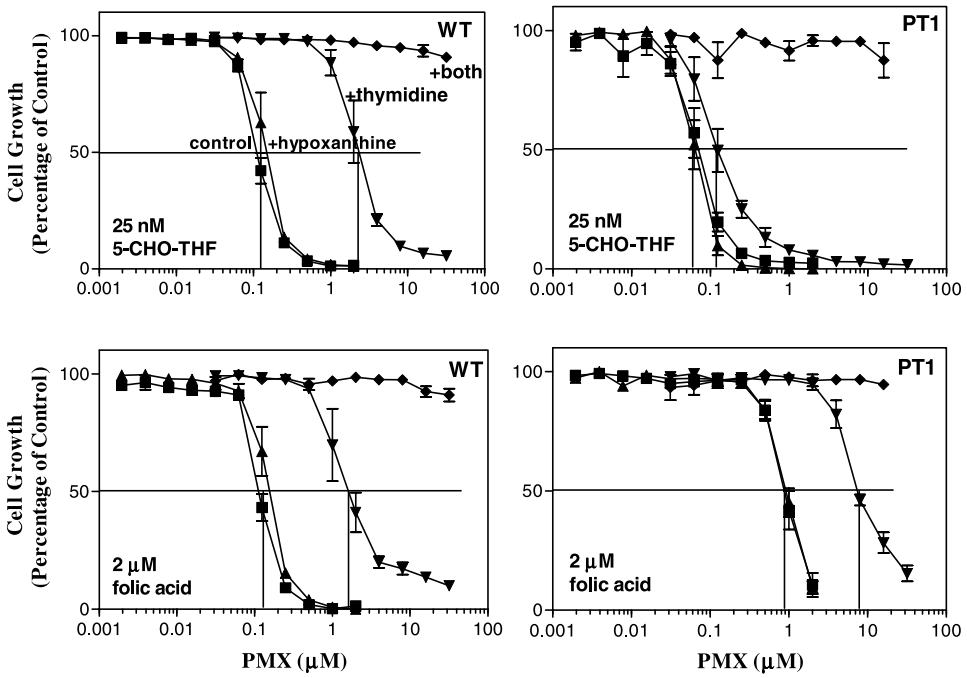

Figure 8. Protection of pemetrexed growth inhibition by purine and pyrimidine nucleosides. Wild-type (*left*) and PT1 cells (*right*) were adapted for at least a week to 10% dialyzed fetal bovine serum containing folate-free RPMI supplemented with 25 nmol/L 5-CHO-THF (*top*) or RPMI containing ~2 μmol/L folic acid (*bottom*). Cells were then seeded in 96-well plates at 1,000 cells/well and exposed to different concentrations of pemetrexed in the presence of 100 μmol/L hypoxanthine (▲), 10 μmol/L thymidine (▼), both thymidine and hypoxanthine (♦), or in the absence of nucleoside protection (■). Cell growth was quantified by the sulforhodamine B assay. *Points,* average of three independent experiments; *bars,* ±SE.

in comparison with methotrexate (21). There was a decrease in the level of cellular folates in PT1 cells by two different assays—32% decrease as assessed by growth in tritiated 5-CHO-THF and a more substantial reduction (60% reduction in total cellular tetrahydrofolate-cofactors) when assessed directly by a TS binding assay. There was no change in the ratio of higher to lower polyglutamate derivatives in PT1 cells. There was, however, ~6.5-fold collateral sensitivity of PT1 cells to trimetrexate, suggesting an even greater degree of folate depletion. Inhibition of DHFR by this agent, which enters cells by passive diffusion and does not undergo polyglutamylation, is highly sensitive to the level of cellular folates (21). This occurs because tetrahydrofolate-cofactors are interconverted to dihydrofolate in the synthesis of thymidylate, which then competes with trimetrexate for DHFR. Hence, the trimetrexate data suggests that, beyond the contraction of cellular folate pools, there may be, in addition, an even greater reduction in the level of cellular folates *available* to sustain tetrahydrofolate-cofactor–dependent reaction.

There is considerable evidence for the compartmentation of cellular folates (25). This may be due to protein binding (26) and/or sequestration in mitochondria (27, 28). A recent report based on modeling of folate reactions suggests that protein binding of folates may serve to maintain reaction velocities when total folates are decreased, thereby contributing to folate homeostasis (29). In L1210 cells, the levels of trimetrexate sufficient to completely block DHFR result in interconversion of only 25% to 30% of reduced folates to dihydrofolate, suggesting that the majority of intracellular folate pools are unavailable for enzymatic reactions (30). Similarly, after exposure of MCF-7 cells to a high concentration of methotrexate, dihydrofolate levels increased from 5% to only 30% of total folate pools (31). Hence, in the current study, the decrease in levels of folates actually available to support biosynthetic processes may be much greater than the measured decline in reduced cellular folates, and is probably best represented by the change in sensitivity of cells to trimetrexate. This apparent marked contraction of cellular folate pools may be the basis for collateral sensitivity to pemetrexed in PT1 cells despite a cellular pemetrexed level of accumulation one-third that of wild-type cells. As shown in Fig. 8, the decrease in folate pools resulted in a substantial increase in inhibition at the level of GARFT, and to a lesser extent at the level of TS, presumably due to decreased competition by the physiologic substrates of these enzymes. Increased GARFT inhibition by pemetrexed due to cellular folate depletion was observed in RFC-null HeLa cells or wild-type HeLa cells grown with very low extracellular folates (23).

The low level of pemetrexed influx in PT1 cells was sufficient to sustain the activity of this drug, but was too low to permit accurate characterization of residual transport properties, and to determine whether this represents residual RFC activity or an RFC-independent transport process. In HeLa cells, there is a pronounced RFC-independent transport activity at physiologic pH with relatively high affinity for pemetrexed (14). RFC-independent transport activity with high affinity for pemetrexed is also detected at low pH in HeLa cells (12). Data suggests that these activities may reflect the same carrier-mediated process, with pH-dependent differences in affinity, because both activities are lost and regained concurrently as methotrexate-selective pressure is applied or withdrawn in an RFC-deficient HeLa cell line selected for further resistance to this drug (32). The low pH activity may play a role in pemetrexed accumulation over the span of 6 days in the growth inhibition studies reported here, when the pH of the medium drops below the physiologic range. It is highly unlikely that the residual transport can be FR-α-mediated. FR-α is expressed at very low levels in PT1 cells, 2 orders of magnitude less than levels detected in HeLa cells, and a recent study from this laboratory indicates that FR-α does not contribute significantly to pemetrexed uptake or activity in HeLa cells whether RFC function is present or absent (13). It is of interest that whereas influx of pemetrexed is markedly reduced in PT1 cells, uptake continued at a low rate to achieve levels 60% that of wild-type cells after 2 hours, and 70% of wild-type cells after 6 days. The basis for this continued accumulation of pemetrexed is attributed to the contraction of cellular folate pools in cells grown with 5-CHO-THF, as described above, resulting in sustained polyglutamylation despite a low level of delivery of pemetrexed into PT1 cells.

The mutant PT1 cells were moderately to highly resistant to methotrexate, ZD1694, and PT632, antifolates that have high affinity for RFC. The activity of these antifolates is less dependent on the levels of intracellular folate pools than trimetrexate and pemetrexed (21), and they are also poorer substrates for RFC-independent alternative routes of entry into the cells—both at low (12) and physiologic pH (14), compared with pemetrexed. For instance, relative K_i's for pemetrexed, ZD1694, methotrexate and PT632 are 1, 6, 6.6, and 14, respectively, for the RFC-independent transport at physiologic pH (14), and 1, 6, 11, and >500, respectively, for transport at pH 5.5 (12). These data, along with the observation that activities of these agents are less modulated by the levels of cellular folate pools, seem to account for their significant loss of activity, compared with pemetrexed, with the loss of RFC function.

The use of 5-CHO-THF versus folic acid as the folate source should be considered within the context of their relevance to physiologic conditions. From the perspective of membrane transport, 5-CHO-THF has an affinity for RFC comparable to that of the major blood folate 5-methyltetrahydrofolate (11). Folic acid, on the other hand, uses a different mechanism of entry into cells, and hence there are only small changes in its uptake when RFC activity is lost (8, 22). The depletion of total intracellular folate pools in PT1 cells compared with the wild-type parental cell line is in good agreement with the role of RFC in 5-CHO-THF transport. It is noteworthy that both the wild-type and PT1

cell lines had very low levels of 10-formyltetrahydrofolate (Table 2) in contrast to several other cell lines in which 10-formyltetrahydrofolate is equal to, or even exceeds, the 5,10-methylenetetrahydrofolate/tetrahydrofolate or 5-methyltetrahydrofolate pools (33–35).

On the other hand, utilization of 5-CHO-THF requires its conversion to 5,10 methenyl-THF, a reaction mediated by methenyltetrahydrofolate synthetase (36). This is the only known step through which 5-CHO-THF can enter the folate cycle and become available for folate-dependent biosynthetic reactions. Hence, 5,10-methenyltetrahydrofolate synthetase might play a role in regulating the level of folate pools when cells are grown with 5-CHO-THF as the folate growth source. However, previous studies with MCF-7 and HCT-116 cells grown at concentrations of 5-CHO-THF from 0.1 to 50 μmol/L showed very low levels of intracellular 5-CHO-THF (<2% of total folates even at the highest extracellular concentrations) indicating that the 5-CHO-THF was efficiently incorporated into cellular folate metabolic pathways (37). Likewise, 5-CHO-THF levels in 5Y neuroblastoma cell cells expressing wild-type levels of 5,10-methenyltetrahydrofolate synthetase were only 3% to 7% of the total cellular folates when cells were exposed to 50 nmol/L [^{3}H]5-CHO-THF for 24 to 36 hours (38). These observations suggest that it is unlikely that 5,10-methenyltetrahydrofolate synthetase is limiting to 5-CHO-THF utilization in these cells.

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The inverse relationship between reduced folate carrier function and Pemetrexed activity in a human colon cancer cell line

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