Loss of Reduced Folate Carrier Function and Folate Depletion Result in Enhanced Pemetrexed Inhibition of Purine Synthesis

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ABSTRACT

Pemetrexed is a novel antifolate with polyglutamate derivatives that are potent inhibitors of thymidylate synthase (TS) and to a lesser extent glycinamide ribonucleotide formyltransferase (GARFT). Conditions that might modulate relative suppression of these sites were assessed by the pattern of hypoxanthine and thymidine protection. When grown with 25 nmol/L racemic 5-formyltetrahydrofolate, thymidine alone fully protected wild-type HeLa cells to at least 1 μmol/L pemetrexed, but protection of a reduced folate carrier (RFC)-null subline required both thymidine and hypoxanthine above a concentration of 30 nmol/L pemetrexed. As medium 5-formyltetrahydrofolate was decreased, protection by thymidine alone decreased, and was further diminished when HeLa cells were grown in dialyzed serum. There was little protection by thymidine of RFC-null HeLa cells under the latter conditions. Thymidine alone was not protective, and hypoxanthine alone produced only a small (2-fold) increase in IC_{50}, in a HeLa-derived line 8-fold resistant to pemetrexed due to a modest increase in TS. Finally, in MCF-7 breast cancer cells there was greater protection with thymidine alone than in HeLa cells when cells were grown in medium containing a low concentration of 5-formyltetrahydrofolate. These observations indicate that as intracellular folates decrease in HeLa cells, due to decreased extracellular reduced folate, or loss of RFC function, pemetrexed inhibition of GARFT increases. These data support the concept that the contribution to pemetrexed activity by inhibition of GARFT, particularly at low folate levels, is a contributing factor to drug activity but relative inhibition of TS and GARFT may vary among human tumors and cell lines.

Received 9/30/04; revised 11/4/04; accepted 11/11/04.
Grant support: NIH grant CA-82621 and Eli Lilly Co.
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INTRODUCTION

Pemetrexed is a new generation antifolate recently approved for the treatment of mesothelioma and non–small cell lung cancer (1, 2). The drug also has activity in the treatment of other solid tumors (3). The tri- and higher polyglutamyl derivatives of pemetrexed are potent inhibitors of human thymidylate synthase ($K_i \sim 1.4$ nmol/K). Their inhibitory potential for mouse glycinamide ribonucleotide formyltransferase (GARFT) is substantially lower, with K_i values for the tri- and pentaglutamates of 380 and 65 nmol/L, respectively (4). Thymidine alone provides protection against pemetrexed growth inhibition at concentrations in the range of the IC_{50} in human and murine leukemia cells *in vitro*, consistent with suppression of thymidylate synthase (TS) alone under these conditions. However, as the pemetrexed concentration is increased beyond this point, both a purine and thymidine are required for full protection, consistent with inhibition at both TS and GARFT (4, 5). On the other hand, when there is a marked increase in the expression of TS, thymidine alone affords no protection, whereas a purine alone provides complete protection at the high concentrations of pemetrexed that are required to achieve growth inhibition (6). Hence, under the latter condition, the actions of the drug can be attributed entirely to suppression of GARFT.

One important determinant of the activity of antifolates is the level of cellular folate cofactors that feedback inhibit the polyglutamation of antifolates (7) and thereby reduce their activity (8, 9). Pemetrexed is one of the antifolates for which this effect is prominent (9). Intracellular folate cofactor levels are directly related to extracellular folate concentration (9). There is a marked contraction of cellular folates when reduced folate carrier (RFC) function is impaired due to decreased transport of 5-formyltetrahydrofolate (5-CHO-THF) into cells (10, 11). Prior studies from this laboratory showed only a modest fall in pemetrexed activity in L1210 murine leukemia cell lines, harboring mutations in RFC that result in markedly impaired function, when the extracellular folate is 5-CHO-THF due to contraction of cellular folate cofactors. However, when cells are grown in folic acid, which is transported largely by an RFC-independent route, folate pools are preserved and there is marked resistance to pemetrexed (11). More recent studies have shown that in a HeLa-derived cell line, R5, in which RFC was deleted from the genome under 4-amino-10-methylpteroylglutamic acid (methotrexate)–selective pressure, there was *collateral sensitivity* to pemetrexed when the cells were grown in 5-CHO-THF associated with marked contraction of cellular folates (12, 13).

This report explores the relationship between conditions that alter cellular folate pools and inhibitory effects of pemetrexed at the level of TS and GARFT, and the extent to which the enhanced activity of pemetrexed in RFC-null R5 cells might be associated with alterations in the pattern of inhibition of these two enzymes. Because of its chemical stability and transport properties that are similar to the physiologic blood

folate (5-methyltetrahyrofolate), 5-CHO-THF was used as folate growth source in these studies.

MATERIALS AND METHODS

Chemicals. Methotrexate was obtained from the National Cancer Institute Developmental Therapeutics Program. Tritiated and nonlabeled pemetrexed were from the (Eli Lilly Co., Indianapolis, IN) ZD1694 and ZD9331 were provided by (AstraZeneca, Wilmington, DE) and AG331 was provided by (Agouron Pharmaceuticals, Inc., La Jolla, CA) 5-CHO-THF or leucovorin, a mixture of diastereoisomers at position 6, was obtained from (Lederle Parenterals, Inc., Carolina, Puerto Rico). All other chemicals were purchased from commercial sources.

Cell Culture Conditions. HeLa cells were purchased from (American Type Culture Collection, Manassas, VA) and MCF-7 cells were obtained from the National Cancer Institute Developmental Therapeutics Program. R5 cells are an RFC-null HeLa clonal derivative obtained under methotrexate-selective pressure with a genomic deletion of the carrier (12). R3 cells are a clonal line derived from HeLa cells under pemetrexed selective pressure and express an increased level of TS (see below). All cells were maintained in RPMI 1640 (Hyclone, Logan, VT) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, 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. For some experiments, cells were transferred to folate-free medium containing 1.6, 4, 10, or 25 nmol/L 5-CHO-THF and grown in this medium for 7 to 10 days. For other experiments, cells were transferred to and grown for 7 to 10 days in folate-free medium that contained different concentrations of 5-CHO-THF and were supplemented with dialyzed calf serum (Life Technologies, Carlsbad, CA). Cell cultures were monitored regularly with a *Mycoplasma* detection kit (American Type Culture Collection) and were shown free of this microorganism.

Selection of R3 Cells. R3 cells were generated by a single step exposure of HeLa cells to 300 nmol/L pemetrexed in DMEM medium (with the same supplements as described above for RPMI 1640) after treatment with 2.4 mmol/L of the chemical mutagen ethylmethanesulfonate for 24 hours, a methodology used previously in this laboratory (12). After it was recognized that the pemetrexed IC_{50} for antifolates was much greater in DMEM than RPMI 1640, likely due to the difference in the folic acid concentrations (12 versus 2.0 μmol/L, respectively), R3 cells were maintained in RPMI 1640 in the presence of 50 nmol/L pemetrexed.

Growth Inhibition by Antifolates. Cells grown in different media were transferred to 96-well plates (1,000 cells per well) and exposed continuously to a spectrum of pemetrexed concentrations for 6 days. To assess the impact of purine and pyrimidine nucleosides on pemetrexed growth inhibition, 10 μmol/L thymidine, 100 μmol/L hypoxanthine, or both were included in the assay media. Cell growth rates were quantified by sulforhodamine B staining (14).

Western Blot Analysis of Thymidylate Synthase Expression Level. HeLa and R3 cells (10^7) were harvested and resuspended in 200 μL of 10 mmol/L Tris-HCl (pH 7.4) with 0.25 mol/L sucrose. Cells were lysed by sonication using three 2- to 3-second bursts and centrifuged at 12,000 rpm for 10 minutes to remove cell debris. Protein concentrations of the supernatants were determined by the bicinchoninic acid assay (Pierce, Rockford, IL). Equal amounts of protein (50 μg) derived from HeLa and R3 cells were loaded and separated on a 12% SDS-PAGE gel, followed by blotting on a nitrocellulose membrane (Hybond-P, Amersham, Piscataway, NJ). Monoclonal anti-human TS antibody (clone TS108, Chemicon, Temecula, CA) was applied to the membrane followed by horseradish peroxidase-conjugated sheep anti-mouse antibody (Sigma). The TS signal was detected using the Enhanced Chemiluminescence Plus protocol (Amersham). After the membranes were stripped at 65°C for 30 minutes in 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, and 100 mmol/L β-mercaptoethanol, they were reprobed with a monoclonal anti-β-actin antibody (clone AC-15, Sigma, St. Louis, MO) as the loading control. Band intensity on X-ray films was quantitated by Kodak Image Station 440.

RESULTS

Effects of Folate Growth Substrate on Pemetrexed Growth Inhibition and Protection by Nucleosides in HeLa Cells and the Derivative R5 Reduced Folate Carrier-Null Cell Line. The R5 cell line is a HeLa variant with a genomic deletion of RFC (12). R5 cells are highly resistant to methotrexate, PT 523, and ZD1694 but minimally resistant to pemetrexed compared with HeLa cells when cells grown in medium containing folic acid (13). However, R5 cells are 2-fold collaterally sensitive to pemetrexed when grown in medium containing 25 nmol/L 5-CHO-THF (13). The effects of 10 μmol/L thymidine, 100 μmol/L hypoxanthine, or both on pemetrexed growth inhibition were assessed. As indicated in

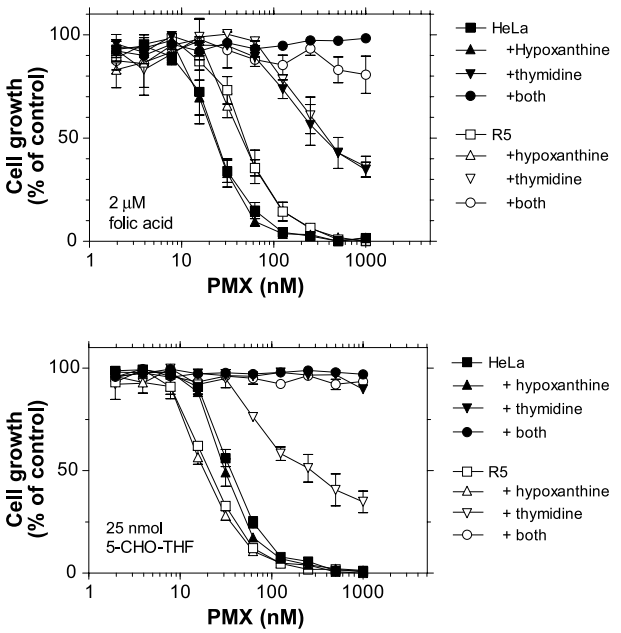

Fig. 1 Effects of thymidine and/or hypoxanthine on pemetrexed (*PMX*) inhibition of the growth of HeLa cells and its RFC-null derivative line, R5, grown in medium containing 2 μmol/L folic acid (*top*) or 25 nmol/L 5-CHO-THF (*bottom*). Both growth media were supplemented with 10% fetal bovine serum. Concentrations of thymidine and hypoxanthine in the media were 10 and 100 μmol/L, respectively. Points, mean from three separate experiments; bars, ±SE.

Fig. 1 (*top*), hypoxanthine alone had no effect at all on pemetrexed activity in either HeLa or R5 cells grown in folic acid medium. Thymidine alone afforded full and comparable protection to a pemetrexed concentration approximately twice the IC_{50} (~80 nmol/L) in both HeLa and R5 cells above which cell growth was progressively inhibited. As expected, thymidine and hypoxanthine together fully protected both cell lines to at least 1 μmol/L pemetrexed. When cells were grown in 5-CHO-THF medium (*bottom*), thymidine alone fully protected HeLa cells to at least 1 μmol/L pemetrexed. However, there was substantially less protection of R5 cells with thymidine with the same pattern as observed with wild-type HeLa cells grown in folic acid medium (*top*). Hence, when RFC-competent cells are grown with 5-CHO-THF, thymidine alone provides substantial protection. However, full protection of RFC-null HeLa cells required the addition of a purine consistent with an added inhibitory effect at the level of GARFT.

Effects of the Media 5-Formyltetrahydrofolate Level on Nucleoside Protection. To determine the extent to which the availability of folates influences pemetrexed suppression of purine and thymidylate biosynthesis, wild-type HeLa cells were grown in medium containing 1.6, 4, or 10 nmol/L 5-CHO-THF for 7 to 10 days, to allow equilibration of cellular folates, following which pemetrexed growth inhibition was assessed. Under these conditions, there is a near-linear relationship between the intracellular folate level and the extracellular concentration of 5-CHO-THF (9). As indicated in Fig. 2 (*left*), there was an inverse relationship between the pemetrexed IC_{50} and the concentration of 5-CHO-THF in the medium as described in previous studies (5, 9). Hypoxanthine alone did not alter pemetrexed activity, whereas both hypoxanthine and thymidine fully protected cells, regardless of the 5-CHO-THF level. At the lowest concentration of 5-CHO-THF (1.6 nmol/L; *top left*), 10 μmol/L thymidine alone had only a modest protective effect. However, as the 5-CHO-THF concentration in the medium was increased thymidine protection was progressively increased so that pemetrexed inhibition was barely perceptible in the presence of thymidine up to a concentration of 1 μmol/L of drug when extracellular 5-CHO-THF was 10 nmol/L (*bottom left*). Hence, as the folate concentration in the medium was reduced, there was progressive failure of thymidine alone to protect HeLa cells consistent with increasing suppression of GARFT.

Effect of Serum Constituents on Pemetrexed Growth Inhibition and Requirements for Nucleoside Protection. Undialyzed serum contains a variety of nucleosides and nucleobases as well as folates (15–17) that can influence the activity of antifolates and, in the case of pemetrexed, this would be expected to alter the degree of growth inhibition by pemetrexed and the pattern of protection by thymidine and hypoxanthine (16, 17). To assess this, studies described in the previous section were repeated using dialyzed bovine calf serum (Fig. 2, *right*). With wild-type HeLa cells, again, hypoxanthine alone did not decrease pemetrexed activity and full protection was achieved by addition of both hypoxanthine and thymidine. There was an inverse relationship between pemetrexed IC_{50} and the concentration of 5-CHO-THF in medium. Whereas the pemetrexed IC_{50} values in cells grown in the dialyzed serum were not very different from those grown in regular serum, the extent to which cells could be protected by thymidine alone was markedly decreased. For example, with 1.6 nmol/L 5-CHO-THF the pemetrexed IC_{50} was increased by only 2-fold (~10-20 nmol/L) with inclusion of 10 μmol/L thymidine (*top right*). This was in contrast to the 23-fold increase (~15 to ~350 nmol/L; *top left*) in cells grown in medium with undialyzed serum. The 5-CHO-THF concentration in the growth medium had a much greater effect on pemetrexed activity in the presence of thymidine than in the presence or absence of hypoxanthine. Hence, when the 5-CHO-THF concentration in the medium was increased from 1.6 to 10 nmol/L (*bottom right*), there was only 3-fold increase in pemetrexed IC_{50} (~10 to ~30 nmol/L) in the presence of hypoxanthine, but there was a ~25-fold increase (~20 to 500 nmol/L) in pemetrexed IC_{50} in the presence of thymidine. These observations suggest that pemetrexed inhibition of purine synthesis is much more sensitive to the level of cellular folates than is inhibition of TS in HeLa cells and this, in turn, is influenced by the level of purine nucleosides and nucleobases in the medium.

Similar experiments were also done with R5 cells grown in medium with 5-CHO-THF supplemented with dialyzed bovine calf serum. Under these conditions, neither 1.6 nor 4 nmol/L 5-CHO-THF supported normal cell growth consistent with the lack of any folate in the dialyzed serum along with loss of RFC activity. However, R5 cells grew normally with 10 or 25 nmol/L 5-CHO-THF (Fig. 3, *top* and *bottom*, respectively). Hypoxanthine did not alter pemetrexed activity and both hypoxanthine and thymidine fully protected R5 cells from pemetrexed inhibition. There was only a small (2-fold) increase in pemetrexed IC_{50} in R5 cells with the inclusion of thymidine when the concentration of 5-CHO-THF in medium was 10 or 25 nmol/L. This observation was similar to what was observed in HeLa cells grown in 1.6 nmol/L 5-CHO-THF (Fig. 2, *top right*) consistent with substantial suppression of purine as well as thymidylate synthesis under these conditions.

Effect of Modest Thymidylate Synthase Overexpression on Pemetrexed Growth Inhibition and Protection by Thymidine and Hypoxanthine. As indicated above, regardless of the sera used or concentration of 5-CHO-THF in the medium, hypoxanthine alone afforded no protection to either HeLa or R5 cells at pemetrexed concentrations close to the IC_{50}. To assess the impact of a modest increase in TS activity on the protective effects of nucleosides on pemetrexed inhibition and the relationship with cellular folate levels, a pemetrexed-resistant HeLa clonal cell line (R3) was developed as described in the Materials and Methods.

As indicated in Table 1, the R3 line was ~8-fold resistant to pemetrexed compared with parental HeLa cells grown in folic acid medium containing fetal bovine serum. Methotrexate sensitivity was not altered in R3 cells, and there was no difference in methotrexate influx between R3 and HeLa cells consistent with intact RFC-mediated transport (data not shown). R3 cells were 14-fold resistant to ZD1694 and ZD9331 and 5.5-fold resistant to AG331 compared with wild-type HeLa cells. Because the structures of both ZD9331 and AG331 preclude the formation of polyglutamate derivatives,

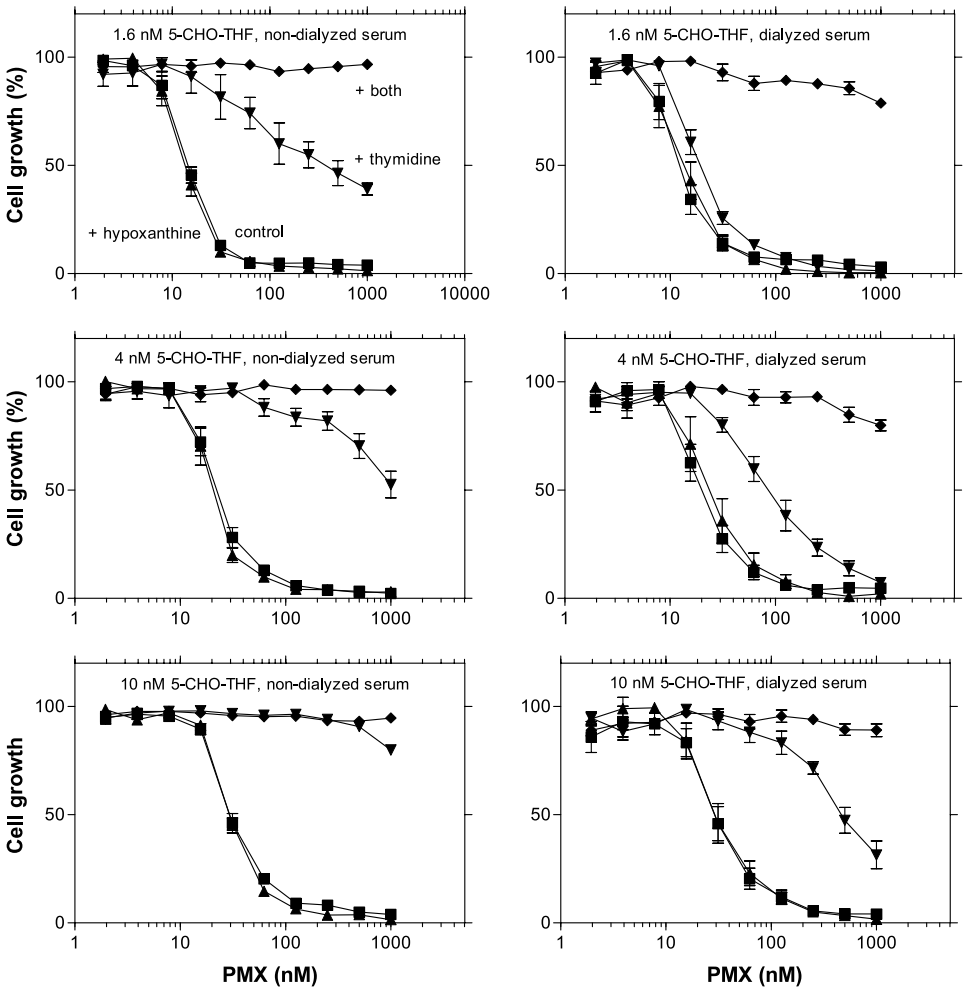

Fig. 2 Impact of the extracellular 5-CHO-THF concentration in the medium on pemetrexed (*PMX*) growth inhibition and protection by thymidine and/or hypoxanthine. Wild-type Hela cells grown and assayed with 10% fetal bovine serum (*left*) or dialyzed bovine calf serum (*right*) in medium containing 1.6 nmol/L (*top*), 4 nmol/L (*middle*), or 10 nmol/L (*bottom*) 5-CHO-THF. Control cells (■) and cells to which hypoxanthine (▲), thymidine (▼), or both of them (♦) were added. Concentrations of thymidine and hypoxanthine in the media were 10 and 100 μmol/L, respectively. Points, mean from three separate experiments; bars, ±SE.

a role for alterations at the level of folylpolyglutamate synthetase in resistance was excluded. Rather, the pattern was consistent with an increase in TS expression as the predominant or sole mechanism of resistance. Western blot analysis confirmed that TS protein in R3 cells was increased compared with that in HeLa cells (Fig. 4). The intensity produced by TS in R3 cells was 6-fold greater than in HeLa cells after normalizing to β-actin.

Pemetrexed growth inhibition as well as the effects of thymidine and hypoxanthine were assessed in R3 cells grown in dialyzed calf serum containing 1.6, 4, or 10 nmol/L 5-CHO-THF. As illustrated in Fig. 5, these cells were ~10-fold resistant to pemetrexed at 1.6 nmol/L 5-CHO-THF compared with wild-type cells at the same concentration (Fig. 2). The pemetrexed IC_{50} was increased as the 5-CHO-THF concentration in the medium was increased, a pattern also seen in HeLa cells. In contrast, however, thymidine alone did not have any effect on the pemetrexed IC_{50}, whereas hypoxanthine alone decreased, though by <2-fold, pemetrexed activity at concentrations of 1.6 nmol/L and to a lesser extent at 4 nmol/L 5-CHO-THF. Thus, with a *modest* increase in TS expression the inhibitory effect of pemetrexed on purine synthesis was initially limiting under conditions in which there were no exogenous nucleosides and nucleobases. However, upon addition of hypoxanthine, thymidylate synthesis became limiting with only a small further increase in pemetrexed concentration so that full protection still required the presence of both a purine and thymidine.

The Effect of Nucleoside Protection on Pemetrexed Growth Inhibition in MCF-7 Breast Cancer Cells. With 1.6 nmol/L 5-CHO-THF and using dialyzed serum, thymidine alone had only minimal effect (2-fold) on pemetrexed growth inhibition in wild-type HeLa cells (Fig. 2). However, this was not the case in wild-type MCF-7 breast cancer cells. As indicated in Fig. 6, thymidine alone increased the pemetrexed IC_{50} in MCF-7 cells by a factor of >10 under the same conditions. On the other hand, thymidine alone did not protect cells completely, even with 10 nmol/L 5-CHO-THF, within the pemetrexed concentration range assessed. In all cases, hypoxanthine alone did not affect pemetrexed growth inhibition, whereas both thymidine and hypoxanthine were required to abolish pemetrexed activity. Hence, inhibition of purine synthesis emerged in MCF-7 cells at much higher pemetrexed levels than in HeLa cells with a low extracellular 5-CHO-THF concentration indicating the potential variability of suppression of pemetrexed target enzymes from one cell line to another.

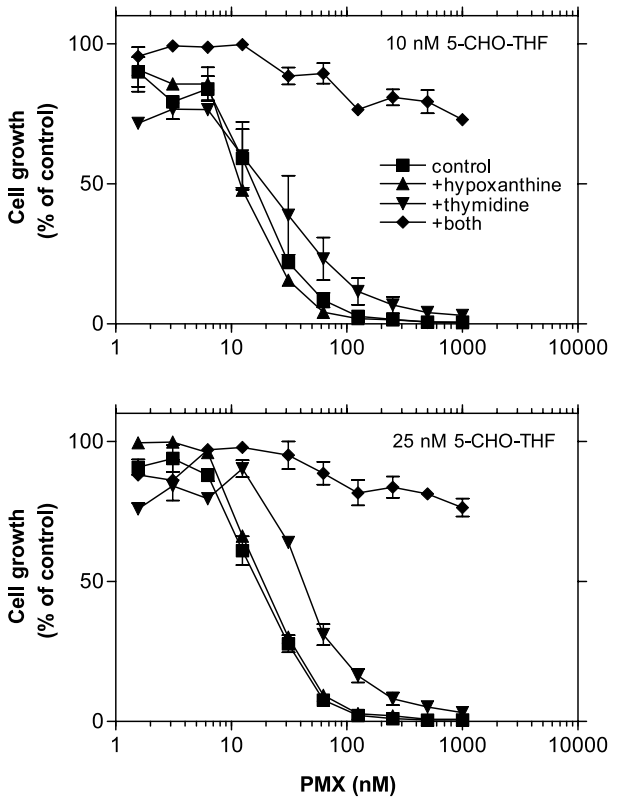

Fig. 3 Thymidine and/or hypoxanthine protection of pemetrexed (*PMX*) growth inhibition in RFC-null R5 cells grown and assayed in medium containing 10 nmol/L (*top*) or 25 nmol/L (*bottom*) 5-CHO-THF supplemented with 10% dialyzed calf serum. Concentrations of thymidine and hypoxanthine in the media were 10 and 100 μmol/l, respectively. Points, mean from three separate experiments; bars, ±SE.

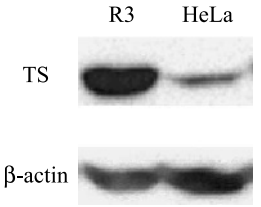

Fig. 4 Western blot analysis of TS expression in HeLa and R3 cells. The soluble fractions of the cell lysates were loaded onto a SDS-PAGE gel and blotted to nitrocellulose membranes. The blots were probed with antibodies targeted to TS and β-actin as described in MATERIALS AND METHODS. Representative of two separate experiments.

DISCUSSION

Pemetrexed is new generation antifolate that in its polyglutamate forms inhibits folate-dependent enzymes. The pentaglutamate of pemetrexed has a similar affinity (~1 nmol/L) for human TS as the tetraglutamate of ZD1694, a TS inhibitor (4, 18). Affinity of pemetrexed pentaglutamate for murine GARFT (65 nmol/L) is much lower than that of the hexaglutamate of DDATHF (0.12 nmol/L), a prototypical GARFT inhibitor, although these values were determined in separate experiments and with different methods (4, 19). Both pemetrexed and pemetrexed pentaglutamate have an affinity for human dihydrofolate reductase (7 nmol/L DHFR) three orders of magnitude lower than that of methotrexate (4 pmol/L) (4, 20). Based upon the spectrum of differences in affinities for these enzymes, pemetrexed seems to be, primarily, an inhibitor of TS. The current study indicates that the determinants of pemetrexed inhibitory effects at its putative targets are complex, multifaceted, and are influenced by the extracellular level and membrane transport of natural folates and, as shown previously, the availability of preformed purine and pyrimidine nucleosides.

The inhibitory effect of pemetrexed at the level of GARFT seems to be secondary to its effects at TS. Hence, growth inhibition by pemetrexed in the range of its IC_{50} can be fully obviated by the presence of thymidine, and as the concentration of drug is increased beyond this level, both thymidine and hypoxanthine are required to protect cells (4, 5). However, because clinical regimens are designed to produce cell kills orders of magnitude above the IC_{50}, requiring pemetrexed blood levels orders of magnitude higher than employed in these studies (21), GARFT are likely to be suppressed under these conditions. The current studies identify the availability of folates as an important determinant of GARFT inhibition by pemetrexed; as cellular folates are contracted, the protective effect of thymidine is progressively diminished. Any factors that lead to changes in the level of cellular folates can modulate the inhibitory effect of pemetrexed on GARFT, such as (i) the extracellular concentration of 5-CHO-THF and/or (ii) the level of RFC activity. The dependence of GARFT inhibition on the level of cellular folates was also observed with DDATHF in murine leukemia cells although this could be attributed in part to enhanced polyglutamation of this agent as folate pools are decreased, a phenomenon also relevant to the polyglutamation of pemetrexed (8–11). The dependence of pemetrexed inhibition of GARFT on cellular folates, with little apparent dependence at TS in HeLa cells, is likely related to the much lower affinity of pemetrexed polyglutamates for the former enzyme.

Table 1 Comparison of antifolate growth inhibition in wild-type HeLa cells and a derivative cell line (R3) with increased expression of thymidylate synthase

Antifolate	(A) IC_{50} in HeLa cells (nmol/L)	(B) IC_{50} in R3 cells (nmol/L)	Fold change (B/A)
PMX	19 ± 1	150 ± 12	7.9
MTX	9.7 ± 0.3	9.7 ± 0.3	1.0
ZD1694	2.4 ± 0.3	33 ± 4	14
ZD9331	107 ± 13	1,470 ± 330	14
AG331	2,800 ± 700	15,300 ± 1,500	5.5

NOTE. Growth inhibition was determined in medium containing 2.0 μmol/L folic acid supplemented with 10% fetal bovine serum. Data are the mean ± SE from three separate experiments.

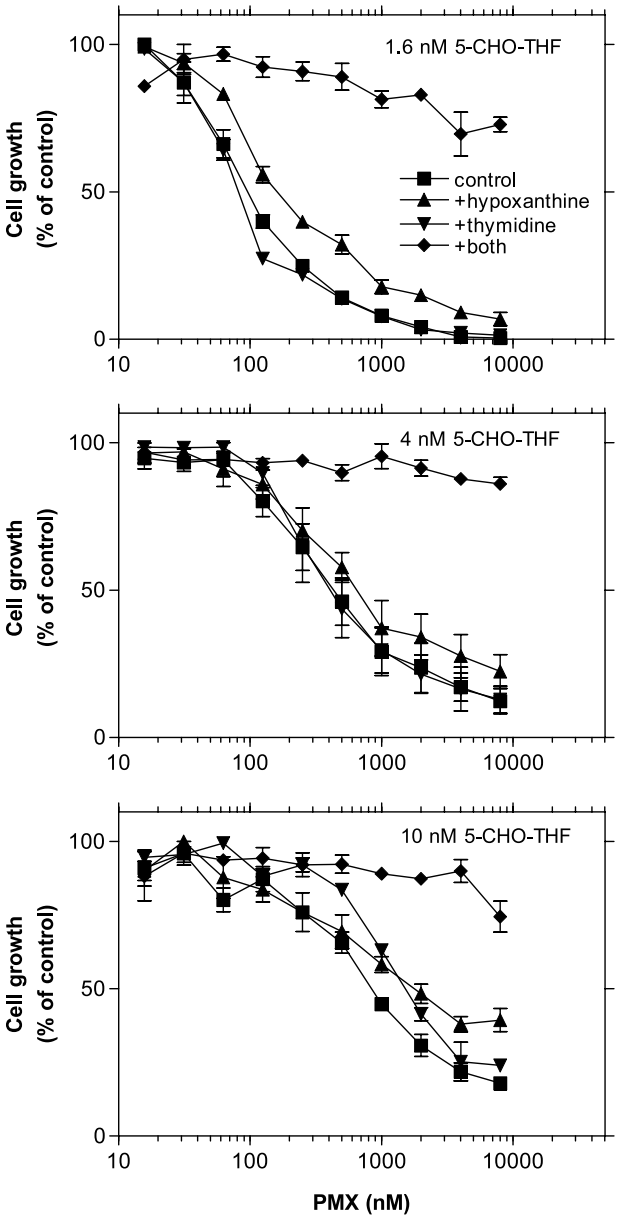

Fig. 5 Effects of increased TS expression on protection by thymidine and/or hypoxanthine. R3 cells with increased TS expression were grown and assayed in medium containing 1.6 nmol/L (*top*), 4 nmol/L (*middle*), or 10 nmol/L (*bottom*) 5-CHO-THF supplemented with 10% dialyzed calf serum. Concentrations of thymidine and hypoxanthine in the media were 10 and 100 μmol/L, respectively. Points, mean from three separate experiments; bars, ±SE.

The contraction of cellular folate pools and the presence of an RFC-independent transport pathway, resulting in the preservation of pemetrexed polyglutamation when RFC activity is lost in R5 cells, were associated with modest enhancement of pemetrexed activity in R5 cells (13). The current study raises the possibility that another element in this phenomenon may enhanced pemetrexed inhibition of GARFT under these conditions. With loss of RFC function, the protective effect of thymidine was markedly decreased consistent with increased inhibition at the level of GARFT. The thymidine and hypoxanthine rescue patterns in RFC-null R5 cells with 25 nmol/L 5-CHO-THF were similar to that observed in wild-type HeLa cells with 1.6 nmol/L 5-CHO-THF. Hence, the observed rescue pattern associated with the marked reduction in cellular folates, that occurs when RFC function is lost, was equivalent to what occurred when the concentration of 5-CHO-THF in the growth medium was decreased by a factor of 16. These observations suggest that loss of RFC function is unlikely to be an important mechanism of primary acquired resistance to

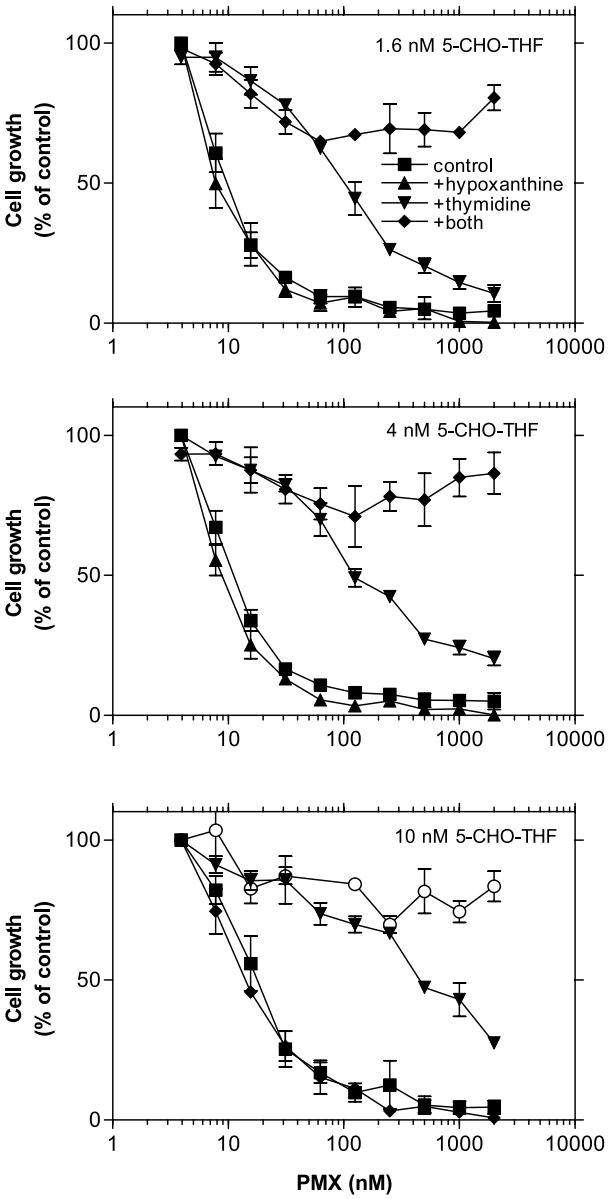

Fig. 6 Thymidine and/or hypoxanthine protection pattern in MCF-7 human breast cancer cells grown and assayed in medium containing 1.6 nmol/L (*top*), 4 nmol/L (*middle*), or 10 nmol/L (*bottom*) 5-CHO-THF supplemented with 10% dialyzed calf serum. Concentrations of thymidine and hypoxanthine in the media were 10 and 100 μmol/L, respectively. Points, mean from three separate experiments; bars, ±SE.

pemetrexed either under experimental conditions or in the clinical setting. This is due to (i) marked contraction of cellular folate pools that tends to preserve polyglutamate formation, (ii) intensified drug inhibition at the level of GARFT, and (iii) the presence of transport activity distinct from RFC and folate receptors that delivers pemetrexed into cells (13).

Another important determinant of pemetrexed effects at its target enzymes is the relative expression of TS and GARFT. With usual levels of TS, in drug-sensitive cells, this enzyme is the primary target. On the other hand, when TS expression is increased, suppression of this site may not be achieved even at high pemetrexed concentrations, GARFT becomes the primary site of action and cells are protected by a purine alone. This was the case in a GC3 colon carcinoma–derived cell line 80-fold resistance to pemetrexed relative to wild-type GC3 cells, with 40-fold higher TS activity (6). The pemetrexed-resistant R3 cells in the current study had a modest increase in TS activity and 8-fold increase in resistance. Under these conditions, the protection pattern was more complex. Thymidine alone did not protect R3 cells at all, whereas hypoxanthine alone had only a small protective effect (Fig. 5) at lower concentrations of 5-CHO-THF. Hence, under these conditions, GARFT became the primary target, but with provision of a purine, TS became limiting at only a modestly increased pemetrexed concentration. Again, pemetrexed growth inhibition in these cells increased substantially as the 5-CHO-THF level was decreased apparently due to inhibition at the level of GARFT.

Because purines and thymidine fully protect cells from the toxic effects of pemetrexed, it is expected that nucleosides and nucleobases in serum influence the activity of pemetrexed and that inhibition of nucleoside transport should enhance the activity of this agent. The latter has been observed *in vitro* with the nucleoside transport inhibitors, dipyridamole and its analogues (22, 23). In the current study, when dialyzed serum, depleted of nucleobases and nucleosides was employed, protection by thymidine was markedly diminished in comparison with HeLa cells grown with undialyzed serum. Hence, the presence of purines in serum enhanced the protective effect of thymidine alone. Likewise, the level of nucleosides/nucleobases in blood will be a determinant of the activity of pemetrexed. In patients with a high tumor burden and catabolic activity resulting in high purine blood levels (15–17), effects of pemetrexed may be due, predominantly, to suppression of TS. Beyond this, the transport processes that deliver these substrates to cells and the metabolic processes that determine the extent to which they are available for nucleotide synthesis will have a profound effect on the activity of pemetrexed. It is of interest that pemetrexed activity was unchanged, irrespective of whether sera was or was not dialyzed either in the presence or absence of hypoxanthine, indicating that thymidine levels in serum were not sufficiently high to influence the block in *de novo* synthesis of this nucleoside.

There are a number of lines of evidence indicating that the pharmacologic effects of pemetrexed are not related to inhibition of DHFR: (i) TS oxidizes 5,10-CH_2-THF to DHF which is reduced to THF by DHFR. In the absence of TS activity, there is no formation of DHF, no THF-cofactor depletion, and therefore no requirement for DHFR (24, 25). The affinity of pemetrexed polyglutamates for TS is so high that soon after administration of the drug, TS activity is abolished and as expected, no cellular DHF is detected after treatment with this drug (26). (ii) Methotrexate suppression of DHFR in cells requires concentrations in the range of 1 to 10 μmol/L due to competition between the drug and DHF at the level of this enzyme. The affinity of pemetrexed and its polyglutamate derivatives for DHFR is three orders of magnitude lower than that of methotrexate and hence pemetrexed is a very weak inhibitor of this enzyme. Consequently, the association between pemetrexed and DHFR is very rapidly reversible in cells and tight binding cannot be detected (11). (iii) With complete inhibition of DHFR, purine and thymidine are required to prevent methotrexate cytotoxicity (27). However, thymidine alone prevents pemetrexed growth inhibition at concentrations in the range of its IC_{50} and when TS is overexpressed, hypoxanthine alone protects cells (4–6).

Finally, in studies extended to MCF-7 breast cancer cells, protection by thymidine alone was much greater than observed in HeLa cells (Figs. 2 and 6) under the same conditions with a low extracellular 5-CHO-THF concentration. The basis for this difference between HeLa and MCF-7 cells is not clear. It may be due to higher cellular folate pools, a higher level of expression of RFC (28), differences in transport and utilization of nucleosides and nucleobases, and/or the ratio of TS to GARFT. However, even in MCF-7 cells, full reversal of pemetrexed growth inhibition at high drug concentrations required both thymidine and hypoxanthine. Hence, there seem to be differences in the extent to which pemetrexed effects are related to inhibition of TS relative to GARFT among different tumor types or cell lines.

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Clin Cancer Res 2005;11:1294-1301.