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# Selective Preservation of Pemetrexed Pharmacological Activity in HeLa Cells Lacking the Reduced Folate Carrier: Association with the Presence of a Secondary Transport Pathway

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## ABSTRACT

**A methotrexate (MTX)-resistant HeLa subline (R5), developed in this laboratory, with impaired transport due to a genomic deletion of the reduced folate carrier (RFC) was only 2-fold resistant to pemetrexed (PMX), but 200- and 400-fold resistant to raltitrexed (ZD1694) and $N^{\alpha}$-(-4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-1-ornithine (PT523), respectively, compared with parental HeLa cells when grown with 2 $\mu$M folic acid. When folic acid was replaced with the more physiological 25 nM 5-formyltetrahydrofolate, R5 cells were 2-fold collaterally sensitive to PMX but still 40- and 200-fold resistant to ZD1694 and PT523, respectively. Sensitivity to PT523 and PMX could be completely restored, and sensitivity to ZD1694 nearly restored, by transfection of RFC cDNA into R5 cells, indicating that the defect in drug transport was the only, or major, factor in resistance. The preserved PMX activity in R5 cells could not be related to the very low expression of folate receptors. Rather, retained PMX activity in R5 cells was associated with residual transport by another process that exhibits good affinity for PMX ($K_t$ = 12 $\mu$M) with much lower affinities for ZD1694, MTX, and PT523 ($K_i$s of ~ 90, 100, and 250 $\mu$M, respectively). PMX transported by this route was rapidly converted to higher polyglutamates and, when grown with 25 nM 5-formyltetrahydrofolate, the rate of formation of these derivatives and their net accumulation in R5 cells was comparable to that of wild-type cells. These data suggest that selective preservation of PMX pharmacological activity in RFC-null R5 cells is due, in part, to partial preservation of transport by secondary process with a higher affinity for PMX than the other antifolates evaluated.**

## INTRODUCTION

Pemetrexed (PMX; Alimta) is a new-generation antifolate with demonstrated clinical activity in Phase II and III studies in the treatment of mesothelioma, non-small cell lung cancer, and other solid tumors (1–3). The activity of the drug requires formation of polyglutamate derivatives in cells that are potent inhibitors of thymidylate synthase (TS; $K_i$ = 1.3 nM) and to a lesser extent glycinamide ribonucleotide formyltransferase ($K_i$ = 65 nM; Ref. 4). Despite its inhibitory potential at two sites, at concentrations in the range of its $IC_{50}$, thymidine alone affords full protection from the cytotoxic effects of this agent in CCRF-CEM human leukemia cells (4, 5). On the other hand, at high PMX concentrations, both thymidine and a purine source are required for protection, consistent with inhibition at two sites (4, 5). Furthermore, in cell lines with acquired resistance to PMX due to increased expression of TS, growth inhibition produced by high concentrations of drug are unaffected by thymidine but are fully prevented by a purine alone, consistent with inhibition solely at glycinamide ribonucleotide formyltransferase under these conditions (6).

There have been reports describing cells with primary resistance to PMX that have greater cross-resistance to raltitrexed (ZD1694; Tomudex) and cells with primary resistance to ZD1694 with lesser cross-resistance to PMX (7, 8). This has been observed whether resistance was due to increased expression of TS or impaired drug accumulation (7, 8). This is, however, not always the case (9). Polyglutamation of ZD1694 is also required for activity, and these derivatives of both agents have comparable TS inhibition constants (4). Likewise, both drugs are comparable, excellent substrates for folypolyglutamate synthase (0.8–1.4 $\mu$M), and have similar affinities for reduced folate carrier (RFC) and folate receptor-$\alpha$ (FR-$\alpha$; Ref. 10, 11). One possible explanation for the preservation of PMX activity relative to ZD1694 is its inhibitory potential at the level of glycinamide ribonucleotide formyltransferase because the site of ZD1694 action is solely at TS.

In a recent report from this laboratory, a methotrexate (MTX)-resistant HeLa cell line, R5, was characterized in which RFC was deleted from the genome (12). This resulted in a large, but incomplete, decrease in MTX influx compared with wild-type cells, consistent with a residual RFC-independent transport pathway. This is unlike what is observed in murine leukemia cells when RFC activity is lost, when virtually all influx ceases (13). The present report explores the cellular pharmacology of PMX in the R5 cell line, the extent to which it is transported by the RFC-independent pathway, and how this impacts on the activity of this agent in comparison to (*a*) the other inhibitors of TS, ZD1694 and AG331 (the latter diffuses into cells and does not undergo polyglutamation), and (*b*) the potent dihydrofolate reductase inhibitor $N^{\alpha}$-(-4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-1-ornithine (PT523), which has a very high affinity for RFC and does not form polyglutamate derivatives.

## MATERIALS AND METHODS

**Chemicals.** [3′,5′,7,9-$^3$H]Folic acid was purchased from Amersham Corp. (Arlington Heights, IL). [$^3$H]PMX (50 Ci/mmol) and unlabeled PMX were provided by the Eli Lilly company (Indianapolis, IN). 5-Methyltetrahydrofolate, 5-formyltetrahydrofolate (5-CHO-THF), and folic acid were obtained from Sigma. ZD1694 was provided by AstraZeneca, and AG331 was provided by Agouron Pharmaceuticals Inc. PT523 and 5,8-dideazaPT523 (PT632) were provided by Dr. Andre Rosowsky, Dana-Farber Cancer Institute (Boston, MA). All radiochemicals were purified by high-pressure liquid chromatography (HPLC) before use (14). The stability of radiochemicals, once purified, was checked by HPLC on a regular basis, and materials were repurified as necessary.

**Cell Culture Conditions and Growth Inhibition Studies.** The HeLa, R5, and RFC-7 (RFC-transfected R5 derivative) cell lines were described in a previous report (12). Cells were maintained in RPMI 1640 (Hyclone) supplemented with 10% fetal bovine serum (Gemini Bio-Products), 2 mM glutamine, 20 $\mu$M 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 $\mu$g/ml) at 37°C in a humidified atmosphere of 5% $CO_2$. The RFC-7 transfectant was maintained in 600 $\mu$g/ml G418. HeLa and R5 cells were also maintained in folate-free RPMI medium (Hyclone) containing 25 nM 5-CHO-THF in addition to the supplements described above. *Mycoplasma* adherent to cells produce folate transport activity with very high affinity for PMX and other folates (15). This is particularly prominent in mesothelioma cells, but a

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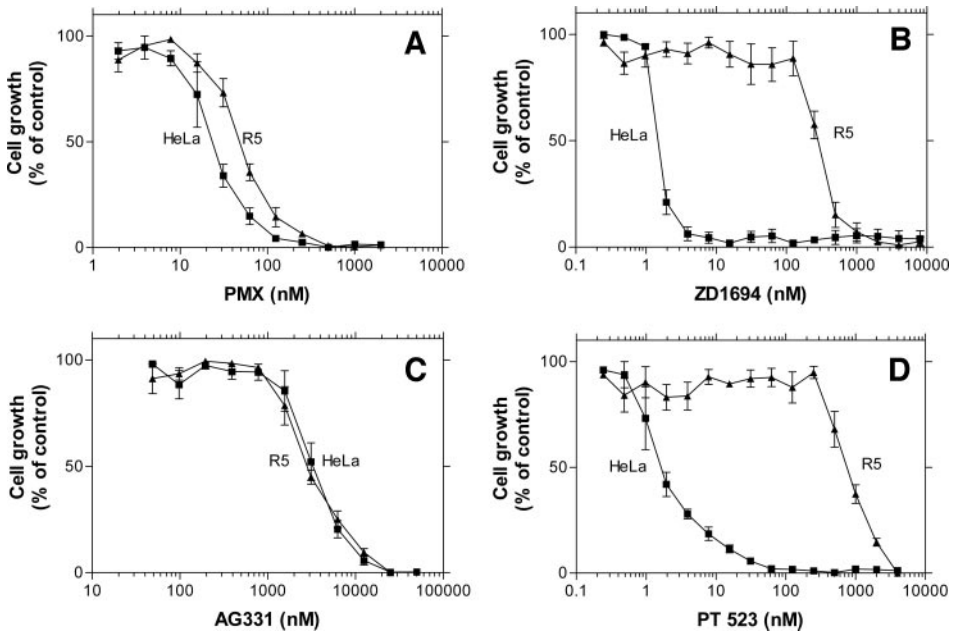


Fig. 1. Growth inhibition by pemetrexed (*PMX*; *A*), raltitrexed (*ZD1694*; *B*), AG331 (*C*), and $N^{\alpha}$-(-4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-1-ornithine (*PT 523*; *D*) in wild-type HeLa and reduced folate carrier-null R5 cells maintained in folic acid-containing medium. HeLa and R5 Cells were grown in RPMI medium containing 2.0 $\mu$M folic acid and exposed continuously to different concentrations of antifolates for 6 days before cell numbers were quantified. The growth rate in the absence of drug was set as 100%. The results in each *panel* represent the mean ± SE (*bars*) from three separate experiments.

low level of activity can also be detected in HeLa cells contaminated with this organism. (15). Because of this, cell cultures were monitored regularly with a *Mycoplasma* detection kit (American Type Culture Collection) and were shown to be free of this microorganism.

For assessment of growth inhibition by antifolates, cells were transferred to 96-well plates (500 cells/well) and exposed continuously to a spectrum of antifolate concentrations for 6 days. G418 was not included in the medium for the RFC-7 line in these experiments. Cell growth rate was quantified by sulforhodamine B staining (16). For measurement of total PMX accumulation, cells were grown either in folic acid or 5-CHO-THF medium containing 50 nM [$^3$H]PMX, 200 $\mu$M glycine, 100 $\mu$M adenine, and 10 $\mu$M thymidine for 1 week. Intracellular tritium was determined as in the transport studies described below.

**Folic Acid-Binding Assay.** Cells near confluence were washed with ice-cold acid buffer [10 mM sodium acetate, 150 mM NaCl (pH 3.5)] followed by a wash with ice cold 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose (pH 7.4; HBS). Cells were incubated for 15 min in ice-cold HBS containing 5 nM [$^3$H]folic acid in the presence and absence of 500 nM nonlabeled folic acid. The cells were then washed three times with ice-cold HBS. [$^3$H]Folic acid bound to the cell surface was released with the acid buffer (0.5 ml) and measured on a liquid scintillation spectrometer. The difference in tritium released in the presence and absence of nonlabeled folic acid represented folic acid specifically bound to the cell surface.

**Transport Studies.** Analysis of PMX transport followed a protocol designed for rapid uptake determinations on cells growing in monolayer cultures (17). Briefly, cells ($3 \times 10^5$) were seeded into 20-ml Low Background Glass vials (Research Products International Corp, Prospect, IL) and grown for 3 days to reach early confluence. The cells were washed twice with HBS and incubated in this buffer at 37°C for 20 min. After removal of the incubation buffer, uptake was initiated by the addition of 0.5 ml of HBS containing radiolabeled folate at the desired concentrations. Uptake was terminated by injection of 5 ml of ice-cold HBS into the vials, after which the adherent cells were washed three times with 5 ml of ice-cold HBS. The cells were then dissolved by incubation in 0.2 M NaOH (0.5 ml) at 65°C for 45 min. Radioactivity in 0.4 ml of the lysate was determined, and 10 $\mu$l were processed for protein determination (BCA; Pierce, Rockford, IL). Cellular uptake is expressed in units of pmol/mg of protein.

**HPLC Analysis of PMX Polyglutamates.** HeLa and R5 cells were grown in either folic acid or 5-CHO-THF medium in 100-mm plates. Cells were washed twice with HBS, exposed to HBS containing 0.5 $\mu$M [$^3$H]PMX for 2 h at 37°C, and then washed three times with cold (0°C) HBS. Cells were then scraped mechanically in 1 ml of ice-cold 50 mM phosphate buffer (pH 6.0) containing 100 mM 2-mercaptoethanol. One portion of this suspension (50 $\mu$l) was used for determination of total PMX accumulation in cells as described above. In this case, protein was determined with the Bio-Rad protein assay because 2-mercaptoethanol was present. The remaining portion was boiled for 10 min to inactivate enzymes, and the supernatant containing radiolabeled PMX and its metabolites was separated by centrifugation. After nonlabeled PMX-monoglutamate, -triglutamate, and -pentaglutamate standards were added, the solution was injected onto a reversed-phase HPLC column (Waters Spherisorb; 5 $\mu$m ODS2; 4.6 × 250 mm) and processed as reported previously (18). The levels of PMX and its polyglutamate derivatives are expressed in units of pmol/mg of protein in the cells.

## RESULTS

**Resistance and Cross-Resistance Patterns in RFC-null HeLa R5 Cells.** Initially, antifolate inhibitory activities were assessed in wild-type HeLa and R5 cells grown in regular RPMI medium containing 2 $\mu$M folic acid. As shown in Fig. 1, R5 cells were 200-fold resistant to ZD1694 (Fig. 1*B*) but only 2-fold resistant to PMX (Fig. 1*A*) compared with the parental HeLa cells. R5 cells were not resistant at all to AG331 (Fig. 1*C*), consistent with a lack of change in the level or properties of TS. In addition, R5 cells were highly (400-fold) resistant to PT523 (Fig. 1*D*). Hence, R5 cells were cross-resistant to two antifolates with different enzyme targets, one of which does (ZD1694) and the other does not (PT523) form polyglutamate derivatives. Both, however, have high affinity for RFC.

Antifolate inhibition was further assessed in cells maintained in RPMI medium with 25 nM 5-CHO-THF as the growth source, a reduced folate substrate with transport and structural properties similar to that of the physiological 5-methyltetrahydrofolate, at a concentration within the physiological range. As indicated in Fig. 2, under these conditions R5 cells were ~2-fold collaterally sensitive to PMX (Fig. 2*A*) and AG331 (Fig. 2*C*), whereas they were still 40- and 250-fold resistant to ZD1694 (Fig. 2*B*) and PT523 (Fig. 2*D*), respectively. Compared with cells grown with 2 $\mu$M folic acid, sensitivity of R5 cells to PMX, AG331, ZD1694, and PT523 was increased by factors of 4, 2, 5, and 2, respectively, in medium containing 25 nM 5-CHO-THF. Hence, the activity of PMX was completely preserved in R5 cells that lacked RFC expression under these physiologically relevant conditions, whereas resistance to ZD1694 and PT523 per-

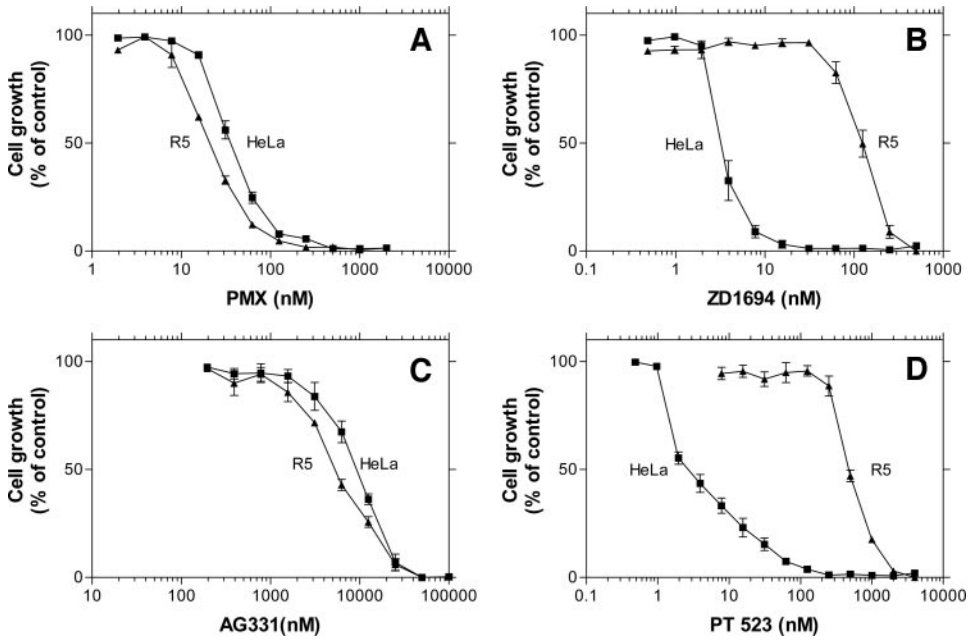


Fig. 2. Growth inhibition by pemetrexed (*PMX*; *A*), raltitrexed (*ZD1694*; *B*), AG331 (*C*), and $N^{\alpha}$-(-4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-1-ornithine (*PT 523*; *D*) in HeLa and R5 cells maintained in 5-formyltetrahydrofolate medium. HeLa and R5 cells were grown in RPMI medium containing 25 nM 5-formyltetrahydrofolate and exposed continuously to different concentrations of antifolates for 6 days before cell numbers were quantitated. The growth rate in the absence of drug was set as 100%. The results in each *panel* represent the mean ± SE (*bars*) from three separate experiments.

sisted, although all of these agents have high affinity for, and are transported by, this carrier.

**Effect of Restoration of RFC Expression in R5 cells on Growth Inhibition by ZD1694, PT523, and PMX.** To verify whether the loss of RFC-mediated transport in R5 cells was the sole mechanism of resistance to these agents, the $IC_{50}$s of these drugs were determined in RFC-7 cells, which were derived by transfection of RFC cDNA into R5 cells (12) and in which RFC function was restored, and the results were compared those obtained in with R5 and HeLa cells under conditions of growth in folic acid. As indicated in Fig. 3, overexpression of RFC nearly fully restored sensitivity to ZD1694 (Fig. 3*A*) and fully restored sensitivity to PT523 (Fig. 3*B*). The low level of PMX resistance in R5 cells was eliminated in the RFC-7 transfectant (Fig. 3*C*). Hence, impaired transport in R5 cells appears to be the major, if not sole, contributor to resistance to all these agents.

**FR Expression: Impact on Transport of PMX.** Specific folic acid binding to the surface of HeLa and R5 cells was assessed under the different growth conditions. As indicated in Table 1, FR expression in wild-type HeLa cells was quite low, and the level in R5 cells was ~40% that of HeLa cells in the folic acid growth medium. When cells were grown in 25 nM 5-CHO-THF, folate acid binding in HeLa and R5 cells was increased by factors of 1.5 and 2.5, respectively, but FR expression in R5 cells was ~60% that of HeLa cells. Hence, FR expression is lower in R5 than HeLa cells during growth in either folate.

Because folic acid has a very high affinity for FR, the presence of folic acid in the growth medium can saturate these receptors and thereby prevent FR-mediated drug internalization. For example, there was a 100-fold increase in the $IC_{50}$ of CB300638, a TS inhibitor that has high affinity for FR-$\alpha$, when 1 $\mu$M folic acid was added to the growth medium compared with growth in 1 or 10 nM 5-CHO-THF (19). The same approach was used to determine whether the low level of FR expression in R5 cells contributes to transport of, and sensitivity to, PMX, which has an affinity for FR-$\alpha$ somewhat greater than that of folic acid (11). As indicated in Fig. 4, when 1 $\mu$M folic acid was included in the growth medium, the PMX $IC_{50}$ in R5 cells (~20 nM) was increased by a factor of only 2, whereas the PMX $IC_{50}$ in HeLa cells (40 nM) was unchanged. As a result, R5 and HeLa cells were equally sensitive to PMX. These concentrations of folic acid are 25- and 50-fold greater, respectively, than the $IC_{50}$ for PMX in these cells and should markedly suppress any FR-mediated transport. The small increase in PMX $IC_{50}$ in R5 cells was likely due to the increase in cellular folate cofactor pools. Hence, FR- mediated delivery of drug does not appear to play a role in PMX activity in these cells.

**Analysis of PMX Initial Uptake and Net Transport Rates.** Because RFC is deleted in R5 cells, for PMX to sustain full, if not increased, activity there must be some secondary process(es) that mediates transport of this agent. To assess this, the initial uptake rate for 0.5 $\mu$M [$^3$H]PMX was evaluated in cells grown in folic acid. As indicated in Fig. 5, uptake was constant over at least 1.5 min in both cell lines, but when the uptake slopes were extrapolated to time zero, there were high ordinate intercepts, consistent with a low level of PMX rapidly bound to the cell surface. On the basis of the uptake slopes, which represent initial uptake rates, PMX influx in R5 cells was decreased by only 55% compared with influx in HeLa cells, consistent with substantial RFC-independent residual transport. Co-addition of 20 $\mu$M folic acid with the [$^3$H]PMX decreased the ordinate intercept in both lines, but there were no changes in initial uptake rates. This reflects inhibition of PMX binding to folate receptors that do not appear to make a measurable contribution to PMX transport across the cell membrane under these conditions. Inclusion of 5 $\mu$M PT632, which has a RFC influx $K_i$ of 0.3 $\mu$M, one-tenth that of PMX (20) and should therefore abolish RFC-mediated PMX influx, decreased PMX influx in HeLa cells to a rate comparable to that of R5 cells. Hence, there appears to be an uptake route for PMX independent of RFC in R5 cells, and this activity is also present in HeLa cells.

Influx and net uptake of PMX in R5 and HeLa cells was monitored for 90 min, and the impact of the folate in the growth medium was assessed (Fig. 6*A*). Again, PMX influx in HeLa cells was approximately twice that in R5 cells; in neither cell line was the initial uptake rate altered by the folate growth sources (Fig. 6*A*, *inset*). Net uptake of PMX beyond 10 min, which reflects synthesis and retention of polyglutamate derivatives [see below and as reported previously (18)], continued at a constant rate that was similar for both cell lines over the period of observation. The absolute difference in the PMX level was constant so that by 90 min the difference between the two lines was negligible when cells were grown in 5-CHO-THF medium. A similar pattern was observed in cells grown in folic acid, but the absolute

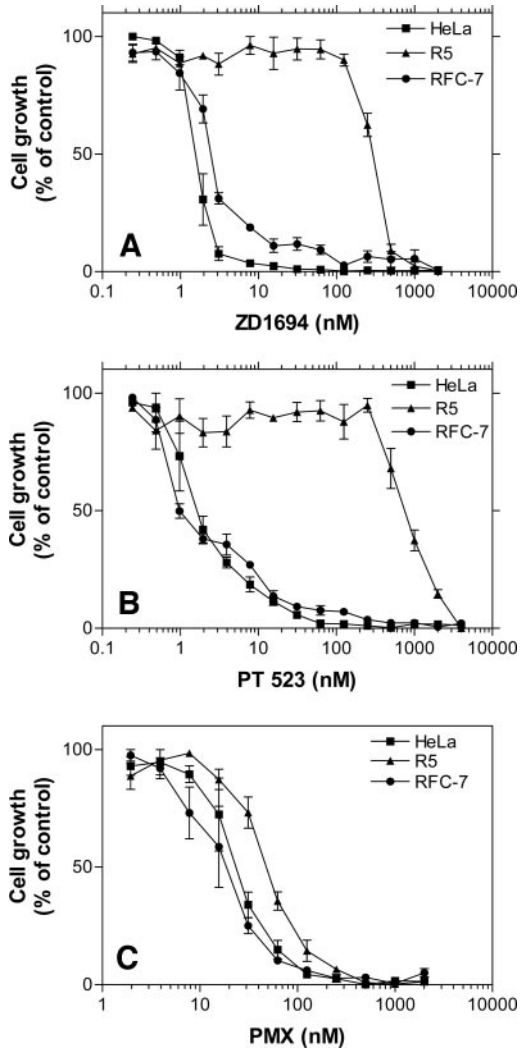


Fig. 3. Comparison of activity of raltitrexed (*ZD1694*; *A*), $N^{\alpha}$-(-4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-1-ornithine (*PT 523; B*) and pemetrexed (*PMX*; *C*) in HeLa, R5, and RFC-7 cells grown in folic acid medium. Cells were grown in RPMI medium containing 2.0 $\mu$M folic acid and exposed continuously to different concentrations of drugs for 6 days before cell numbers were determined. The growth rate in the absence of drug was set as 100%. The RFC-7 line was derived from R5 cells by transfection of RFC cDNA and expresses a high level of this carrier. Data are the mean ± SE (*bars*) from three separate experiments for all three panels.

difference in total PMX uptake was much greater. Hence, although the PMX initial uptake rate in R5 cells was less than half that of HeLa cells, this appears to sustain a rate of PMX polyglutamation similar to that of wild-type cells irrespective of the folate growth source.

**Polyglutamation of PMX.** The formation of polyglutamate derivatives was analyzed by HPLC after a 2-h incubation with 0.5 $\mu$M [$^3$H]PMX. As indicated in Fig. 6*B*, no major radioactive peaks were detected other than PMX and its polyglutamate derivatives; these could be clearly distinguished and quantitated. Consistent with the data presented in Fig. 6*A*, total PMX accumulation was greater in cells grown in 5-CHO-THF than in folic acid medium (Table 2). In 5-CHO-THF medium, ~95% of total PMX represented polyglutamate derivatives, largely the higher derivatives, but in R5 cells grown in folic acid, only 75% of cell PMX was polyglutamates, and the level of derivatives at the triglutamate and higher was 50% that of wild-type cells. Under all conditions, long-chain polyglutamates (pentaglutamate and above) were the major metabolites of PMX along with smaller percentages of di-, tri-, and tetraglutamates.

**Intracellular PMX Accumulation under Growth Conditions.** Total PMX accumulation in R5 and HeLa cells, which represents it polyglutamate derivatives, was determined after 1 week of growth in folic acid or 5-CHO-THF medium containing 50 nM [$^3$H]PMX. We also included 200 $\mu$M glycine, 100 $\mu$M adenine, and 10 $\mu$M thymidine in the medium to circumvent the metabolic effects of the drug. With folic acid medium, accumulation PMX and its polyglutamates in R5 cells (19.3 ± 1.3 pmol/mg of protein) was half that in HeLa cells

Table 1 *Specific folic acid binding in R5 and HeLa cells*

Folic acid binding was assessed by exposing cells to 5 nM [$^3$H]folic acid in the presence or absence of 500 nM nonlabeled folic acid at 4°C for 15 min. Folic acid bound to the cell surface was released by pH 3.5 buffer. Specific folic acid bound is the difference in tritium released in the presence and absence of 500 nM unlabeled folic acid. Data are expressed in units of pmol/mg of protein based on the average of three independent experiments ± SE.

| Cell line | [$^3$H]Folic acid bound (*A*) | [$^3$H]Folic acid bound in the presence of 500 nM non-labeled folic acid (*B*) | Specific [$^3$H]folic acid bound (*A* − *B*) |
|---|---|---|---|
| HeLa in folic acid medium | 0.38 ± 0.06 | 0.014 ± 0.002 | 0.37 |
| R5 in folic acid medium | 0.15 ± 0.03 | 0.009 ± 0.001 | 0.14 |
| HeLa in 5-CHO-THF[a] medium | 0.59 ± 0.09 | 0.02 ± 0.002 | 0.57 |
| R5 in 5-CHO-THF medium | 0.37 ± 0.05 | 0.014 ± 0.001 | 0.36 |

[a] 5-CHO-THF, 5-formyltetrahydrofolate.

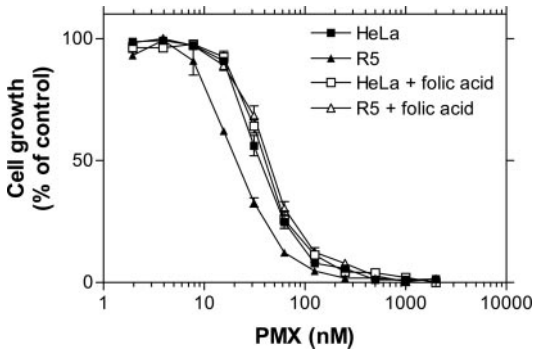


Fig. 4. Effect of 1 $\mu$M folic acid on pemetrexed (*PMX*) growth inhibition in HeLa and R5 cells maintained in 5-formyltetrahydrofolate medium. HeLa and R5 cells were grown in RPMI medium containing 25 nM 5-formyltetrahydrofolate and exposed continuously to different concentrations of pemetrexed in the absence or presence of 1 $\mu$M folic acid for 6 days before cell numbers were quantitated. The growth rate in the absence of drug was set as 100%. Data are the mean ± SE (*bars*) from three separate experiments.

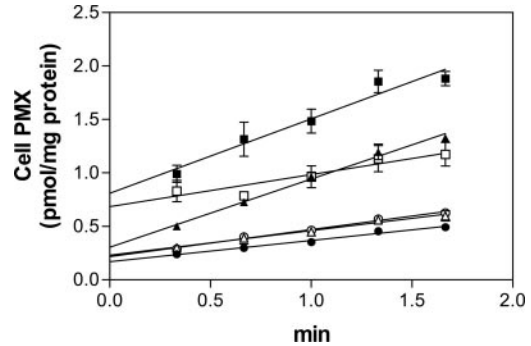


Fig. 5. Effect of 20 $\mu$M folic acid or 5 $\mu$M 5,8-dideaza-$N^{\alpha}$-(-4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-1-ornithine on pemetrexed (*PMX*) influx in HeLa and R5 cells grown in folic acid medium. [$^3$H]Pemetrexed uptake was assessed at a concentration of 0.5 $\mu$M in the absence (*squares*) or presence of 20 $\mu$M folic acid (*triangles*) or 5 $\mu$M PT632 (*circles*) in wild-type HeLa (*filled symbols*) or R5 (*open symbols*) cells grown in RPMI medium containing 2 $\mu$M folic acid. Data are the mean ± SE (*bars*) from three experiments. When the SE was smaller than the symbol used, the *bars* are not visible.

(37.7 ± 0.3 pmol/mg of protein) based on three independent experiments. With 5-CHO-THF medium, PMX accumulation in R5 cells (43 ± 3.0 pmol/mg of protein) was comparable to that of HeLa cells (47.0 ± 2.6 pmol/mg of protein). These data are consistent with the pattern of PMX net uptake determined over shorter intervals (Fig. 6). Hence, under conditions of growth in a physiological folate, there is only a negligible decrease in accumulation of PMX polyglutamates in R5 cells despite the loss of RFC function.

**Kinetics of PMX Influx Mediated by a RFC-Independent Route.** PMX influx kinetics were determined in R5 cells grown in folic acid medium. Unlabeled folic acid (20 $\mu$M) was included to block PMX binding to FRs to minimize the ordinate intercept and thereby to increase the accuracy of influx measurements (see Fig. 5). PMX initial uptake was saturable with a $K_t$ of 12 ± 6 $\mu$M and a $V_{max}$ of 5.6 ± 1.4 pmol/mg protein/min determined from a nonlinear regression fit to the Michaelis–Menten equation (Fig. 7), or 18 $\mu$M and 6.9 pmol/mg protein/min, respectively, determined from a Lineweaver–Burk plot analysis (Fig. 7, *inset*).

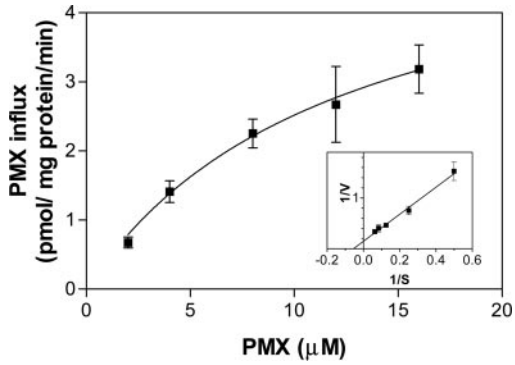


Fig. 7. Determination of pemetrexed (*PMX*) influx kinetics in R5 cells grown in folic acid medium. R5 cells grown in folic acid medium were exposed to the indicated concentrations of [$^3$H]pemetrexed in the presence of 20 $\mu$M folic acid. Influx is based on the slope of four to five initial uptake points assessed within 75 s after addition of drug. The *line* is a nonlinear regression fit to the Michaelis–Menten equation. *Inset*, Lineweaver–Burk plot of the same measurements. Data are the mean ± SE (*bars*) from six independent experiments.

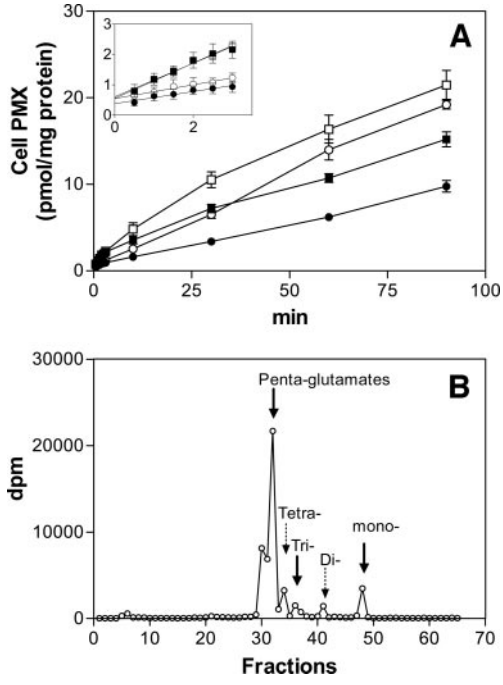


Fig. 6. Comparison of initial and net uptake of pemetrexed (*PMX*) in HeLa and R5 cells grown in either folic acid or 5-formyltetrahydrofolate medium (5-CHO-THF) (*A*) and a representative high-pressure liquid chromatographic analysis of PMX and its polyglutamate derivatives that accumulated within cells (*B*). *A*, uptake was assessed with 0.5 $\mu$M [$^3$H]PMX in HeLa (*squares*) and R5 cells (*circles*) grown in RPMI medium containing 2 $\mu$M folic acid (*filled symbols*) or 25 nM 5-CHO-THF (*open symbols*). Initial uptake data from the same experiments are shown in the *inset*. Data are the mean ± SE (*bars*) from three separate experiments. *B*, representative high-pressure liquid chromatographic analysis of PMX and its polyglutamate derivatives that accumulate in R5 cells grown in 5-CHO-THF medium after 2 h of incubation with 0.5 $\mu$M [$^3$H]PMX. *Solid arrows* indicate radioactive peaks identified by comigration of nonlabeled standards; *dashed arrows* indicate predicted peaks.

Relative affinities of other folates and antifolates for this RFC-independent process were assessed based on inhibition of 0.5 $\mu$M [$^3$H]PMX influx by these substrates at an inhibitor concentration of 40 $\mu$M. The extent to which PMX influx was inhibited varied among different folates, with greatest suppression by PMX and least inhibition by PT523 (Fig. 8). On the basis of the measured PMX influx $K_t$ of 12 $\mu$M and assuming that all of these agents inhibit PMX influx competitively, influx $K_i$s were calculated and are indicated at the end of the columns in Fig. 8. The PMX influx $K_i$ determined in this way was comparable to the influx $K_t$ determined directly with tritiated drug as indicated above. The folic acid $K_i$ (92 $\mu$M) was consistent with a lack of appreciable inhibition of PMX influx by 20 $\mu$M folic acid (Fig. 5). This transport route also has a low affinity for ZD1694 and PT523 (~7- and 14-fold less than for PMX, respectively), to which R5 cells were highly resistant in either folic acid or 5-CHO-THF medium. The affinities for 5-CHO-THF and 5-methyltetrahydrofolate (racemic), were 3 and 5 times lower, respectively, than that for PMX. Hence, this transport process favors PMX compared with the other folate and antifolate substrates tested.

## DISCUSSION

The HeLa-derived R5 cell line is a valuable human solid tumor model for studying the impact of the loss of RFC function on the activity of antifolates that use this carrier. The *RFC* gene was deleted from the genome of R5 cells by chemical mutagenesis followed by MTX selective pressure, thereby providing a stable defect. The lack of cross-resistance to trimetrexate ruled out additional changes at the level of dihydrofolate reductase (12). Furthermore, R5 cells were not cross-resistant to AG331, an antifolate TS inhibitor that diffuses into cells and requires neither RFC nor folypolyglutamate synthase for activity, excluding significant changes at the level of TS. PMX was as

Table 2 *Distribution of Pemetrexed and pemetrexed polyglutamate derivatives in R5 and HeLa cells grown either in folic acid or 5-formyltetrahydrofolate medium*

Cells were exposed to 0.5 $\mu$M premetrexed for 2 h at 37°C. The values are expressed in unit of pmol/mg of protein. *Numbers* in *parentheses* indicate percentage of total premetrexed accumulation. Data are the average of two independent experiments.

| | Total PMX[a] accumulation | PMX monoglutamate | PMX diglutamate | PMX triglutamate | PMX tetraglutamate | PMX pentaglutamate and higher |
|---|---|---|---|---|---|---|
| R5 in folic acid medium | 15.2 | 3.8 (25.0%) | 0.9 (5.9) | 1.0 (6.6%) | 1.4 (9.2%) | 8.1 (53.3%) |
| HeLa in folic acid medium | 23.1 | 1.7 (7.4%) | 0.6 (2.6%) | 0.7 (3.0%) | 1.0 (4.3%) | 19.1 (82.7%) |
| R5 in 5-CHO-THF medium | 35.0 | 2.3 (6.6%) | 1.0 (2.9%) | 1.4 (4.0%) | 1.9 (5.4%) | 28.4 (81.1%) |
| HeLa in 5-CHO-THF medium | 41.1 | 1.7 (4.1%) | 1.0 (2.4%) | 0.9 (2.2%) | 1.6 (3.9%) | 35.9 (87.3%) |

[a] PMX, Pemetrexed; 5-CHO-THF, 5-formyltetrahydrofolate.

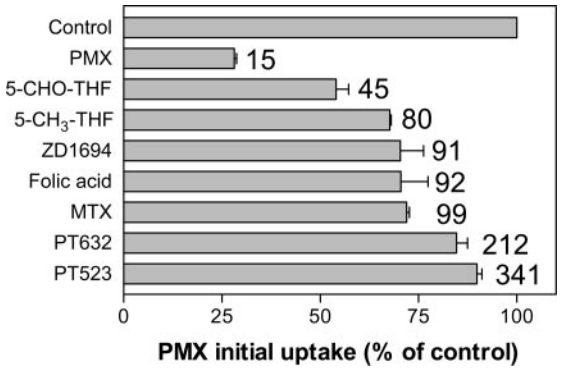


Fig. 8. Inhibition of pemetrexed (*PMX*) influx by various folates and antifolates and their computed influx $K_i$s determined in R5 cells grown in folic acid medium. R5 cells grown in folic acid medium were exposed to 0.5 $\mu$M [$^3$H]PMX and 20 $\mu$M unlabeled folic acid for 5 min in the presence and absence of 40 $\mu$M inhibitor. As a result, the final concentration of folic acid was 60 $\mu$M when folic acid was used as an inhibitor. *Control* indicates uptake in the absence of inhibitor but in the presence of 20 $\mu$M folic acid. Data are the mean ± SE (*bars*) from three separate experiments. The *numbers* next to columns are the influx $K_i$ ($\mu$M) for each inhibitor calculated from the measured [$^3$H]PMX influx $K_t$ and the extent of inhibition by each folate/antifolate. *5-CHO-THF*, 5-formyltetrahydrofolate; *5-CH$_3$-THF*, 5-methyltetrahydrofolate; *ZD1694*, raltitrexed; MTX, methotrexate; *PT632*, 5,8-dideaza-$N^{\alpha}$-(-4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-1-ornithine; *PT523*, $N^{\alpha}$-(-4-amino-4-deoxypteroyl)-$N^{\delta}$-hemiphthaloyl-1-ornithine.

efficiently converted into polyglutamate derivatives in R5 cells as in HeLa cells, consistent with preservation of folypolyglutamate synthase activity. The most compelling evidence that the transport defect in R5 cells was the sole, or predominant, mechanism of resistance to ZD1694 and PT523 was the observation that PMX and PT523 activity was completely, and ZD1694 almost completely, restored in R5 cells transfected with RFC cDNA.

Despite the loss of RFC, PMX activity in R5 cells was preserved; indeed, cells were collaterally sensitive to this agent when 5-CHO-THF was the folate growth source. This preservation of PMX activity could be attributed to two factors. First, there was an alternative transport route for this agent. Indeed, ~45% of the initial PMX uptake rate was preserved in R5 cells despite the lack of RFC. This residual transport activity could not be attributed to expression of folate receptors in R5 cells because FR expression was very low, addition of folic acid did not alter PMX influx, and inclusion of 1 $\mu$M folic acid in the growth medium only minimally decreased PMX activity in R5 cells. This is in contrast to the 100-fold decrease in the activity of CB300638, a TS inhibitor that is an excellent substrate for FR-$\alpha$ but not RFC in human epidermoid A430 cells that express high levels of FR-$\alpha$ (19). Second, as indicated previously, when R5 cells are grown in 5-CHO-THF there is a contraction of cellular folate cofactor pools (12). This was shown in murine leukemia cells to augment PMX polyglutamation by decreasing feedback inhibition by cellular folate cofactors at the level of folypolyglutamate synthase, thereby compensating for the loss of PMX transport (18, 21, 22).

The data indicate the presence of a novel folate transporter in R5 cells with an influx $K_t$ for PMX of ~12 $\mu$M. Other antifolates had a much lower affinity for this transporter, with $K_i$s of 340, 99, and 91 $\mu$M for PT523, MTX, and ZD1694, respectively. The affinity of this transporter for three naturally occurring folate substrates, folic acid, 5-CHO-THF, and 5-methyltetrahydrofolate was also lower with $K_i$s of 92, 45, and 80 $\mu$M, respectively. This transporter does not distinguish among folic acid, ZD1694, and MTX as does RFC (with an affinity for folic acid two orders lower than for these antifolates) and does not distinguish MTX from folic acid, as does FR-$\alpha$ (with a 100-fold higher affinity for folic acid; Ref. 11). The data suggest that this transport activity is also present in wild-type HeLa cells because addition of folic acid, to abolish possible FR- mediated transport, along with PT632, to abolish RFC-mediated transport, left substantial residual transport activity that was comparable to PMX transport in R5 cells. It is not clear what role this transporter may play in PMX activity when RFC is intact. However, because this is a lower affinity process than RFC, it could contribute to the delivery of PMX and other antifolates into cells at the high initial concentrations that follow bolus administration of drugs *in vivo*, when RFC would be saturated.

The identity of this RFC-independent transport pathway in HeLa cells is unclear, nor is it clear as to the extent to which it might be expressed in other human solid tumor cell lines. There are a variety of organic anion transporters of the SLC21 and SLC22 superfamilies of facilitative carriers largely expressed in hepatic and renal tissues (23, 24), some of which, such as OAT-K1-2 (25, 26), LST-2 (27), and hOAT (28), transport MTX; and LST-2 has been detected in human gastrointestinal tumors (27). However, initial studies suggested that the secondary pathway in R5 cells is not related to these processes. This laboratory described a transport activity with high affinity ($K_t$ = 30 nM) for PMX and low affinity for MTX (~100 $\mu$M) in human mesothelioma cell lines. Recent studies, however, have clarified that this activity is associated with the presence of *Mycoplasma* in cell cultures and likely reflects transport into this microorganism (15). This activity is also present, although at a much lower level (<10-fold) in HeLa cell lines contaminated with *Mycoplasma*. However, in the present study, cells were tested regularly with a PCR-based *Mycoplasma* detection kit and were shown to be free from any contamination by this organism.

The high affinity of this secondary pathway for PMX in HeLa cells explains, in part, the preservation of PMX activity relative to ZD1694 and PT523 when RFC is deleted because the latter agents have much lower affinities for this process. In addition, previous studies have demonstrated that a decrease in cell folate cofactor pools has a much greater salutatory effect on the activity of PMX than ZD1694 and virtually no effect at all on the activity of PT523 (21). Relative preservation of PMX activity compared with that of ZD1694 has been observed previously under conditions in which transport is impaired. For example, 4-fold resistance to PMX was accompanied by 64-fold resistance to ZD1694 in ZR-75-1 MTX$^R$ breast cancer cells with impaired RFC function grown in 5-CHO-THF (4). Likewise, 41M: ZD1694 human ovarian cells with impaired MTX uptake were ~7-fold resistant to PMX but 123-fold resistant to ZD1694 (8). In addition, in H630TDX cells in which "uptake" was impaired but TS activity was unaltered, there was 4-fold resistance to PMX but 7500-fold resistance to ZD1694 (7, 29). Hence, preservation of PMX activity relative to ZD1694, despite impaired RFC-mediated transport, appears to have general relevance, although the extent to which this is due to the presence of a secondary transport pathway, as described in this report, is not clear.

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