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Dihydrofolate Reductase Binding and Cellular Uptake of Nonpolyglutamatable Antifolates: Correlates of Cytotoxicity Toward Methotrexate-Sensitive and -Resistant Human Head and Neck Squamous Carcinoma Cells

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SUMMARY

Several mechanisms have been demonstrated to be independently involved in methotrexate (MTX) resistance, including increased dihydrofolate reductase (DHFR) activity, decreased membrane transport, and decreased conversion to nonefluxing polyglutamates by folylpolyglutamate synthetase. We conducted the present study to test the hypothesis that nonpolyglutamatable antifolates with an N^{δ}-hemiphthaloyl-L-ornithine side chain could be more potent than MTX against MTX-sensitive and -resistant human carcinoma cells via tighter DHFR binding, more efficient cellular uptake, the ability to bypass defective polyglutamation, or a combination. Two nonpolyglutamatable antifolates, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine (PT523) and the new B-ring analogue N^{α}-[4-[*N*-(2,4-diamino-5-chloroquinazolin-6-yl)methyl]amino] benzoyl-N^{δ}-hemiphthaloyl-L-ornithine (PT619), were tested as inhibitors of purified recombinant human DHFR and were found to bind somewhat better to the enzyme than MTX as determined by competitive radioligand binding assay. PT523 and PT619 were 9- and 14-fold, respectively, more active than MTX as inhibitors of parental SCC25 human and neck squamous carcinoma cell growth in 72-hr cultures. Moreover, there was an even greater increase in relative potency against two previously described MTX-resistant cell lines with an increased DHFR content and a decreased ability to convert MTX to polyglutamates: SCC25/R1 (selected with MTX) and SCC25/CP (selected with cisplatin but collaterally resistant to MTX). Both PT523 and PT619 very efficiently inhibited [^{3}H]MTX uptake by SCC25 cells in a 1-hr assay, with PT523 being 11-fold more potent and PT619 being 17-fold more potent than MTX. Greater inhibition of [^{3}H]MTX uptake with PT523 and PT619 than with MTX was also observed in SCC25/R1 and SCC/CP cells. However, the increase in activity of PT523 and PT619 relative to MTX in uptake experiments was less than that in growth-inhibition assays, especially for SCC25/CP cells. This suggested that additional cytotoxicity determinants may exist in these resistant cells.

MTX is one of the first agents shown to be effective in cancer chemotherapy, and it continues to play a central role in the treatment of clinical human malignancies (1, 2). MTX is best known for its efficacy against choriocarcinoma and hematological malignancies and is considered to be one of the first choices for treatment of head and neck squamous carcinomas when used either as a single agent or in combination with other drugs, such as CDDP or bleomycin (2). A major obstacle in the clinical use of MTX, as with many other anticancer drugs, is the ability of tumor cells to rapidly become resistant. This problem is compounded by the very steep dose-response curve of MTX, which restricts the physician's ability to overcome resistance by administering an increased dose. Thus, an urgent need exists for new antifolates with greater potency, better selectivity, and a broader spectrum of activity than MTX, especially against resistant tumors.

The mechanism of action of MTX is related to its potent inhibition of the enzyme DHFR, resulting in depletion of cellular reduced folates and blockade of *de novo* thymidylate and purine nucleotide synthesis. MTX enters cells via transport systems that are concentrative, energy dependent, and carrier mediated (3). Intracellular MTX is converted to a

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ABBREVIATIONS: DHFR, dihydrofolate reductase; MTX, methotrexate; PT523, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine; PT619, N^{α}-[4-[(2,4-diamino-5-chloroquinazolin-6-yl)methyl]amino]benzoyl-N^{δ}-hemiphthaloyl-L-ornithine; H_2PteGlu, 7,8-dihydrofolate; H_4PteGlu, 5,6,7,8-tetrahydrofolate; CDDP, *cis*-diamminedichloroplatinum(II) (cisplatin); PBS, phosphate-buffered saline; FBS, fetal bovine serum.

polyglutamated species whose chain length in a given cell line appears to depend on the respective anabolic and catabolic activities of two enzymes, folylpolyglutamate synthetase and folylpolyglutamate hydrolase (4–9). Due to the polyanionic character of MTX polyglutamates, efflux of these species is markedly reduced in comparison with the parent monoglutamate (10). Moreover, the longer polyglutamates of MTX bind at least as tightly as the parent drug to DFHR but dissociate more slowly from the enzyme (11, 12). Therefore, changes in amount or ligand binding affinity of the target enzyme DHFR, in membrane transport and intracellular metabolism of the drug, or in one or more of these can alter the sensitivity of tumor cells to MTX. Because there are no known selective, nontoxic modulators with which to overcome antifolate resistance by targeting these changes, as has been possible for multidrug resistance (13), the development of new side chain-modified antifolates whose ability to inhibit growth is independent of the capacity of a tumor to form polyglutamates has been attractive as a drug design strategy (14–16). Among the numerous types of side chain-modified analogues that we have previously synthesized, the most promising have been those in which L-glutamic acid is replaced by N^5-hemiphthaloyl-L-ornithine (17–20). In the present study, the mode of action of two of the most potently cytotoxic compounds, PT523 and PT619 (Fig. 1), was examined with the use of the previously described MTX-sensitive human squamous cell carcinoma line SCC25 (21) and its MTX-resistant sublines SCC25/R1 (22) and SCC25/CP (23–26).

Materials and Methods

Cell lines and chemicals. The parental human squamous cell carcinoma line SCC25 was originally established at the Dana-Farber Cancer Institute (21). The SCC25/R1 subline was obtained by continuous exposure to increasing concentrations of MTX in the culture medium, and its phenotype has been characterized (22). The SCC25/CP subline was developed by repetitive pulse treatment of SCC25 cells with CDDP (23–26). The SCC25/CP cells have also been called SCC25/CP[1] cells (25). SCC25/R1 cells were treated with 0.9 μM MTX for one week every other week and SCC25/CP cells were treated with 0.1 mM CDDP for 30 min once a month to maintain the resistant phenotype. All experiments were carried out with cells kept in drug-free medium for at least one passage (usually four or five generations). The parental and resistant cells were grown under 6% CO_2 at 37° in monolayer culture in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 20% FBS (Sigma Chemical Co., St. Louis, MO), penicillin/streptomycin, and hydrocortisone (0.4 μg/ml). [3′,5′,7-^{3}H]MTX was purchased from Moravek Biochemicals (Brea, CA) and had a specific activity of 1 mCi/ml (19.3 Ci/mmol) and a purity of >99% by high-performance liquid chromatography. MTX was a gift from the National Cancer Institute. CDDP was obtained as a solution (1 mg/ml) from Bristol-Myers Squibb Company (Princeton, NJ). PT523 was synthesized in our laboratory as described (17, 27). The synthesis of PT619 by condensation of N^α-(4-aminobenzoyl)-N^5-phthaloyl-L-ornithine with 2,4-diamino-5-chloroquinazoline-6-carbonitrile and alkaline opening of the phthalimide ring will be reported separately. Stock solutions of 1 mM MTX, 0.5 mM PT523, and 0.5 mM PT619 were made up in PBS, pH 7.5, and kept at −20° in the absence of light. Total protein content of the cells was measured with the Bradford assay (28).

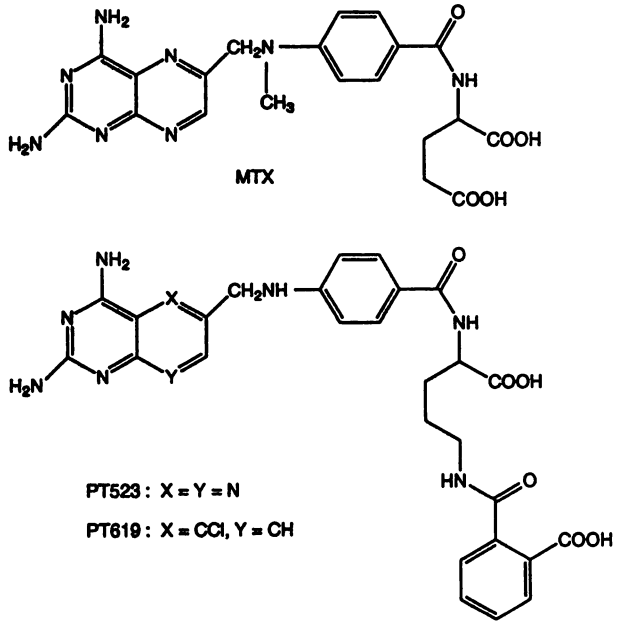

Fig. 1. Structures of MTX, PT523, and PT619.

Isolation and purification of human recombinant DHFR. *Escherichia coli* strain JM107 cells transfected with plasmid pDFR, containing a restriction fragment of human DHFR cDNA and the expression vector pKK-223–3 with a hybrid *tac* promoter (29), were kindly provided by Dr. M. Ratnam (Medical College of Ohio, Toledo, OH). The enzyme was purified essentially as described (29) except that MTX-agarose was used in the affinity chromatography step (30). The final recovery of enzyme after MTX-agarose chromatography, ultrafiltration (Amicon YM-10 membrane), and gel filtration (Sephadex G-75) contained ~60% of the activity in the crude cell extract. The final fraction used in binding and inhibition assays gave a single band on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining (Bio-Rad, Hercules, CA).

DHFR activity assay. Functional DHFR activity was assessed spectrophotometrically (29). The decrease in molar absorbance at 340 nm due to reduction of H_2PteGlu and concomitant oxidation of NADPH was assumed to be 12,300 mol/cm (31). Standard conditions for the enzyme purification and titration were 50 mM Tris·HCl/0.15 M KCl, pH 7.5, containing 60 μM NADPH and 50 μM H_2PteGlu at 22° in a volume of 1 ml. The enzyme was kept on ice with NADPH for ~30 min and then transferred to the cuvette with a 10-μl aliquot of inhibitor solution or drug-free control. After a 2-min preincubation in the cuvette at 22°, the reaction was initiated by the addition of the H_2PteGlu substrate (29). The three inhibitors were tested in parallel to allow comparison under identical conditions, and a total of five replicate analyses were performed on different days.

To compare DHFR activity among the three cell lines, cultures in exponential growth were trypsinized, and 10^7 trypsinized cells were washed three times with cold PBS containing 1 mg/ml BSA at 4° (180 × *g*, 5 min). Cell suspensions were then sonicated on ice (3 × 10 sec) at a power setting of 35% (model 300, Fisher Sonic Dismembranator). After centrifugation (13,000 × *g*, 15 min), the supernatant was stored at −80° and thawed as needed. To improve the sensitivity of the measurement of DHFR activity in tumor cells, the assay conditions were modified so that the stock buffer (50 mM Tris·HCl/0.15 M KCl, pH 7.5) contained 100 μM NADPH, 20 μM H_2PteGlu, and 4.5 mM 2-mercaptoethanol, and the reaction was carried out at 30° (32). The absorbance change at 340 nm was recorded for 1 min for the activity assay during purification of the recombinant enzyme, 2 min for the inhibition assays with antifolates, and 5 min for the assay of enzymic activity in cell extracts. Uncatalyzed NADPH oxidation was negligible under these conditions. One unit of enzyme activity was defined in each case as the amount of enzyme required to convert 1.0 μmol H_2PteGlu to H_4PteGlu in 1 min.

Competitive DHFR binding assay. The assay, which measured binding of [^{3}H]MTX and nonlabeled antifolates to DHFR, with re-

moval of excess nonbound drugs on charcoal, was performed according to an improved separation procedure with disposable filters (33) instead of centrifugation (34). The assay, which has been previously described (33), was modified slightly. Briefly, to each Eppendorf tube, we added sequentially the following solutions: (a) 150 μl water containing 19 nCi [^{3}H]MTX, (b) 200 μl aqueous solutions containing different dilutions of nonlabeled inhibitors, and (c) 50 μl 0.5 M potassium phosphate buffer, pH 7.2, containing 2.7 munits DHFR and 800 μM NADPH. After equilibration of the mixture at room temperature for 10 min, 50 μl charcoal suspension was added (made up from 10 g activated charcoal, 2.5 g bovine serum albumin, and 0.1 g high-molecular-weight dextran in 100 ml water, pH 7.2). One minute after vortexing, the mixture was filtered into a counting vial through a Gelman Acro-LC13 filter (0.45 μm, Gelman Scientific, Ann Arbor, MI). Radioactivity was counted in duplicate with a Beckman LS700 instrument in 10 ml Ready-Safe scintillation fluid (Beckman, Fullerton, CA). Data were linearized by logit transformation (34), with logit values of 0.0 and −2.2 corresponding to the IC_{50} and IC_{90}, respectively. The significance of differences between mean IC_{50} and IC_{90} values for competitive inhibition of [^{3}H]MTX binding by PT523, PT619, and nonlabeled MTX was analyzed with a one-tailed, two-sample Wilcoxon test using S Plus statistical software.

Cytotoxicity assay. The ability of MTX, PT523, and PT619 to inhibit the growth of cells was evaluated as described (35). Cells from logarithmically growing cultures were seeded at a density of 5 × 10^3/0.1 ml per well into a 96-well plate in Dulbecco's modified Eagle's medium supplemented with 20% dialyzed FBS. Drugs were added after 24 hr, and incubation was continued for an additional 72 hr. At least 4 wells were used for each treatment. After incubation, the medium was aspirated and replaced with 0.1 ml of medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma). The plates were then incubated for 4 hr at 37°, the formazan crystals on the bottom of the wells were dissolved in HCl/isopropanol, and absorbance at 560 nm was determined with a Titertek Multiskan Plate Reader. Mean absorbances in replicate wells were calculated, the mean value of relative absorbance (percentage of control) from 8–10 independent experiments was plotted on semi-log paper, and the IC_{50} was determined as a function of concentration.

[^{3}H]MTX transport. For measurement of competitive inhibition of [^{3}H]MTX accumulation by nonlabeled MTX, PT523, and PT619, cells in logarithmic phase of growth were detached by trypsinization and washed twice with cold PBS containing 1 mg/ml bovine serum albumin. The cell suspension was then filtered through a cell strainer (model 2350, Falcon, Lincoln Park, NY) to remove cell clumps. Cell pellets were resuspended at a density of 1 × 10^6/0.5 ml in Richter's folate-free medium containing 20% dialyzed FBS, 2 mM L-glutamine, and 0.4 μg/ml hydrocortisone. To each tube we added an additional 0.5 ml Richter's folate-free medium containing various dilutions of the unlabeled inhibitors and sufficient [^{3}H]MTX to bring the final concentration to 0.2 μM. The rack containing the tubes was shaken several times manually and placed into a shaking water bath for 1 hr at 37°. To stop drug uptake, 4 volumes of ice-cold PBS were added to each tube, and the cells were washed three times with cold PBS (470 × *g*, 4°, 5 min). The pellets were then resuspended in 1 ml of 1 N NaOH and digested overnight. After neutralization with an equal volume of 1 N HCl, 0.8 ml of each extract was added to a scintillation vial containing 20 ml Ready-Safe, and radioactivity was counted in duplicate as described.

Results

Characteristics of the sensitive and resistant cells. Several phenotypic traits of the wild-type SCC25 and the resistant SCC25/R1 and SCC25/CP cells have been determined previously in this laboratory, including DHFR content, kinetics of MTX uptake, and sensitivity to MTX during continuous exposure for ~2 weeks (23, 26). As shown in Table 1, the IC_{50} obtained for inhibition of SCC25 cell growth by MTX during a 72-hr incubation was 32 nM. The SCC25/R1 cells (IC_{50}, 1100 nM) were 33-fold resistant, and the SCC25/CP cells (IC_{50}, 370 nM) were 12-fold resistant. DHFR activity in lysates of the three cell lines, assayed spectrophotometrically at 340 nm at pH 7.5 and 30° in the presence of NAPDH, H_2PteGlu, and 2-mercaptoethanol according to Domin *et al.* (31), was found to be 0.62, 2.5, and 0.75 munits/min/mg protein in the SCC25, SCC25/R1, and SCC25/CP cells, respectively. However, when activity was expressed on a per-cell basis to correct for differences in protein concentration, these values were 0.15, 0.75, and 0.17 munits/min/10^6 cells for SCC25, SCC25/R1, and SCC25/CP cells, respectively (data not shown). Thus, when expressed in this manner, DHFR activity was 4–5-fold greater in SCC25/R1 cells than in the parental SCC25 cells, which is in qualitative agreement with the previously determined 3.5-fold increase in DHFR content based on radioligand binding (22). [^{3}H]MTX accumulation in the three cell lines was measured at 1 hr, a time previously found to be near the end of the plateau of the exponential uptake phase (22). The parental SCC25 cells were found to contain 0.84 pmol/mg protein (0.20 pmol/10^6 cells) compared with 1.1 pmol/mg (0.33 pmol/10^6 cells) for the SCC25/R1 cells and 1.2 pmol/mg (0.27 pmol/10^6 cells) for SCC25/CP cells. Thus, uptake of MTX after 1 hr was slightly greater in the two resistant sublines, although the difference was <1.5-fold.

Interaction of the antifolates with DHFR. PT523 and PT619 were compared initially with MTX in a standard spectrophotometric assay of dihydrofolate reduction in the presence of NADPH and purified human recombinant DHFR (31) and then with the use of a competitive radioligand binding

TABLE 1

Characteristics of MTX-sensitive (SCC25) and MTX-resistant (SCC25/R1, SCC25/CP) human squamous carcinoma cells

DHFR activity, MTX accumulation, and MTX IC_{50} values are compared for each cell line (see Materials and Methods).

Cells	Protein content[a]	DHFR activity[b]	MTX uptake, 1 hr[c]	MTX IC_{50}[d]
	μg/10^6 cells	*munits/min/mg protein*	*pmol/mg protein*	*nM*
SCC25	240 ± 9	0.6 ± 0.1 (1.0)	0.8 ± 0.1 (1.0)	32 ± 1.4 (1.0)
SCC25/R1	300 ± 37	2.5 ± 0.4 (4.0)	1.1 ± 0.2 (1.3)	1100 ± 190 (34)
SCC25/CP	220 ± 12	0.8 ± 0.1 (1.2)	1.2 ± 0.1 (1.4)	370 ± 30 (12)

[a] Measured by the Bradford assay (28) on lysates of exponentially growing cells. Data are mean ± standard error (n = 6).

[b] Activity was monitored spectrophotometrically at 340 nm in an assay mixture containing 20 μM H_2PteGlu, 100 μM NADPH, and 4.5 mM 2-mercaptoethanol in 0.05 M Tris · HCl/0.15 M KCl buffer, pH 7.5, at 30°. Data are mean ± standard error (n = 5). Numbers in parentheses are normalized relative to the parental SCC25 cells.

[c] Total intracellular radioactivity was determined after treating a suspension of trypsinized cells (10^6/ml) in Richter's folate-free medium with 0.2 μM [^{3}H]MTX for 1 hr at 37°. Data are mean ± standard error (n = 6). Numbers in parentheses are normalized relative to the parental SCC25 cells.

[d] Cells were plated at an initial density of 10^5/ml and incubated for 72 hr. Data are mean ± standard error (n = 6). Numbers in parentheses are normalized relative to the parental SCC25 cells.

assay (33), which is performed without the presence of functional substrates. The IC_{50} values of the compounds in the spectrophotometric assay of enzyme activity were approximately the same (Table 2 and Fig. 2). In the competitive inhibition assay of [^{3}H]MTX binding (Fig. 3), the IC_{50} of MTX was greater than that of PT523 at a statistical significance level of $p = 0.014$ (one-tailed, two-sample Wilcoxon test) and was also greater than that of PT619 but only at $p = 0.057$. The IC_{90} of MTX was also higher than that of either PT523 ($p = 0.057$) or PT619 ($p = 0.014$).

Cell growth inhibition by PT523 and PT619. As shown in Fig. 4 and Table 3, the IC_{50} values of PT523 and PT619 as inhibitors of the growth of wild-type SCC25 cells in a 72-hr assay were 3.5 and 2.2 nM, respectively. The corresponding values against SCC25/R1 cells were 46 and 34 nM, and those against SCC25/CP cells were 7.3 and 5.2 nM. The increase in potency of the analogues against the parental cells, relative to MTX, was 9-fold for PT523 and 14-fold for PT619. In contrast to the parental cells, PT523 was 23-fold more potent than MTX against SCC25/R1 cells and 51-fold more potent against SCC25/CP cells. Similarly, PT619 was 31-fold more potent than MTX against SCC25/R1 cells and 72-fold more potent against SCC25/CP cells. As a result, although the SCC25/R1 cells were found to be 34-fold resistant to MTX, their cross-resistance to PT523 and PT619 was only 13- and 15-fold, respectively. Similarly, SCC25/CP cells were 12-fold resistant to MTX, but their cross-resistance to PT523 and PT619 was only 2-fold.

Inhibition of [^{3}H]MTX uptake by PT523 and PT619. To assess the relative ability of PT523 and PT619 to inhibit MTX accumulation in cells, the amount of [^{3}H]MTX in SCC25, SCC25/R1, and SCC25/CP cells was measured after 1 hr of coincubation in the presence of different concentrations of the nonlabeled drugs (Fig. 5). The extracellular [^{3}H]MTX concentration chosen for the experiment was 0.2 μM. The 1-hr point was chosen because this was previously found to be near the uptake plateau in SCC25 cells (22).

As shown in Table 3, the IC_{50} for inhibition of [^{3}H]MTX uptake by unlabeled MTX in the parental SCC 25 cells was 2.9 μM, a value consistent with the K_m/K_i values obtained for MTX in various human tumor cell lines in culture (reviewed in Ref. 36). In comparison, the IC_{50} values of PT523 and PT619 against SCC25 cells were 0.26 and 0.17 μM respectively; thus, these compounds were 11- and 17-fold better inhibitors than MTX of [^{3}H]MTX accumulation. The IC_{50} values obtained for MTX with SCC25/R1 and SCC25/CP cells were 3.0 and 1.1 μM, respectively. When relative abilities of MTX and PT523 to compete with [^{3}H]MTX uptake were compared, PT523 was 18-fold more active than MTX in SCC25/R1 cells and 25-fold more active in SCC25/CP cells. Similarly, PT619 was 26-fold better than MTX in SCC25/R1 cells and 31-fold better in SCC25/CP cells.

TABLE 2

Interaction of PT523 and PT619 with purified human DHFR

Inhibition of enzyme activity was measured spectrophotometrically; enzyme binding in the absence of dihydrofolate substrate was measured by competitive radioligand binding assay with [^{3}H]MTX (see Materials and Methods).

Drug	Spectrophotometric assay[a]	Competitive binding assay[b]	
		IC_{50}	IC_{90}
	nM	nM	
MTX	23 ± 7 (1.0)	30 ± 5 (1.0)	170 ± 27 (1.0)
PT523	21 ± 8 (1.1)	21 ± 2 (1.4)	130 ± 22 (1.3)
PT619	18 ± 8 (1.3)	15 ± 2 (2.0)	110 ± 22 (1.5)

[a] IC_{50} values were determined from spectrophotometric titration curves with data points (mean ± standard deviation) plotted from five independent experiments for each drug (compare with Fig. 2). Activity was monitored at 340 nm in the presence of 50 μM H_2PteGlu, 60 μM NADPH, and 0.15 M KCl in 50 mM Tris · HCl, pH 7.5, at 22°. Numbers in parentheses are normalized relative to MTX. DHFR activity in the absence of inhibitor was 7.7 ± 1.2 munits/ml. The difference in the IC_{50} values among the three inhibitors did not reach statistical significance in this assay ($p > 0.05$, Student's t test).

[b] IC_{50} and IC_{90} values were determined with logit plots to linearize the pooled binding data in Fig. 3. Four independent experiments were performed with each drug, and statistical significance was assessed with a one-tailed, two-sample Wilcoxon test.

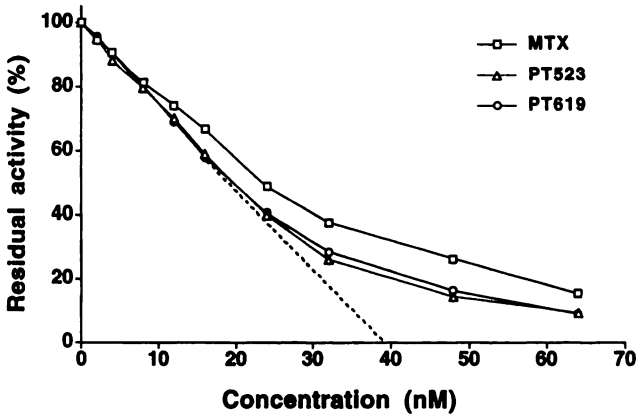

Fig. 2. Inhibition of human DHFR activity by MTX, PT523, and PT619. *Points*, mean ± standard error from five independent experiments.

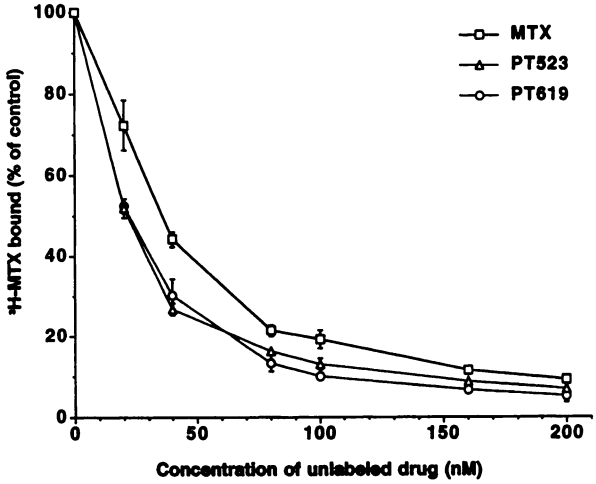

Fig. 3. Inhibition of [^{3}H]MTX binding to human DHFR by MTX (nonlabeled), PT523, and PT619. *Points*, mean ± standard error from four independent experiments.

Discussion

Possible factors that may contribute to the higher potencies of PT523 and PT619 relative to MTX in SCC25 cells include tighter binding to DHFR and more efficient uptake. Tighter DHFR binding was indicated by the lower IC_{50} and IC_{90} of these nonpolyglutamatable analogues in the competitive radioligand binding assay with [^{3}H]MTX (Table 2 and Fig. 3). Interestingly, both the IC_{50} and IC_{90} of PT619 in the ligand binding assay were ~20% lower than those of PT523, suggesting that replacement of the 2,4-diaminopteridine moiety by the 2,4-diamino-5-chloroquinazoline moiety was a favorable modification where binding to DHFR was con-

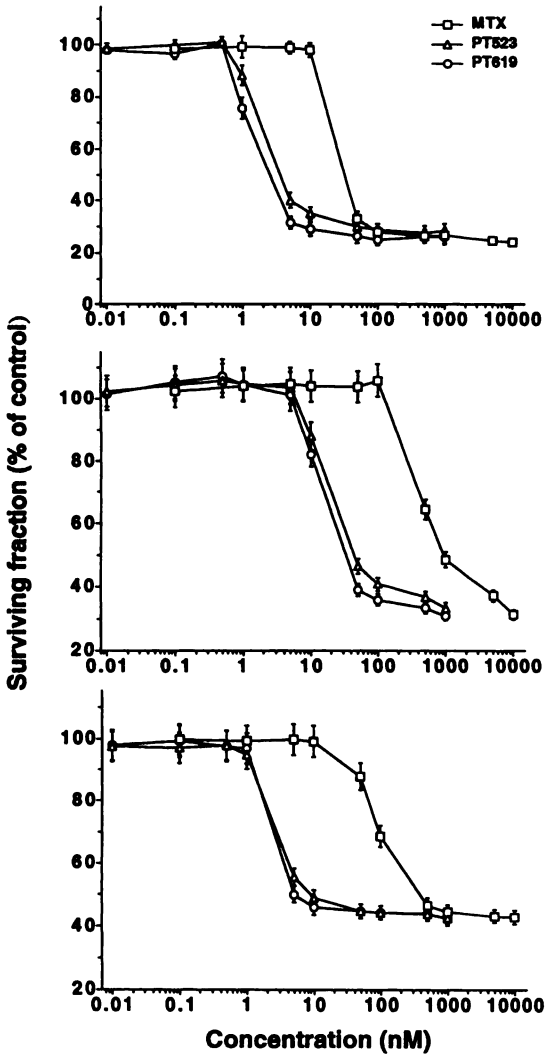

Fig. 4. Growth inhibition of SCC25, SCC25/R1, and SCC25/CP cells by MTX, PT523, and PT619. *Points*, mean ± standard error from 9 or 10 independent experiments.

cerned. Because it is well recognized that effective blockade of cellular dTMP and purine nucleotide synthesis via depletion of tetrahydrofolates requires that inhibition of DHFR activity greatly exceed 90% (37), even a small incremental difference in binding at or above the IC_{90} could be very meaningful. It is noteworthy in this regard that the K_i of the corresponding molecule with a glutamate side chain against DHFR from murine L1210 leukemia cells is reported to be 32-fold lower than that of aminopterin and 43-fold lower than that of MTX (38). The stronger binding of PT523 and PT619 to DHFR in comparison with MTX is almost certainly due to the N^{δ}-hemiphthaloyl-L-ornithine side chain. Although the exact reason for this difference is still not known, we believe that it may reflect differences in the formation and isomerization of the ternary complexes of the inhibitors with DHFR and NADPH (39). As indicated in Fig. 1, the N^{δ}-hemiphthaloyl-L-ornithine moiety is more hydrophobic than the glutamate side chain in classic antifolates. It is tempting to speculate that after the 2,4-diaminopteridine moiety of PT523 or the 2,4-diaminoquinazoline moiety of PT619 binds to the active site, additional favorable interactions can occur between the N^{δ}-hemiphthaloyl-L-ornithine moiety and hydrophobic residues just outside the active site. These additional interactions may cause the inhibitor to dissociate less readily from the active site, thereby allowing more time for isomerization to a stable complex. It is also possible that the hemiphthaloyl COOH group, which is further away from the rest of the molecule than is the γ-COOH group of MTX (see Fig. 1), can more easily form an ionic bond with a basic lysine or arginine residue in the DHFR.

TABLE 3

Effect of PT523 and PT619 on SCC25, SCC25/R1, and SCC25/CP cell growth and MTX accumulation

The concentrations of drug required to achieve 50% inhibition of cell growth after 72 hr and 50% inhibition of [^{3}H]MTX accumulation after 1 hr were compared (see Materials and Methods).

Cell/drug	Growth inhibition		[^{3}H]MTX uptake inhibition		RP ratio[d]
	IC_{50}[a]	RP[b]	IC_{50}[c]	RP	
SCC25	*nM*		*μM*		
MTX	32 (1.0)		2.9		
PT523	3.5 (1.0)	9	0.26	11	1.2
PT619	2.2 (1.0)	14	0.17	17	1.2
SCC25/R1					
MTX	1100 (34)		3.0		
PT523	46 (13)	23	0.17	18	0.8
PT619	34 (15)	31	0.12	26	0.8
SCC25/CP					
MTX	370 (12)		1.1		
PT523	7.3 (2.1)	51	0.046	25	0.5
PT619	5.2 (2.4)	72	0.037	31	0.4

[a] Data are average values from 9 or 10 independent experiments. The difference between MTX and PT523 and between MTX and PT619 was significant in all three cell lines ($p < 0.05$); however, statistically significant difference (Student's *t* test) was achieved between PT523 and PT619 only in SCC25 cells.
[b] RP = relative potency, calculated by dividing the IC_{50} of PT523 or PT619 into the IC_{50} of MTX against the same cell line.
[c] IC_{50} values were obtained from the plots shown in Fig. 5.
[d] The RP ratio was calculated by dividing the RP for 72-hr growth inhibition into the RP for 1-hr MTX uptake inhibition.

Of the two possibilities just discussed, more efficient uptake is supported by the following considerations: (a) PT523 and PT619 are more active than MTX as inhibitors of [^{3}H]MTX accumulation in MTX-sensitive as well as MTX-resistant cells (Table 3 and Fig. 5); (b) other studies have demonstrated a correlation between the cytotoxicity of PT523 and its ability to block the cellular uptake of an antifolate, in this case, (6*R*)-DDATHF, in MTX-sensitive and -resistant cells with a transport/polyglutamation defect (19); and (c) a general correlation between the uptake of antifolates and their therapeutic efficacy has been demonstrated in a number of tumor cell lines both *in vitro* and *in vivo* (39).

Even when allowance is made for the fact that PT523 and PT619 bind better than MTX to purified DHFR (Table 2), their ability to compete with MTX for uptake into SCC25 cells is ~10-fold greater (Table 3 and Fig. 5). Moreover, this difference persists, and may even be accentuated, in MTX-resistant SCC25/R1 and SCC25/CP cell lines with increased DHFR activity. In a previous study, we showed that unidirectional influx of 0.2 μM MTX was slightly higher in SCC25/R1 cells than in the parental line (22). Polyglutamation was also defective, with only 69% nonpolyglutamated MTX found in SCC25/R1 cells at 24 hr compared with 13% in SCC25 cells. In addition, SCC25/R1 cells exhibited a 3.5-fold increase in DHFR content as measured by [^{3}H]MTX binding assays on cell lysates. In SCC25/CP cells, a 2-fold increase in DFHR and a decrease in polyglutamation (35% versus 10%

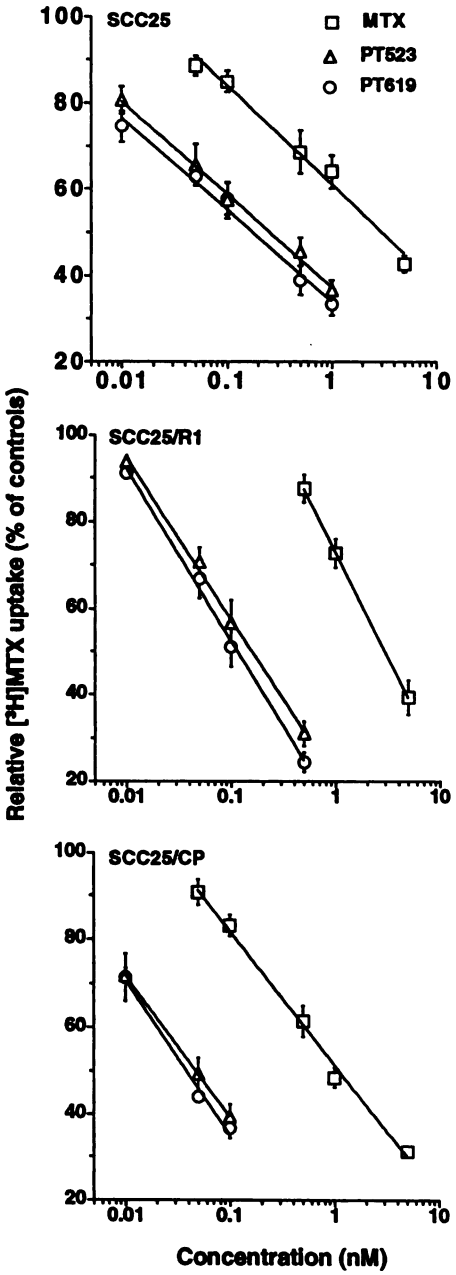

Fig. 5. Inhibition of [^{3}H]MTX uptake (1 hr) by MTX, PT523, and PT619 in SCC25, SCC25/R1, and SCC25/CP cells. *Points*, mean ± standard error from five independent experiments.

unchanged MTX) were observed, although unidirectional influx in the presence of [^{3}H]MTX was nearly the same as in parental SCC25 cells (26). MTX resistance was therefore ascribed in both cell lines to a combination of increased DHFR activity and decreased polyglutamation, the latter of which would decrease inhibition of several key folate-dependent enzymes (40–44). The present findings suggest that very efficient cellular uptake of PT523 and PT619 in comparison with MTX may enable these compounds to substantially overcome MTX resistance based on increased DHFR. Moreover, because they are incapable of forming polyglutamates and their activity does not require polyglutamation, their ability to inhibit cell growth is not affected by low folylpolyglutamate synthetase activity.

The results in Table 3 and Fig. 5 showing that PT523 efficiently inhibits cellular accumulation of MTX are consistent with the hypothesis that PT523 uptake, like that of MTX, uses the reduced folate carrier (18). The fact that PT619 is also a potent inhibitor of MTX uptake suggests that this compound may likewise use this transport route. However, it remains to be determined whether PT523 and PT619 are taken up exclusively via this route, whether there exists an alternative pathway not shared by MTX, and whether tight binding can occur to a still-unidentified cellular compartment. Because decreased intracellular [^{3}H]MTX is linearly correlated with the concentration of all three nonlabeled antifolates in each cell line (Fig. 5), we lean toward the first possibility, namely, that they are more efficiently taken up via the MTX/reduced-folate carrier. Differences in cellular accumulation among classic antifolates with a glutamate side chain are known to be associated with differences in influx (36, 38). However, compounds with similar influx rates can also differ considerably in their rate of efflux. A notable example is 5-chloro-5,8-dideazaaminopterin, the glutamate analogue of PT619, whose influx kinetics are not unusual but whose first-order rate constant for efflux is exceptionally low (38). Although the structural basis for this phenomenon is not known, it is possible that slow efflux rather than rapid influx is the critical determinant of rapid accumulation in the N^5-hemiphthaloyl-L-ornithine derivatives used in the present study. Direct kinetic measurements with radiolabeled PT523 or PT619, alone or in the presence of different concentrations of nonlabeled MTX, would help answer this question and shed light on whether a second transport route exists for these compounds that does not use the MTX/reduced folate carrier.

A major objective in the development of PT523 and related compounds in our laboratory has been to bypass polyglutamation while retaining potent activity against DHFR. It is anticipated that this class of agents will be useful against tumors with either intrinsic or acquired MTX resistance, especially where defective polyglutamation plays a major role in the resistance phenotype (6, 45–50). Both SCC25/R1 and SCC25/CP cells show defective polyglutamation activity (22, 26). Therefore, these cell lines were chosen to test the hypothesis that PT523 and PT619 might be more active than MTX because they can circumvent the polyglutamation defect. Each compound proved to be more potent than MTX against both cell lines (Table 3 and Fig. 4). Because defective polyglutamation in the resistant cells can influence the activity of MTX but not that of PT523 or PT619, the greater potency of the latter compounds against SCC25/R1 and SCC25/CP cells in comparison with MTX is most likely due to this fundamental difference in mode of action.

The potencies of PT523 and PT619 as inhibitors of [^{3}H]MTX accumulation were likewise greater than that of MTX in the MTX-resistant cells (Fig. 5), further attesting to the importance of improved uptake as a determinant of cytotoxicity. However, a slightly greater difference in relative potency was observed for PT523 and PT619 as inhibitors of cell growth than in their relative potency as inhibitors of [^{3}H]MTX accumulation. This was reflected in the relative potency *ratio*, which was calculated by dividing the relative potency for growth inhibition by the relative potency for MTX uptake inhibition (Table 3), and presumably reflects the fact that growth was measured over 72 hr, whereas MTX uptake

was measured after only 1 hr, when polyglutamation typically has not yet reached the same stage (50). Interestingly, the elevated DHFR activity in SCC25/R1 cells did not affect the concentration of MTX required to inhibit [^{3}H]MTX accumulation at 1 hr by 50% (Table 3), whereas only ~65% as much PT523 or PT619 was sufficient to produce the same effect. Similarly, although the MTX concentration required to achieve 50% inhibition of [^{3}H]MTX uptake at 1 hr in SCC25/CP cells was 40% of the MTX concentration in the parental cell line (1.1 μM versus 2.9 μM), the IC_{50} of PT523 or PT619 in SCC25/CP cells was only ~20% of the IC_{50} in SCC25 cells (0.037 μM versus 0.17 μM).

Changes in sensitivity to MTX have been observed in several tumor cell lines selected for resistance to CDDP and may include either decreased responsiveness (collateral resistance) (25, 26, 51, 52) or increased responsiveness (collateral sensitivity) relative to the parental cells (53). Because combinations of MTX and CDDP are used clinically (2), it is important to be aware of this potential drug interaction and to identify or design antifolates that are less likely to be involved in the collateral cross-resistance phenotype. Interestingly, PT523 and PT619 are much more potent than MTX against the MTX cross-resistant line SCC25/CP. This cell line was derived by selection with CDDP (23), and neither the parental SCC25 cell line nor the patient from whom it was derived was ever treated with an antifolate. The 2-fold greater relative potency of PT523 and PT619 versus MTX against SCC25/CP cells compared with SCC25/R1 cells is probably due in part to more efficient transport, as indicated by the higher relative potency (Table 3). The mechanisms for MTX resistance in most cases are multifactorial, and the relative contributions of these mechanisms may vary with the degree of resistance (45). Because the increase in DHFR activity in SCC25/CP cells is only marginal (Table 1), the 12-fold resistance of these cells to MTX (Table 3) is probably due to defective polyglutamation. The enhanced PT523 and PT619 sensitivities of SCC25/CP cells in comparison with SCC25/R1 cells could indicate that the relative contribution of defective polyglutamation to the resistance phenotype of the two cell lines is not the same.

With regard to other possible cellular properties of SCC25/CP cells that could account for their MTX resistance, a 2-fold increase in total protein sulfhydryl groups has been observed in these cells relative to parental SCC25 cells, along with a similar increase in glutathione transferase activity; however, glutathione levels were not different in the two cell lines (25). Free protein sulfhydryl groups on the cell membrane are believed to be essential for MTX transport (54), and elevated cellular glutathione levels are reported to be associated with increased MTX accumulation (55). However, these studies (54, 55) are limited to hepatocytes. Thus, a causal relationship between the higher total protein sulfhydryl groups and the greater activity of MTX, PT523, and PT619 against SCC25/CP cells is possible but remains unproved.

In conclusion, the present study demonstrates that (a) PT523 and PT619, two novel nonpolyglutamatable DHFR inhibitors, are 9- and 14-fold, respectively, more potent than MTX against the human head and neck squamous carcinoma cell line SCC25; (b) the potency of these compounds relative to MTX is further increased (~2-fold) against the subline SCC25/R1 (selected with MTX) and is likewise increased (~5-fold) against the subline SCC25/CP (selected with CDDP and cross-resistant to MTX); and (c) the higher potency of PT523 and PT619 against the parental cell line may be due to a combination of more efficient uptake and tighter binding to DHFR, whereas preferential cytotoxicity in the resistant sublines may be related to the ability to bypass defective polyglutamation.

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References

1. Jukes, T. Searching for magic bullets: early approaches to chemotherapy: antifolates, methotrexate (The Bruce F. Cain Memorial Award Lecture). *Cancer Res.* **47**:5528–5536 (1987).
2. Bertino, J. R., and A. Romanini. Folate antagonists, in *Cancer Medicine* (J. F. Holland, E. Frei III, R. C. Bast, D. W. Kufe, D. L. Morton, and R. R. Weichselbaum, eds.). Lea & Febiger, Philadelphia, 689–711 (1993).
3. Henderson, G. B. Transport of folate compounds into cells, in *Folates and Pterins, Vol. 3* (R. L. Blakley and M. V. Whitehead, eds.). Academic Press, New York, 207–250 (1984).
4. Fry, D. W., L. A. Anderson, M. Borst, and I. D. Goldman. Analysis of the role of membrane transport and polyglutamylation of methotrexate in gut and Ehrlich tumor *in vivo* as factors in drug sensitivity and selectivity. *Cancer Res* **43**:1087–1092 (1983).
5. Johnson, T. B., M. G. Nair, and J. Galivan. Role of folylpolyglutamate synthetase in the regulation of methotrexate polyglutamate formation in H35 rat hepatoma cells. *Cancer Res* **48**:2426–2431 (1988).
6. Pizzorno, G., Y. M. Chang, J. J. McGuire, and J. R. Bertino. Inherent resistance of human squamous carcinoma cell lines to methotrexate as a result of decreased polyglutamylation of this drug. *Cancer Res.* **49**:5275–5280 (1989).
7. Barredo, J., and R. G. Moran. Determinants of antifolate cytotoxicity: folylpolyglutamate synthetase activity during cellular proliferation and development. *Mol. Pharmacol* **42**:687–694 (1992).
8. Barrueco, J. R., D. F. O'Leary, and F. M. Sirotnak. Metabolic turnover of methotrexate polyglutamates in lysosomes derived from S180 cells. *J. Biol. Chem.* **266**:15356–15361 (1992).
9. Rhee M. S., Y. Wang, M. G. Nair, and J. Galivan. Acquisition of resistance to antifolates caused by enhanced γ-glutamyl hydrolase activity. *Cancer Res.* **53**:2227–2230 (1993).
10. Balinska M, J. Galivan, and J. K. Coward. Efflux of methotrexate and its polyglutamate derivatives from hepatic cells *in vitro*. *Cancer Res.* **41**:2751–2756 (1981).
11. Fry, D. W., J. C. Yalowich, and I. D. Goldman. Rapid formation of poly-γ-glutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high-pressure liquid chromatography in the Ehrlich ascites tumor cell *in vitro*. *J. Biol. Chem.* **257**:1890–1896 (1982).
12. Jolivet, J., and B. A. Chabner. Intracellular pharmacokinetics of methotrexate polyglutamates in human breast cancer cells: selective retention and less dissociable binding of 4-NH_2-10-CH_3-pteroylglutamate$_4$ and $_5$ to dihydrofolate reductase. *J. Clin. Invest.* **72**:773–778 (1983).
13. Yang, C. P., L. M. Greenberger, and S. B. Horwitz. Reversal of multidrug resistance in tumor cells, in *Synergism and Antagonism in Chemotherapy* (T. C. Chou and D. C. Rideout, eds.). Academic Press, New York, 311–338 (1991).
14. Rosowsky, A. Development of new antifolate analogs as anticancer agents. *Am. J. Pharm. Educ.* **56**:453–463 (1992).
15. L. L. Habeck, T. A. Leitner, K. A. Shackelford, L. S. Gossett, R. M. Schultz, S. L. Andis, C. Shih, G. B. Grindey, and L. G. Mendelsohn. A novel class of monoglutamated antifolates exhibits tight-binding inhibition of human glycinamide ribonucleotide formyltransferase and potent activity against solid tumors. *Cancer Res.* **54**:1021–1026 (1994).
16. Jackman, A. L., G. W. Aherne, R. Kimbell, L. Brunton, A. Hardcastle, J. W. Wardleworth, T. C. Stephens, and F. T. Boyle. A non-polyglutamatable quinazoline thymidylate synthase (TS) inhibitor. *Proc. Am. Assoc. Cancer Res.* **35**:301 (1994).
17. Rosowsky, A, H. Bader, C. A. Cucchi, R. G. Moran, W. Kohler, and J. H. Freisheim. Methotrexate analogues. 33. N^{δ}-Acyl-N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine derivatives: synthesis and *in vitro* antitumor activity. *J. Med. Chem.* **31**:1332–1337 (1988).
18. Rhee, M. S., J. Galivan, J. E. Wright, and A. Rosowsky. Biochemical studies on PT523, a potent nonpolyglutamatable antifolate in cultured cells. *Mol. Pharmacol.* **45**:783–791 (1994).
19. Rosowsky, A., H. Bader, J. E. Wright, K. Keyomarsi, and L. H. Matherly. Synthesis and biological activity of N^{ω}-hemiphthaloyl-α,ω-diaminoalkanoic acid analogues of aminopterin and 3′,5′-dichloroaminopterin. *J. Med. Chem.* **37**:2167–2174 (1994).

20. Rosowsky, A., H. Bader, G. Chen, C. E. Mota, C. Vaidya, and J. E. Wright. Dihydrofolate reductase inhibition and *in vitro* antitumor activity of nonpolyglutamatable analogs of aminopterin against human non-small cell lung carcinoma. *Proc. Am. Assoc. Cancer Res.* **35:** 376 (1995).
21. Rheinwald, J. G., and M. A. Beckett. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinoma. *Cancer Res.* **41:**1657–1663 (1981).
22. Rosowsky, A., J. E. Wright, C. A. Cucchi, J. A. Lippke, R. Tantravahi, T. J. Ervin, and E. Frei III. Phenotypic heterogeneity in cultured human head and neck squamous cell carcinoma lines with low-level methotrexate resistance. *Cancer Res.* **45:**6205–6212 (1985).
23. Frei, E., III, C. A. Cucchi, A. Rosowsky, R. Tantravahi, S. Bernal, T. J. Ervin, R. M. Ruprecht, and W. A. Haseltine. Alkylating agent resistance: *in vitro* studies with human cell lines. *Proc. Natl. Acad. Sci. USA* **82:**2158–2162 (1985).
24. Teicher, B. A., C. A. Cucchi, J. B. Lee, J. L. Flatow, A. Rosowsky, and E. Frei III. Alkylating agents: *in vitro* studies of cross-resistance patterns in human cell lines. *Cancer Res.* **46:**4379–4383 (1986).
25. Teicher, B. A., S. A. Holden, M. J. Kelley, T. C. Shea, C. A. Cucchi, A. Rosowsky, W. D. Henner, and E. Frei III. Characterization of a human squamous carcinoma cell line resistant to cis-diamminedichloroplatinum(II). *Cancer Res.* **47:**388–392 (1987).
26. Rosowsky, A., J. E. Wright, C. A. Cucchi, J. A. Flatow, D. Trites, B. A. Teicher, and E. Frei III. Collateral methotrexate resistance in a human head and neck squamous cell carcinoma line selected for resistance to cis-diamminedichloroplatinum (II). *Cancer Res.* **47:**5913–5918 (1987).
27. Rosowsky, A, H. Bader, and R. A. Forsch. Synthesis of the folylpolyglutamate synthetase inhibitor N^{α}-pteroyl-L-ornithine and its N^{δ}-benzoyl and N^{δ}-hemiphthaloyl derivatives, and an improved synthesis of N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine. *Pteridines* **1:**91–98 (1989).
28. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254 (1976).
29. Prendergast, N. J., T. J. Delcamp, P. L. Smith, and J. H. Freisheim. Expression and site-directed mutagenesis of human dihydrofolate reductase. *Biochemistry* **27:**3664–3671 (1988).
30. Kaufman, B. T. Methotrexate-agarose in the purification of dihydrofolate reductase. *Methods Enzymol.* **34:**272–281 (1974).
31. Hillcoat, B. L., P. F. Nixon, and R. L. Blakley. Effect of substrate decomposition on the spectrophotometric assay of dihydrofolate reductase. *Anal. Biochem.* **21:**178–189 (1967).
32. Domin B. A., Cheng, Y.-C., and M. T. Hakala. Properties of dihydrofolate reductase from a methotrexate-resistant subline of human KB cells and comparison with enzyme from KB parent cells and mouse S180 AT/3000 cells. *Mol. Pharmacol.* **21:**231–238 (1982).
33. Drake, J., Allegra, C. A., and B. A. Chabner. A re-evaluation of the competitive protein binding assay for methotrexate binding to dihydrofolate reductase. *Biochem. Pharmacol.* **35:**1212–1214 (1986).
34. Myers, C. E., M. E. Lippman, H. M. Eliot, and B. A. Chabner. Competitive protein binding assay for methotrexate. *Proc. Natl. Acad. Sci. USA* **72:**3683–3686 (1975).
35. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65:**55–63 (1983).
36. Sirotnak, F. M. Obligate genetic expression in tumor cells of a fetal membrane property mediating "folate" transport: biological significance and implications for improved therapy of human cancer. *Cancer Res.* **45:**3992–4000 (1985).
37. Jackson, R. C., L. I. Hart, and K. R. Harrap. Intrinsic resistance to methotrexate of cultured mammalian cells in relation to the inhibition kinetics of their dihydrofolate reductase. *Cancer Res.* **36:**1991–1997 (1976).
38. Sirotnak, F. M., P. L. Chello, J. I. DeGraw, J. R. Piper, and J. A. Montgomery. Membrane transport and the molecular basis for selective antitumor action of folate analogs, in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (A. C. Sartorelli, J. S. Lazo, and J. R. Bertino, eds.). Academic Press, New York, 349–384 (1981).
39. Appleman, J. R., N. Prendergast, T. J. Delcamp, J. H. Freisheim, and R. L. Blakley. Kinetics of the formation and isomerization of methotrexate complexes of recombinant human dihydrofolate reductase. *J. Biol. Chem.* **263:**10304–10313 (1988).
40. Allegra, C. J., J. C. Drake, J. Jolivet, and B. A. Chabner. Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolate polyglutamates. *Proc. Natl. Acad. Sci. USA* **82:**4881–4885 (1985).
41. Allegra, C. J., B. A. Chabner, R. Drake, D. Lutz, D. Rodbard, and J. Jolivet. Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. *J. Biol. Chem.* **260:**9720–9726 (1985).
42. Allegra, C. J., K. Hoang, G. C. Yeh, J. C. Drake, and J. Baram. Evidence for direct inhibition of *de novo* purine synthesis in human MCF-7 breast cells as a principal mode of metabolic inhibition by methotrexate. *J. Biol. Chem.* **262:**13520–13526 (1987).
43. Baram, J., B. A. Chabner, J. C. Drake, A. L. Fitzhugh, P. W. Sholar, and C. J. Allegra. Identification and biochemical properties of 10-formyldihydrofolate, a novel folate found in methotrexate-treated cells. *J. Biol. Chem.* **263:**7105–7111 (1989).
44. Jolivet, J., R. L. Schilsky, B. D. Bailey, J. C. Drake, and B. A. Chabner. Synthesis, retention, and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. *J. Clin. Invest.* **70:**351–360 (1982).
45. Frei, E., III, A. Rosowsky, J. E. Wright, C. A. Cucchi, J. A. Lippke, T. J. Ervin, J. Jolivet, and W. A. Haseltine. Development of methotrexate resistance in a human squamous cell carcinoma of the head and neck in culture. *Proc. Natl. Acad. Sci. USA* **81:**2873–2877 (1984).
46. Cowan, K. H., and J. Jolivet. A methotrexate-resistant human breast cancer cell line with multiple defects including diminished formation of methotrexate polyglutamates. *J. Biol. Chem.* **259:**10793–10800 (1984).
47. Curt, G. A., J. Jolivet, D. N. Carney, B. D. Bailey, J. C. Drake, N. J. Clendeninn, and B. A. Chabner. Determinants of the sensitivity of human small-cell lung cancer cell lines to methotrexate. *J. Clin. Invest.* **76:**1323–1329 (1985).
48. Pizzorno, G., E. Mini, M. Coronnello, J. J. McGuire, B. A. Moroson, A. R. Cashmore, T. J. Dreyer, T. Lin, T. Mazzei, P. Periti, and J. R. Bertino. Impaired polyglutamation of methotrexate as a cause of resistance in CCRF-CEM cells after short-term, high-dose treatment with this drug. *Cancer Res.* **48:**2149–2155 (1988).
49. Li, W. W., J. T. Lin, W. P. Tong, T. M. Tripett, M. F. Brennan, and J. R. Bertino. Mechanisms of natural resistance to antifolates in human soft tissue sarcomas. *Cancer Res.* **52:**1434–1438 (1992).
50. Samuels, L. L., D. M. Moccio, and F. M. Sirotnak. Similar differential for polyglutamation and cytotoxicity among various folate analogues in human and murine tumor cells *in vitro*. *Cancer Res.* **45:**1488–1495 (1985).
51. Newman, E. M., Lu, Y., M. Kashani-Sabet, V. Kesevan, and K. J. Scanlon. Mechanisms of cross-resistance to methotrexate and 5-fluorouracil in an A2780 human ovarian carcinoma subline resistant to cisplatin. *Biochem. Pharmacol.* **37:**443–447 (1988).
52. Fram, R. J., B. A. Woda, J. M. Wilson, and N. Robichaud. Characterization of acquired resistance to cis-diamminedichloroplatinum(II) in BE human colon carcinoma cells. *Cancer Res.* **50:**72–77 (1990).
53. Hill, B. T., S. A. Shellard, L. K. Hosking, W. C. M. Dempke, A. M. Fichtinger-Schepman, A. M. Tone, K. J. Scanlon, and W. D. H. Shelan. Characterization of a cisplatin-resistant human ovarian carcinoma cell line expressing cross-resistance to 5-fluorouracil but collateral sensitivity to methotrexate. *Cancer Res.* **52:**3110–3118 (1992).
54. Gewirtz, D. A., J. C. White, J. K. Randolph, and I. D. Goldman. Transport, binding, and polyglutamation of methotrexate in freshly isolated rat hepatocytes. *Cancer Res.* **40:**573–578 (1980).
55. Leszczynska, A., and E. Pfaff. Activation by reduced glutathione of methotrexate transport into isolated rat liver cells. *Biochem. Pharmacol.* **31:**1911–1918 (1982).

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