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Biochemical Studies on PT523, a Potent Nonpolyglutamatable Antifolate, in Cultured Cells

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SUMMARY

Studies on the mode of action of PT523 [N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine], a potent nonpolyglutamatable antifolate, were carried out in sensitive and resistant H35 rat hepatoma cell lines in culture, to compare it with other antifolates, including three dihydrofolate reductase (DHFR) inhibitors, i.e., methotrexate (MTX), γ-fluoro-MTX, and trimetrexate (TMQ), two thymidylate synthase inhibitors, i.e., N^{10}-propargyl-5,8-dideazafolate (PDDF) and 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolate (dmPDDF), and the glycinamide ribonucleotide formyltransferase inhibitor 5,10-dideaza-5,6,7,8-tetrahydrofolate. PT523 was the most active compound in this group against the parental H35 cells, with an IC_{50} ranging from 2.5 nM for 72 hr of treatment to 0.21 μM for 2 hr of treatment. Sublines resistant to MTX by virtue of a transport defect or a combination of defective transport and increased DHFR activity were resistant to PT523 and MTX but not to PDDF, whereas sublines resistant to fluoropyrimidines by virtue of increased thymidylate synthase activity were resistant to PDDF but not to PT523, TMQ, or MTX. Inhibition of H35 cell growth by PT523 was associated with a concentration- and time-related decrease in *de novo* dTMP and purine biosynthesis. Growth inhibition by PT523, MTX, and TMQ was prevented by leucovorin or a combination of thymidine (dThd) and hypoxanthine but not by dThd or hypoxanthine alone; in contrast, growth inhibition by dmPDDF was prevented by dThd alone. Intracellular reduced folate polyglutamate pools were markedly altered by PT523 treatment, with the most pronounced effect being an increase in 7,8-dihydrofolate mono- and polyglutamates and a decrease in 5,10-methylene-5,6,7,8-tetrahydrofolate mono- and polyglutamates, 5,6,7,8-tetrahydrofolate mono- and polyglutamates, and 10-formyl-5,6,7,8-tetrahydrofolate mono- and polyglutamates. This pattern was qualitatively similar to that observed with MTX and TMQ but different from that observed with dmPDDF or 5,10-dideaza-5,6,7,8-tetrahydrofolate, which resulted in little or no change in the folate species. Uptake of [^{3}H]MTX and [^{3}H]folinic acid, but not [^{3}H]folic acid, by H35 cells was inhibited in a dose-related manner by PT523, suggesting that penetration of the cell probably involves, at least in part, active transport by the MTX/reduced folate carrier. To determine whether the potent cellular effects of PT523 might be due to chemical or enzymic cleavage to N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine, a potent inhibitor of folylpolyglutamate synthetase, the formation of [^{3}H]MTX polyglutamates in CCRF-CEM lymphoblasts pulsed with [^{3}H]MTX after preincubation with PT523 was examined. The MTX polyglutamate profile was not markedly altered by PT523 treatment, suggesting that inhibition of DNA precursor synthesis and cell growth is not due to any significant perturbation of cellular folylpolyglutamate synthetase activity. The results of these studies support the view that cell growth inhibition by PT523 is due to DHFR inhibition.

PT523 (Fig. 1) is a novel antifolate with potent activity against tumor cell lines in culture, including cells that are 20–30-fold resistant to MTX by virtue of either a defect in transport or an increase in DHFR content (1–4). PT523 has been found to be 10–100-fold more potent than MTX against most of the cell lines in the *in vitro* human solid tumor panel of the National Cancer Institute, with IC_{50} values generally in the range of 0.1–10 nM (4). *In vivo* activity has also been observed against L1210 leukemia (3) and M5076 ovarian reticular sarcoma in mice (4). The latter tumor is inherently refractory to MTX, primarily because of impaired transport, along with a

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ABBREVIATIONS: PT523, N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine; MTX, methotrexate; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; APA-Orn, N^{α}-(4-amino-4-deoxypteroyl)-L-ornithine; dThd, thymidine; Hx, hypoxanthine; TMQ, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methylamino]quinazoline (trimetrexate); PDDF, N^{10}-propargyl-5,8-dideazafolate; dmPDDF, 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolate; DDATHF, 5,8-dideaza-5,6,7,8-tetrahydrofolate; dUrd, 2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylic acid; Gly, glycine; GARFT, glycinamide ribonucleotide formyltransferase; TS, thymidylate synthase; 5,10-CH_2H_4-$PteGlu_n$, 5,10-methylene-5,6,7,8-tetrahydrofolate mono- and polyglutamates; $H_4PteGlu_n$, 5,6,7,8-tetrahydrofolate mono- and polyglutamates; $H_2PteGlu_n$, 7,8-dihydrofolate mono- and polyglutamates; 10-$HCOH_4PteGlu_n$, 10-formyl-5,6,7,8-tetrahydrofolate mono- and polyglutamates; 10-$HCOH_2PteGlu_n$, 10-formyl-7,8-dihydrofolate mono- and polyglutamates; HBSS, Hanks' balanced salt solution; dIno, 2′-deoxyinosine; HPLC, high performance liquid chromatography.

modest elevation in DHFR content (5). The greater *in vitro* potency of PT523, relative to MTX, cannot be explained merely on the basis of tighter DHFR binding, because the IC_{50} values of the two compounds against purified human enzyme differ by <2-fold (1). Moreover, the absence of a glutamate side chain in PT523 excludes polyglutamylation as a basis for enhanced activity.

This paper presents the results of experiments with PT523 in hepatoma cell lines previously well characterized with respect to the action of MTX and other clinically used antifolates (6–13). Both dTMP biosynthesis and purine nucleotide biosynthesis were inhibited by PT523 and, as expected for a DHFR inhibitor, tetrahydrofolate cofactor pools were depleted, whereas the dihydrofolate pool was expanded. These effects were dose and time dependent and were seen at lower concentrations of PT523 than MTX. Uptake of MTX and folinic acid, but not folic acid, was decreased by PT523, suggesting that the latter behaves more like classical than nonclassical antifolates in terms of transport across the cell membrane. Also presented are the results of experiments suggesting that intracellular loss of the phthaloyl group, with formation of the potent FPGS inhibitor APA-Orn (14), is probably not responsible for the high activity of PT523.

Materials and Methods

Reagents. Swim's S-77 medium, horse serum, and fetal calf serum were obtained from GIBCO (Grand Island, NY). Plastic tissue culture dishes were from Falcon/Becton Dickinson (Lincoln Park, NJ). dThd, Hx, and folic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Folinic acid [(6*S*)-5-formyl-5,6,7,8-tetrahydrofolic acid], as the calcium salt, and MTX, as the disodium salt, were generous gifts from Lederle Laboratories (Pearl River, NY). Folinic acid, folic acid, and MTX were purified by ion exchange column chromatography on DEAE-cellulose (6). PT523 was synthesized at the Dana-Farber Cancer Institute by Dr. Henry Bader and was purified to >95% purity by preparative HPLC on C_{18} silica gel, as described (2). TMQ, as the glucuronate salt, was kindly provided by Drs. David Fry and Robert Jackson (Warner Lambert, Ann Arbor, MI), PDDF (CB3717) and dmPDDF (ICI 198583) by Dr. M. G. Nair (University of South Alabama, Mobile, AL), DDATHF (as the 6*R*/6*S* mixture) by Dr. Suresh Kerwar (Lederle), and γ-fluoro-MTX (as the *erythro*/*threo* mixture) by Dr. James Coward (University of Michigan, Ann Arbor, MI). [3′,5′,7-^{3}H]MTX, (6*S*)-5-formyl-5,6,7,8-[3′,5′,7,9-^{3}H]tetrahydrofolic acid ([^{3}H]folinic acid), [3′,5′,7,9-^{3}H]folic acid, [6-^{3}H]dUrd, and [6-^{3}H]FdUMP were purchased from Moravek Biochemicals (Brea, CA). [2-^{14}C]Gly was from NEN Research Products (Boston, MA). All labeled compounds were >99% radiochemically pure by thin layer chromatography. *Lactobacillus casei* TS and chicken liver GARFT for the folate pool assays were gifts from Dr. Gladys Maley (New York State Department of Health, Albany, NY) and Dr. John Whiteley (Scripps Clinic and Research Foundation, La Jolla, CA), respectively.

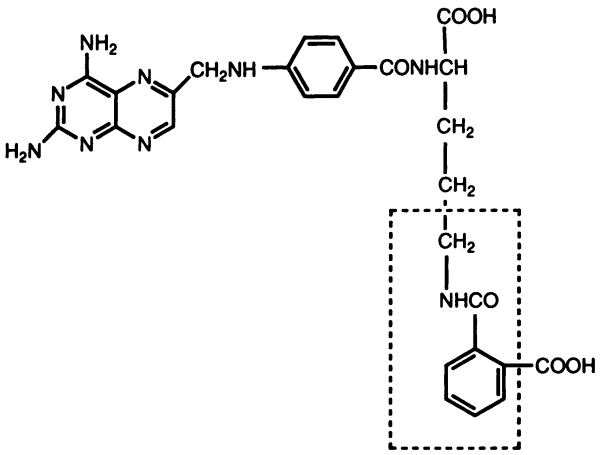

Fig. 1. Structure of PT523. *Dotted lines*, area of major structural difference between this molecule and MTX.

Cell culture. Monolayers of H35 cells (6), the MTX-resistant sublines $H35R_{0.3}$ (selected in medium containing 0.3 μM MTX; transport defect) (6) and $H35R_{10}$ (selected in medium containing 10 μM MTX; transport defect with 11-fold increase in DHFR activity) (7), and the PDDF-resistant and dmPDDF-resistant subline H35FF (selected in medium containing 0.1 μM 5-fluoro-2′-deoxyuridine and 25 μM folinic acid; 60–70-fold increase in TS activity) (12) were grown at 37° in a 5% CO_2 atmosphere in Swim's S-77 medium supplemented with 20% horse serum, 5% fetal calf serum, and 4 mM L-glutamine. All cell lines were determined to be free of *Mycoplasma* (8) and were grown in the absence of the selection agent(s) for 1 week before use, for all experiments. CCRF-CEM human lymphoblasts were grown at 37° in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, L-glutamine, and antibiotics, as described previously (15).

Growth inhibition and cytotoxicity assays. In the growth inhibition assays, cells were plated in 96-well dishes at a density of 1×10^4 cells/200 μl of medium/well and were counted at designated times by the modified methylene blue staining procedure described previously (11). The cells were counted either after 72 hr of continuous drug treatment or after a protocol of pulse treatment consisting of 24 hr without drug followed by 2, 4, 6, or 24 hr with drug and finally 48 hr without drug. In the cytotoxicity assays based on clonal growth, cells were plated at 2×10^5 cells/60-mm dish, appropriate drug dilutions were added 3 hr later, and incubation was continued for 48 hr. The cells were then trypsinized and replated at 800 cells/4 ml of medium/60-mm plate in drug-free medium. After 10 days of incubation at 37°, the colonies (>75 cells) were washed, stained with crystal violet, and counted.

Protection studies. To determine whether H35 cells can be protected from the growth-inhibitory effect of PT523 and other antifolates, H35 cells were grown for 72 hr in the presence of enough drug to produce approximately 90% inhibition of growth in the absence of protection or with the same concentration of drug plus 20 μM dThd, 50 μM Hx, 20 μM dThd and 50 μM Hx, or 100 μM folinic acid in the medium.

Thymidylate and purine nucleotide biosynthesis. Incorporation of [^{3}H]dUrd and [^{14}C]Gly, respectively, into the acid-precipitable DNA of H35 cells was used to assess the effect of PT523 and other antifolates on dTMP and purine synthesis. [^{3}H]dUrd and [^{14}C]Gly incorporation was measured according to a previously described adaptation of literature methods (9). Cells were either treated with PT523 or other antifolates for the last 4 hr or 18 hr of a 72-hr incubation. In both protocols, [^{3}H]dUrd was added for the last 0.5 hr and [^{14}C]Gly for the last 2 hr of the incubation. Total cell protein in this and all other experiments was assayed according to the method of Lowry *et al.* (16).

Reduced folate pools. A previously described procedure (12, 17, 18) based on the formation of a [^{3}H]FdUMP-TS-5,10-$CH_2H_4PteGlu_n$ ternary complex was used to determine how PT523 alters reduced folate pool sizes as a function of time. The H35 cells in these experiments were grown in standard medium, and the antifolate was added for either the last 4 hr or the last 18 hr of the incubation. In the case of MTX, the drug was added after 54 hr and was either kept in the medium for 18 hr or removed after 2 hr followed by 16 hr of in drug-free medium. The 5,10-$CH_2H_4PteGlu_n$ in cell lysates was measured directly by adding excess *L. casei* TS and [^{3}H]FdUMP to the cell extract. The $H_4PteGlu_n$ was determined by subtracting the amount of this complex from the amount formed in the presence of excess formaldehyde, and the $H_2PteGlu_n$ was determined by subtracting the amount of complex formed in the presence of excess formaldehyde

without addition of DHFR from the amount formed with DHFR added. 10-$HCOH_4PteGlu_n$ was measured after conversion to $H_4PteGlu_n$ in the presence of added GARFT, and 10-$HCOH_2PteGlu_n$ was measured after conversion to $H_4PteGlu_n$ in the presence of both GARFT and DHFR. The molar concentration of each reduced folate in the cells was calculated by using the previously determined cellular water volume of 3.15 μl/mg of protein for H35 cells (6). Control experiments were performed for each reduced folate with polyglutamate standards containing five or seven glutamate residues, and recoveries were estimated to be >75%.

Drug uptake. H35 cells were grown in medium with 20% horse serum and 5% fetal calf serum for 96 hr and then in serum- and folate-free medium for the next 24 hr, to decrease cellular folates. The cells were then washed and incubated at 37° in HBSS in the presence of various concentrations of PT523 and either 5 μM [^{3}H]MTX for 4 min, 2 μM [^{3}H]folinic acid for 10 min, or 5 μM [^{3}H]folic acid for 20 min. Uptake was terminated by washing the cells several times with phosphate-buffered saline at 0°. The cells were then extracted with 1 N NaOH, aliquots of the digest were assayed for protein (16), and total radioactivity was determined by liquid scintillation counting.

Effects of PT523 on MTX uptake and polyglutamylation. H35 cells were grown in Swim's S-77 medium containing 20% horse serum and 5% fetal calf serum for 96 hr and then in folate-free medium with insulin (10 milliunits/ml) for an additional 24 hr (total time, 120 hr). The resultant stationary cells were then treated sequentially with PT523 (0.2 or 2.0 μM) for 2 hr or 24 hr and with 10 μM [^{3}H]MTX for 4 hr (total treatment time, 6 hr or 28 hr). Cytosols were extracted and the MTX polyglutamates were analyzed by HPLC on a C_{18} silica gel column, with a linear 0–12% acetonitrile gradient in 0.1 N sodium acetate (11). For the analysis of MTX polyglutamylation in CCRF-CEM lymphoblasts, late-logarithmic phase cells in Dulbecco's modified Eagle's medium were incubated for 24 hr with PT523 (0.2 or 2.0 μM), along with 10 μM dThd and 100 μM dIno to maintain viability. Control cultures contained dThd and dIno but no PT523. At the end of the 24-hr incubation, the cells were treated with 2.0 μM [^{3}H]MTX for another 2 hr or 24 hr (total treatment time, 26 hr or 48 hr). Cytosols were analyzed for unchanged [^{3}HMTX and [^{3}H]MTX polyglutamates by HPLC on a C_{18} silica gel column, with a linear 0–5% acetonitrile gradient in 0.1 M ammonium acetate, pH 6.0 (15).

Results

H35 cell growth inhibition by PT523 and other antifolates. The ability of PT523 to inhibit the growth of parental H35 rat hepatoma cells was compared with that of MTX, TMQ, γ-fluoro-MTX, and dmPDDF (ICI 198583). TMQ was chosen as an example of a glutamate-lacking nonclassical antifolate whose action is independent of the ability of the cell to form polyglutamates (9–12, 19–22), γ-fluoro-MTX as an example of a classical antifolate whose glutamate side chain is altered to prevent polyglutamylation (20–24), and dmPDDF as an example of a classical antifolate targeted against TS rather than DHFR (25, 26). IC_{50} values for growth inhibition of H35 cells during continuous incubation with PT523, MTX, TMQ, γ-fluoro-MTX, and dmPDDF for 72 hr are given in Table 1; IC_{50} values comparing the effect of shorter treatment (2, 4, 6, or 24 hr) with PT523, MTX, and γ-fluoro-MTX are likewise shown. The IC_{50} of PT523 ranged from 0.0025 μM (72 hr) to 0.21 μM (2 hr), whereas the IC_{50} of MTX ranged from 0.015 μM (72 hr) to 0.78 μM (2 hr) and that of γ-fluoro-MTX ranged from 0.12 μM (72 hr) to 700 μM (2 hr). The IC_{50} values for TMQ and dmPDDF in the 72-hr incubation were 0.0075 and 0.055 μM, respectively. Thus, PT523 was more potent in short as well as long incubations.

The IC_{50} of PT523 in clonal growth experiments was found to be 1.6 nM versus 18 nM for MTX (data not shown). Thus, PT523 was more cytotoxic than MTX as well as more cytostatic.

TABLE 1

Activity of PT523 and other antifolates against cultured H35 rat hepatoma cells

Cells were incubated for the indicated times in the presence of drugs (see Materials and Methods). Data shown are the means of two or more replicate experiments and have a limit of variability of <15%.

Compound	IC_{50}[a]				
	72 hr[b]	24 hr	6 hr	4 hr	2 hr
	μM				
PT523	0.0025	0.016	0.074	0.17	0.21
MTX	0.015	0.037	0.14	0.36	0.78
γ-Fluoro-MTX	0.12	0.4	10	34	700

[a] Cells exposed to the antifolates for <72 hr were kept in drug-free medium for 24 hr, treated with drug for 2, 4, 6, or 24 hr, and returned to drug-free medium for another 48 hr.

[b] The IC_{50} values for TMQ and dmPDDF (72-hr incubation) were 0.0075 and 0.055 μM, respectively.

TABLE 2

Effect of PT523 on the growth of resistant sublines of H35 rat hepatoma

Cells were incubated for 72 hr in the presence of drugs (see Materials and Methods). Data shown are the mean of two or more replicate experiments and have a limit of variability of <15%.

Compound	IC_{50}			
	H35	$H35R_{0.3}$	$H35R_{10}$	H35FF
	μM			
PT523	0.002 (1.0)[a]	3.0 (1,500)	50 (25,000)	0.003 (1.5)
MTX	0.01 (1.0)	1.0 (100)	100 (10,000)	0.01 (1.0)
TMQ	0.0075 (1.0)	0.009 (1.2)	0.8 (100)	0.002 (0.3)
PDDF	3.9 (1.0)	8.0 (2.1)	13.3 (3.3)	>1,400 (>360)

[a] Numbers in parentheses are the approximate fold resistance of each compound, relative to the H35 parental line (1.0).

Growth inhibition of resistant H35 sublines by PT523 and other antifolates. The resistance patterns of $H35R_{0.3}$, $H35R_{10}$, and H35FF cells with respect to PT523, MTX, TMQ, and PDDF were compared. The $H35R_{0.3}$ subline of H35 rat hepatoma is 100-fold resistant to MTX because of a transport defect (6), the $H35R_{10}$ subline is 10,000-fold resistant to MTX by virtue of impaired transport along with increased DHFR activity (7), and the H35FF subline is sensitive to MTX but >360-fold resistant to PDDF by virtue of increased TS activity (12). As shown in Table 2, the $H35R_{0.3}$ and $H35R_{10}$ sublines were both resistant to PT523, with IC_{50} values of 3 and 50 μM versus 0.003 μM for the parental H35 cells, but were only slightly resistant to PDDF. In contrast, only the $H35R_{10}$ subline was resistant to TMQ (IC_{50} of 0.8 versus 0.0075 μM). The H35FF subline was not resistant to PT523 or the other DHFR inhibitors (MTX and TMQ) but was resistant to PDDF (IC_{50} >1400 versus 3.9 μM).

Protection of H35 cells from growth inhibition by PT523. The ability of dThd, Hx, and folinic acid to protect H35 rat hepatoma cells from the growth-inhibitory effects of a 72-hr treatment with PT523 and several other antifolates was compared. The antifolates were used at concentrations 10-fold higher than their IC_{50} values for 72-hr continuous exposure. These concentrations were 20 nM for PT523 (83% inhibition), 100 nM for MTX (95% inhibition), 75 nM for TMQ (91% inhibition), and 550 nM for dmPDDF (88% inhibition). Thus, the order of potency of the antifolates was the same for 85–95% inhibition as for 50% inhibition, i.e., PT523 > TMQ > MTX > dmPDDF. As indicated in Table 3, 20 μM dThd

TABLE 3

Protection of H35 rat hepatoma cells from PT523 and other antifolates

Cells were cultured for 72 hr (see Materials and Methods). Protection is expressed as a percentage of the growth of control cultures containing the protectant but no drug. dThd, Hx, and folinic acid concentrations were 20, 50, and 100 μM, respectively. The results shown are the means of two or more replicate experiments, with a limit of variability of <15%.

Compound	Cell growth				
	No protectant	dThd	Hx	dThd + Hx	Folinic acid
	% of control				
PT523 (20 nM)	7	22	7	98	88
MTX (100 nM)	5	20	20	100	91
TMQ (75 nM)	9	57	10	97	83
dmPDDF (550 nM)	12	98	0	100	99

completely protected the cells from dmPDDF but was only partially protective for PT523, MTX, and TMQ. Hx (50 μM) was essentially devoid of protective activity with all the antifolates, but a combination of 20 μM dThd and 50 μM Hx was fully protective, as was 100 μM folinic acid. Full protection was also achieved with 10μM folinic acid, whereas half-maximal protection was achieved at a concentration of 0.5 μM (data not shown).

Effects of PT523 on *de novo* DNA precursor synthesis in H35 cells. Incorporation of [^{3}H]Urd and [^{14}C]Gly into the acid-insoluble fraction of cells provides a convenient measure of dTMP and purine nucleotide biosynthesis in the presence of antifolates. Fig. 2 compares the effect of treating H35 rat hepatoma cells with various concentrations of PT523 for the last 4 hr (Fig. 2A) or last 18 hr (Fig. 2B) of a 72-hr incubation. The IC_{50} values in both assays were approximately 10 nM for the 4-hr treatment and 4 nM for the 18-hr treatment. The concentration of PT523 needed to achieve >90% inhibition was 40 nM after 4 hr and 10 nM after 18 hr. Thus, the effect of PT523 on dTMP and purine nucleotide synthesis was both dose and time dependent.

Table 4 presents the results of a similar experiment comparing the potencies of PT523, MTX, TMQ, dmPDDF, and DDATHF as inhibitors of dTMP and purine nucleotide synthesis in H35 cells. dmPDDF and DDATHF were chosen as examples of antifolates acting only at the level of dTMP (25, 26) and purine biosynthesis (9, 27, 28), respectively. Data on the activity of dmPDDF and DDATHF against H35 cell lines have not been published previously. The IC_{50} of PT523 for [^{3}H]dUrd incorporation was 0.009 μM versus 0.22 μM for MTX, 0.02 μM for TMQ, 0.18 μM for dmPDDF, and >20 μM for DDATHF. The IC_{50} of PT523 for [^{14}C]Gly incorporation was 0.012 versus 0.24 μM for MTX, 0.08 μM for TMQ, 10 μM for dmPDDF, and 1.5 μM for DDATHF. Thus, the order of potency of these antifolates as inhibitors of *de novo* DNA precursor synthesis in H35 cells was PT523 > TMQ > MTX = dmPDDF > DDATHF for dTMP synthesis and PT523 > TMQ > MTX > DDATHF > dmPDDF for purine nucleotide synthesis. The IC_{50} difference between PT523 and the TS inhibitor dmPDDF as dTMP synthesis inhibitors was approximately 800-fold, and the IC_{50} difference between PT523 and the GARFT inhibitor DDATHF as purine nucleotide synthesis inhibitors was >2000-fold.

Effects of PT523 and other antifolates on reduced folate pools in H35 cells. A comparison of the effects of PT523 and other antifolates on reduced folate pools in H35 cells is presented in Table 5. The concentrations of PT523 and TMQ sufficient to cause at least 90% inhibition of dTMP and purine nucleotide synthesis during short treatment were the same (40 nM, 4 hr), whereas those of MTX (10 μM, 2 hr), dmPDDF (0.5 μM, 4 hr), and DDATHF (20 μM, 4 hr) were higher. When drug treatment was extended to 18 hr, these IC_{90} values decreased to 5 nM for PT523, 20 nM for TMQ, and 0.2 μM for dmPDDF but did not change for DDATHF. Treatment of the cells with TMQ at the IC_{90} for dTMP and purine nucleotide synthesis decreased the total pool of 5,10-$CH_2H_4PteGlu_n$, $H_4PteGlu_n$, and 10-$HCOPteGlu_n$ from 8.9 μM to 0.7 μM after the 4-hr treatment and to 0.55 μM after the 18-hr treatment, whereas 2-hr treatment with MTX at the IC_{90}

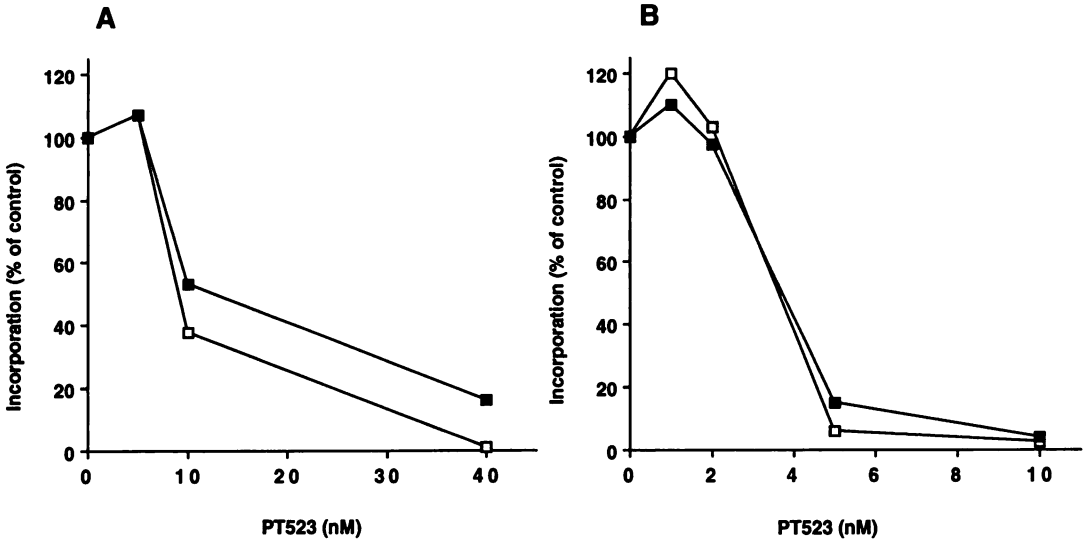

Fig. 2. Effect of PT523 on thymidylate and purine nucleotide synthesis in H35 rat hepatoma cells. Cells were exposed to drug for the last 4 hr (A) or 18 hr (B) of a 72-hr culture, and [^{3}H]dUrd (□) and [^{14}C]Gly (■) incorporation was determined (see Materials and Methods). Each *point* is the average of several measurements; error bars were omitted for the sake of clarity. [^{3}H]dUrd and [^{14}C]Gly incorporation in untreated controls was 264 ± 14.9 × 10^3 and 16.3 ± 1.41 × 10^3 dpm/mg of protein, respectively.

TABLE 4

Effect of PT523 and other antifolates on thymidylate and purine nucleotide biosynthesis in cultured H35 rat hepatoma cells

Incorporation of [^{3}H]dUrd and [^{14}C]Gly into the acid-precipitable fraction of H35 cells was determined after treatment with each compound for the last 4 hr of a 72-hr culture (see Materials and Methods). The data shown are means of two independent replicate experiments and have a limit of variability of <15%.

Compound	IC_{90}	
	[^{3}H]dUrd	[^{14}C]Gly
	μM	
PT523	0.009 (1.0)[a]	0.012 (1.0)
TMQ	0.02 (0.5)	0.08 (0.2)
MTX	0.22 (0.04)	0.24 (0.05)
dmPDDF	0.18 (0.05)	10 (0.001)
DDATHF	>20 (<0.0005)	1.5 (0.008)

[a] Numbers in parentheses indicate approximate relative potencies (PT523 = 1.0).

decreased this pool to 1.2 μM after 2-hr incubation in drug-free medium and to 0.9 μM when the latter period was extended to 16 hr. The $H_2PteGlu_n$ pool in the TMQ-treated cells increased from 0.7 μM to 4.0 μM at 4 hr and to 4.3 μM at 18 hr. Treatment with dmPDDF did not significantly change the pool size of the three tetrahydrofolate cofactors at either 4 hr or 18 hr but did expand the $H_2PteGlu_n$ pool slightly, from 0.7 to 1.5 μM, when used at the IC_{90} (18 hr) for dTMP synthesis. A detectable concentration of 10-$HCOH_2PteGlu_n$ was also produced under these conditions. DDATHF had little effect on any of the reduced folate cofactor pools even at the IC_{90} (18 hr) for purine nucleotide synthesis.

Effects of PT523 on MTX, folinic acid, and folic acid uptake. Although detailed kinetic analyses were not done, a preliminary experiment comparing the ability of PT523 to inhibit the uptake of [^{3}H]MTX, [^{3}H]folinic acid, and [^{3}H]folic acid into H35 cells was carried out to determine whether PT523 uses the MTX/reduced folate transport pathway. As indicated in Fig. 3, uptake of 5 μM [^{3}H]MTX during a 4-min incubation or of 2 μM [^{3}H]folinic acid during a 10-min incubation at 37° in serum-free HBSS was inhibited in a dose-dependent manner, with IC_{50} values of 4.0 and 4.25 μM, respectively; in contrast, uptake of [^{3}H]folic acid (5 μM, 20 min) was unaffected by up to 50 μM PT523.

Effects of PT523 on MTX uptake and polyglutamylation. This experiment was carried out to assess whether PT523 might be acting as a prodrug from which the hemiphthaloyl group is removed via intracellular cleavage, to give the potent FPGS inhibitor APA-Orn (14). PT523 was added at two concentrations (0.2 and 2.0 μM) to either stationary H35 cells or CCRF-CEM cells in late-logarithmic phase of growth, with dThd and dIno added in the latter instance to prevent cell killing as a result of DHFR inhibition. After 2 hr or 24 hr, the capacity of both cell lines to polyglutamylate MTX was determined *in situ*. The 24-hr pretreatment period with PT523 was chosen because we felt that this should be long enough for loss of the hemiphthaloyl group to occur by either chemical or enzymic hydrolysis. As shown in Fig. 4, addition of 10 μM [^{3}H]MTX to H35 cells for the last 4 hr of a 6-hr incubation or the last 24 hr of a 26-hr incubation with 2.0 μM PT523 resulted in some reduction in total MTX uptake but no striking effect on the distribution of polyglutamates, as measured by changes in each species relative to the total or by changes in each species in treated versus nontreated cells. In a similar experiment using CCRF-CEM cells to which 2.0 μM [^{3}H]MTX was added for the last 2 hr of a 26-hr incubation or the last 24 hr of a 48-hr incubation with 2.0 μM PT523, a somewhat greater reduction in total MTX uptake was seen, but again with no striking change in the distribution of polyglutamates. With both cell lines, the effect of 2.0 μM PT523 treatment was only marginally greater than the effect of 0.2 μM PT523 treatment. These results made it seem very unlikely that hydrolysis of PT523 within cells forms enough APA-Orn to inhibit FPGS.

Discussion

The results of this study support previous findings that PT523 is a very potent antifolate despite the lack of a glutamate

TABLE 5

Effect of PT523 and other antifolates on reduced folate pools in H35 rat hepatoma cells

Cells were incubated in the presence of the antifolate for the last 4 or 18 hr of a 72-hr culture, except for MTX, which was present for only 2 hr in the short-treatment experiment (see Materials and Methods). The concentration of each compound was the minimum level required for >90% inhibition of thymidylate and purine nucleotide biosynthesis. Data shown are the means ± standard deviations for three independent experiments.

Compound	Concentration				
	Tetrahydrofolates			Dihydrofolates	
	5,10-$CH_2PteGlu_n$	$H_4PteGlu_n$	10-$HCOH_4PteGlu_n$	$H_2PteGlu_n$	10-$HCOH_2PteGlu_n$
			μM		
None	2.1 ± 0.23 (1.0)[a]	2.0 ± 0.17 (1.0)	4.8 ± 0.86 (1.0)	0.7 ± 0.06 (1.0)	—[b]
4-hr treatment					
PT523 (0.04 μM)	0.6 ± 0.16 (0.3)	0.5 ± 0.18 (0.25)	1.2 ± 0.1 (0.3)	2.8 ± 0.2 (4.0)	—
TMQ (0.04 μM)	0.2 ± 0.01 (0.1)	0.2 ± 0.01 (0.10)	0.3 ± 0.02 (0.06)	4.0 ± 0.48 (5.7)	0.1 ± 0.01
MTX (10 μM)[c]	0.4 ± 0.02 (0.2)	0.3 ± 0.01 (0.15)	0.5 ± 0.02 (0.1)	4.7 ± 0.51 (6.7)	0.1 ± 0.02
dmPDDF (0.5 μM)	1.8 ± 0.21 (0.9)	1.4 ± 0.11 (0.70)	5.8 ± 1.21 (1.2)	0.1 ± 0.01 (0.1)	—
DDATHF (20 μM)	2.3 ± 0.24 (1.1)	2.1 ± 0.17 (1.0)	3.6 ± 0.48 (0.8)	0.6 ± 0.04 (0.9)	—
18-hr treatment					
PT523 (0.005 μM)	0.22 ± 0.05 (0.1)	0.24 ± 0.04 (0.1)	0.2 ± 0.09 (0.04)	4.7 ± 1.2 (6.7)	—
TMQ (0.02 μM)	0.2 ± 0.01 (0.1)	0.15 ± 0.01 (0.1)	0.2 ± 0.01 (0.04)	4.2 ± 0.61 (6.0)	0.1 ± 0.01
MTX (10 μM)[d]	0.3 ± 0.02 (0.1)	0.2 ± 0.01 (0.1)	0.4 ± 0.02 (0.08)	4.6 ± 0.55 (6.6)	0.3 ± 0.02
dmPDDF (0.2 μM)	2.5 ± 0.29 (1.2)	1.8 ± 0.14 (0.9)	3.9 ± 0.31 (0.8)	1.5 ± 0.09 (2.1)	0.7 ± 0.03
DDATHF (20 μM)	2.7 ± 0.38 (1.3)	2.4 ± 0.19 (1.2)	4.2 ± 0.50 (0.9)	0.4 ± 0.02 (0.6)	—

[a] Numbers in parentheses are expressed as an approximate fold change over untreated controls (1.0).
[b] —, not detected.
[c] MTX was added after 68 hr of culture, kept in the medium for 2 hr, and removed for the balance of the 72-hr incubation.
[d] MTX was added after 54 hr of culture, kept in the medium for 2 hr, and removed for the balance of the 72-hr incubation.

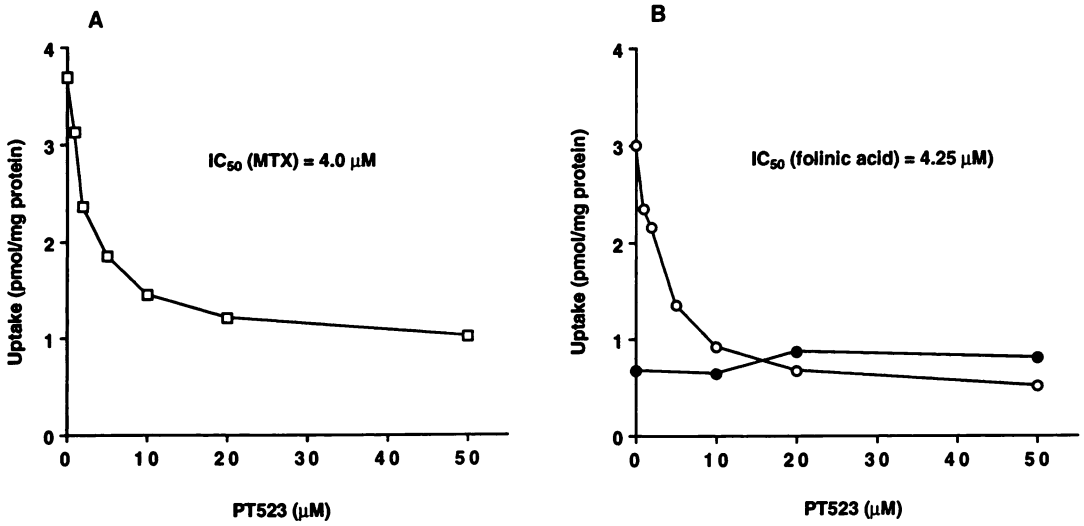

Fig. 3. Inhibition of the uptake of MTX (A), folinic acid (B), and folic acid (B) by H35 rat hepatoma cells in the presence of PT523. Cells were grown in medium with 20% horse serum and 5% fetal calf serum for 96 hr and then in serum-free medium for 24 hr to decrease cellular folates. Uptake of labeled MTX (□), folic acid (●), and folinic acid (○) was determined in HBSS as described in Materials and Methods.

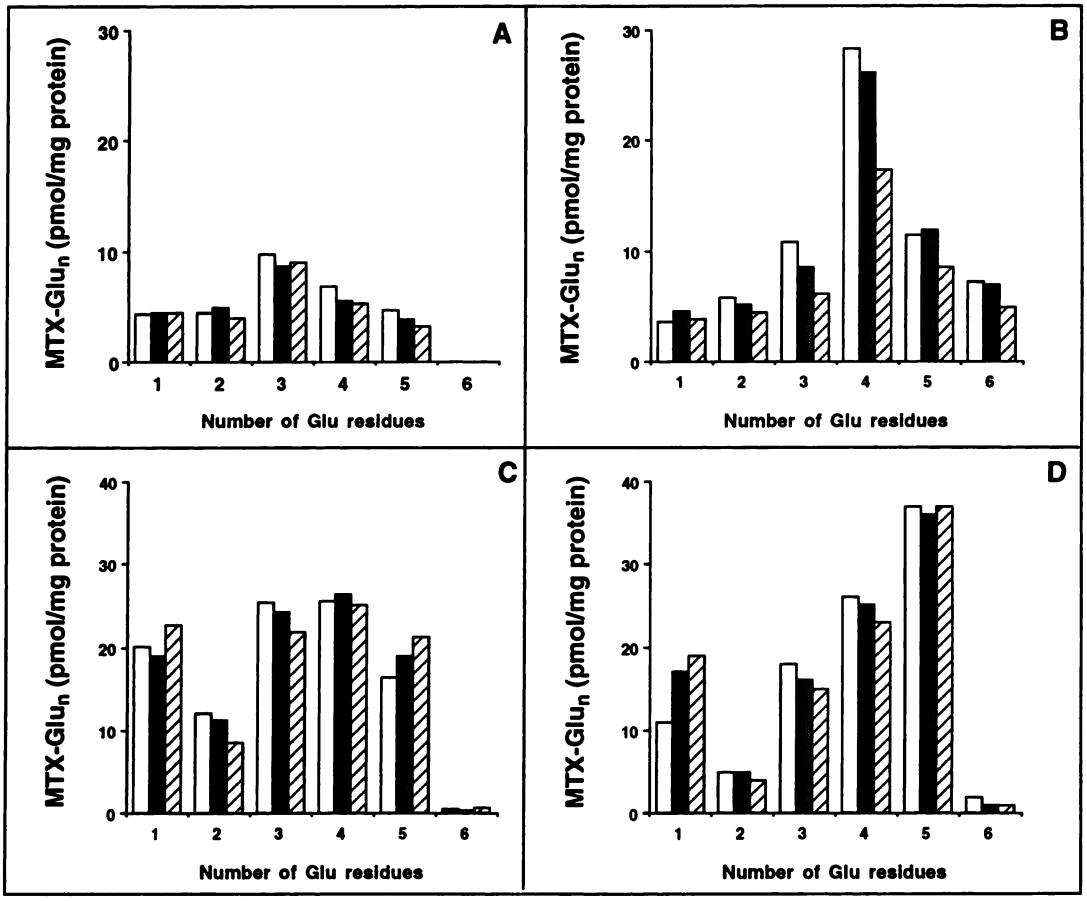

Fig. 4. Lack of effect of PT523 on MTX polyglutamylation in H35 cells and CCRF-CEM cells. A and B, H35 cells in stationary phase were incubated for 2 hr without drug (□) or with PT523 [0.2 μM (■) or 2.0 μM (▨)], after which [^{3}H]MTX (10 μM) was added for another 4 hr (A) or 24 hr (B). C and D, CCRF-CEM cells in late logarithmic phase were incubated for 2 hr (C) or 24 hr (D) without drug (□) or with PT 523 [0.2 μM (■) or 2.0 μM (▨)], after which [^{3}H]MTX (2.0 μM) was added for another 24 hr. Unchanged [^{3}H]MTX and [^{3}H]MTX polyglutamates were analyzed by HPLC (see Materials and Methods).

side chain (1, 3, 4). In H35 cell growth inhibition assays in which drug was present continuously for 72 hr, PT523 was 6-fold more potent than MTX and 22-fold more potent than dmPDDF, despite the fact that the latter compound forms polyglutamates whose activity against enzymes of one-carbon metabolism can be as much as 100-fold greater than that of the parent drug (29, 30). Moreover, PT523 appeared to retain superior activity even during shorter incubations. For example, whereas the nonpolyglutamable analogue γ-fluoro-MTX showed a nearly 6000-fold decrease in potency when treatment was shortened from 72 hr to 2 hr, this decrease for MTX and PT523 was only 52-fold and 84-fold, respectively. Thus, PT523 more closely resembled MTX than γ-fluoro-MTX in this respect, even though it cannot form polyglutamates. It is worth noting that PT523 was more active than MTX even when treatment was for only 2 hr, at which time noneffluxing MTX polyglutamates are already present (8, 31).

Resistance patterns among several H35 sublines were examined with a view to gaining some information about the uptake mechanism and target enzymes of PT523. We reasoned that if, for example, a subline resistant to MTX by virtue of a defect in active transport were still sensitive to PT523, as has been shown for TMQ (9, 19–22, 32–34), then an alternative transport route for PT523 might be involved. By the same token, if a subline resistant to fluoropyrimidines by virtue of increased TS activity were cross-resistant to PT523, then it might be concluded that PT523 owes at least part of its activity to TS inhibition. As shown in Table 2, both the 100-fold MTX-resistant $H35R_{0.3}$ cells and the 1000-fold MTX-resistant $H35R_{10}$ cells were more resistant to PT523 than to MTX. Moreover, the $H35R_{0.3}$ cells remained sensitive to TMQ and to PDDF. Because levels of DHFR and TS in $H35R_{0.3}$ cells are normal, these results confirmed that PT523 resistance in these cells is not due to increased DHFR or TS activity. The >1400-fold resistance of H35FF cells to PDDF and their lack of resistance to PT523 as well as to MTX and TMQ indicated that PT523 does not act at the level of TS and that it might be possible to use PT523 synergistically with TS inhibitors that do not compete with it at the level of membrane transport. Such compounds could include either fluoropyrimidines (35) or lipophilic nonclassical TS inhibitors targeted to the 5,10-$CH_2H_4PteGlu_n$ binding site (Refs. 36 and 37; see also references cited in Ref. 37).

The fact that $H35R_{10}$ cells were more resistant than $H35R_{0.3}$ cells to both PT523 and MTX (Table 2) was expected, given that this subline has increased DHFR activity as well as a transport defect (7). However, having previously found that another transport-defective cell line, SCC15/R1, derived by stepwise selection with MTX from a human squamous cell carcinoma of the head and neck (SCC15) (38), was collaterally sensitive to PT523 (1), we were surprised to find resistance in $H35R_{0.3}$ cells to be so high. However, it should be noted that the SCC15/R1 cells in which collateral sensitivity was observed were only 15-fold resistant to MTX, whereas an approximately 200-fold resistant cell line, derived from human leukemic lymphoblasts (CCRF/CEM), was also resistant to PT523 (1). Moreover, whereas the rate of initial MTX influx was lower in the SCC15/R1 subline than in the parental SCC15 cells, the plateau levels after 2 hr were almost the same. In contrast, the MTX influx rates in CEM and CEM/MTX cells were similar, but the plateau level in the resistant cells was much lower. Thus, it appears that the ability of PT523 to overcome transport-based MTX resistance depends on the degree of resistance and the kinetic characteristics of MTX transport in a particular cell line. Although our understanding of the determinants of low level (<20-fold) versus higher level (>100-fold) transport resistance in cultured cells is far from complete, it is possible that the level of resistance reflects a set of qualitative as well as quantitative differences among multiple components of the transport phenotype, such as the amount and binding affinity of membrane-associated carrier proteins, the fluidity of the plasma membrane, and the kinetics of energy-dependent efflux. It is also important to stress that only low level resistance is likely to be relevant to the use of MTX in patients, because tumors tend to become clinically refractory long before 100-fold resistance to the drug has a chance to develop. Thus, low activity of PT523 against cells with >100-fold transport-based resistance would not necessarily exclude it from consideration as a clinical candidate.

The ability of H35 rat hepatoma cells to be protected from PT523 by dThd, Hx, and folinic acid was examined to establish that growth inhibition is not due to something other than an antifolate effect. As shown in Table 3, H35 cells treated with the DHFR inhibitors PT523, MTX, and TMQ at concentrations that inhibited growth by >95% in a 72-hr culture were only partially protected by 20 μM dThd; in contrast, growth inhibition by the pure TS inhibitor dmPDDF was completely prevented. Thus, TS inhibition could not, by itself, explain the potent action of PT523. The presence of 50 μM Hx alone afforded only minimal protection (<20%) from PT523, MTX, or TMQ and none at all from dmPDDF. Thus, the effect of PT523 on cell growth could not be due exclusively to purine depletion. On the other hand, almost complete protection from PT523 occurred with 20 μM dThd and 50 μM Hx, showing that processes other than one-carbon metabolism do not contribute in any major way to the effect of PT523 on cell growth. Whereas complete protection was also achieved with 100 μM folinic acid, it should be noted that at this high concentration folinic acid is likely not only to circumvent the anti-DHFR effect of PT523 but also to diminish uptake of the drug (see Fig. 3).

As shown in Fig. 2, the effect of PT523 on *de novo* dTMP and purine nucleotide synthesis in H35 cells appeared to depend on both concentration (C) and time (t). For example, treatment with 10 nM PT523 for 4 hr ($C \times t$ = 40 nM-hr) led to 38% incorporation of [^{3}H]dUrd into DNA, relative to untreated controls (Fig. 2A), whereas treatment with 10 nM PT523 for 18 hr ($C \times t$ = 180 nM-hr) led to only 7% incorporation (Fig. 2B), a 5-fold increase in effect with a 4-fold increase in $C \times t$. These results suggested that the major intracellular species responsible for the observed effect is probably intact PT523.

Additional evidence of the potency of PT523 as a DNA synthesis inhibitor is seen in Table 4, which shows that the concentration of drug needed to decrease [^{3}H]dUrd incorporation by 50% over a 4-hr period is 25-fold lower than that of MTX and 20-fold lower than that of the TS inhibitor dmPDDF. Among the compounds tested, PT523 was also the best inhibitor of *de novo* purine nucleotide synthesis, being nearly 200-fold more potent than the GARFT inhibitor DDATHF. It was also 5-fold more potent than TMQ and 20-fold more potent than MTX. On the other hand, it should be noted that, when the IC_{50} ratios for [^{3}H]dUrd versus [^{14}C]Gly incorporation were compared for PT523 and MTX, they were found to be almost

identical (i.e., 1:1 for both drugs). Thus, even though PT523 is more potent that MTX, there does not seem to be a qualitative difference between the two compounds in terms of a preference for the purine pathway versus dTMP synthesis.

It is noteworthy that PT523, like TMQ, cannot form polyglutamates and yet is more potent than MTX, dmPPDF, or DDATHF. The fact that PT523 does not require polyglutamylation to rapidly block dTMP and purine nucleotide biosynthesis has potential clinical significance, because there is growing evidence that inefficient polyglutamylation is associated with resistance to classical antifolates (24, 26, 39–41) (see Ref. 39 for a review of earlier literature).

The idea that the effect of PT523 on dTMP and purine nucleotide synthesis, and ultimately cell growth, is due to DHFR inhibition was further supported by reduced folate pool measurements using the Priest assay (11, 12). As shown in Table 5, treatment of H35 cells with concentrations of PT523 giving >90% inhibition of dTMP and purine nucleotide synthesis led to marked depletion of the 5,10-$CH_2H_4PteGlu_n$, $H_4PteGlu_n$, and 10-$HCOH_4PteGlu_n$ pools and expansion of the $H_2PteGlu_n$ pool, in a pattern consistent with that of other potent DHFR inhibitors. In contrast, the TS inhibitor dmPDDF and the GARFT inhibitor DDATHF had minimal effects on $H_2PteGlu_n$ and 10-$HCHOH_4Pte$-Glu_n pools, respectively, and these effects were observed only after 18 hr of treatment. Because PT523 inhibits TS only at concentrations orders of magnitude higher than the concentration needed to inhibit cell growth, it seems likely that the smaller tetrahydrofolate cofactor pool in the presence of this drug is primarily due to DHFR inhibition. These results are consistent with the 2,4-diamino structure of PT523 and with the finding that both dThd and Hx, or folinic acid alone, are required for full protection.

An unanswered question with regard to the mode of action of PT523 is why this compound is more potent than MTX against a large number of different cells even though its potency as a DHFR inhibitor is similar. Initial interest in PT523 was sparked by the finding that it could overcome MTX resistance not only in cells with impaired transport but also in cells with a 20-fold increase in DHFR. To be active at low nanomolar or subnanomolar concentrations and to possess the ability to circumvent resistance based on defective transport as well increased DHFR production, PT523 would have to either act at a site other than DHFR or accumulate more efficiently in the cell, giving a higher concentration of free drug and less dissociation of bound drug from the enzyme. The findings reported in this paper argue against TS, GARFT, or FPGS as the primary targets. However, improved accumulation remains a possibility. This could come about if PT523 is a better substrate for the active transport pathway used by MTX and reduced folates, is able to utilize an alternative pathway, such as facilitated diffusion, or effluxes more slowly. The data in Fig. 3, showing a 50% decrease in [^{3}H]MTX and [^{3}H]folinic acid, but not [^{3}H]folic acid, uptake by H35 cells in the presence of an approximately equimolar concentration of PT523, suggest that some transport of PT523 probably occurs via the MTX/reduced folate carrier. The data in Fig. 4 suggest that utilization of the MTX/reduced folate carrier by PT523 probably also occurs in CEM cells, because the total cellular [^{3}H]MTX (mono- and polyglutamates) level was lower in cells incubated with PT523 and [^{3}H]MTX than in cells incubated with [^{3}H]MTX alone. It should be noted that, in both experiments, inhibition of [^{3}H]MTX or [^{3}H]folinic acid uptake by PT523 does not rule out partial use by PT523 of some alternative transport route(s) not shared with MTX and reduced folates. Pilot experiments using radiolabeled PT523 indicate that its uptake is rapid even in cells with impaired MTX transport.[1] However, more extensive studies are needed to determine how the kinetics of uptake of PT523 differ from those of MTX, whether inhibition of PT523 influx by MTX is competitive, and whether an alternative mechanism exists for efficient accumulation in the cell. It is possible that the acidity of the aromatic COOH group in the hemiphthaloyl moiety contributes to retention. It is also conceivable that PT523 binds to an as yet unidentified intracellular compartment, providing in effect the functional equivalent of polyglutamylation.

The possibility that PT523 is converted to APA-Orn, a potent inhibitor of FPGS (IC_{50} of 0.15 μM) (14) that could conceivably interfere with reutilization of reduced folate cofactors if a high enough intracellular concentration were achieved, seems to be ruled out. No substantial change in intracellular [^{3}H]MTX polyglutamate distribution was observed in either H35 cells or CEM lymphoblasts even after treatment with 2.0 μM PT523 for 48 hr (Fig. 4). The MTX polyglutamylation profiles in the two cell lines were similar regardless of whether they were in stationary phase (H35 cells) or late logarithmic phase (CEM cells). These experiments seem to rule out FPGS as a target for PT523. It should also be noted that, unlike PT523, whose IC_{50} for cell growth inhibition is in the low nanomolar range, APA-Orn inhibits cell growth only at micromolar concentrations (14). It thus appears that APA-Orn arising by loss of the phthaloyl group is not likely to contribute to the antifolate effects of PT523 *in vitro* or *in vivo*.

In summary, PT523, a water-soluble, nonpolyglutamatable analogue of aminopterin, has been shown in this study to be highly active against cultured H35 rat hepatoma cells, with a potency greater than that of several well known classical antifolates including the DHFR inhibitors MTX and TMQ, the TS inhibitors PDDF and dmPDDF, and the GARFT inhibitor DDATHF. Results obtained thus far support the conclusion that the primary target of PT523, like that of MTX and TMQ, is DHFR rather than TS, GARFT, or FPGS. Inhibition of dTMP and purine nucleotide *de novo* synthesis appears to be due to depletion of tetrahydrofolate polyglutamate cofactors and expansion of the dihydrofolate pool. The high potency of PT523, combined with the fact that it does not require polyglutamylation to inhibit cell growth, suggests that its best therapeutic use may be against tumors that are inefficient in polyglutamylating classical antifolates.

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