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METHOTREXATE ANALOGUES—27

DUAL INHIBITION OF DIHYDROFOLATE REDUCTASE AND FOLYLPOLYGLUTAMATE SYNTHETASE BY METHOTREXATE AND AMINOPTERIN ANALOGUES WITH A γ-PHOSPHONATE GROUP IN THE SIDE CHAIN

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Abstract—γ-Phosphonate analogues of methotrexate (MTX) and aminopterin (AMT) were synthesized from 4-amino-4-deoxy-N^{10}-methylpteroic acid and 4-amino-4-deoxy-N^{10}-formylpteroic acid, respectively, by reaction with methyl D,L-2-amino-4-phosphonobutyrate followed by gentle alkaline hydrolysis. The products were compared with the corresponding D,L-homocysteic acid derivatives as inhibitors of dihydrofolate reductase and folylpolyglutamate synthetase, and as inhibitors of cell growth in culture. The γ-phosphonates were somewhat less active than either the γ-sulfonates or the parent drugs as inhibitors of murine dihydrofolate reductase. The MTX γ-sulfonate and γ-phosphonate analogues were equally inhibitory toward mouse liver folylpolyglutamate synthetase (K_i = 190 μM), but in the AMT series the γ-phosphonate (K_i = 8.4 μM) was more potent than the γ-sulfonate (K_i = 45 μM). The AMT analogues were consistently more inhibitory than the MTX analogues against cultured L1210 murine leukemia cells, but neither the γ-phosphonates nor the γ-sulfonates were as potent as their respective parent drugs. The γ-phosphonate analogue of MTX was three times more potent than MTX against the MTX-resistant mutant line L1210/R81, but the AMT γ-phosphonate was less potent than AMT; however, these differences were small in comparison with the level of resistance to all these compounds in the L1210/R81 line. The results suggest than N^{10}-methyl and N^{10}-unsubstituted compounds altered at the γ-position do not necessarily follow identical structure–activity patterns in every test system.

Interest in folylpolyglutamate synthetase (FPGS) has grown in recent years [1–5] because of the evidence that this enzyme plays a pivotal role in the biochemistry of reduced folates in mammalian cells [6–13], in the action of classical antifolates in these cells [14–29], and in the therapeutic efficacy and, perhaps, the selectivity of leucovorin rescue as an adjunct to antifolate therapy [21, 30–32].

The role of polyglutamylation in the biochemistry of endogenous reduced folates in mammalian cells is 2-fold. First, since polyglutamates with more than three glutatmate residues are very inefficiently transported across the cell membrane [33], the ability to form these polyanionic metabolites may represent an evolutionary mechanism by which cells have learned to conserve reduced folate cofactors [34]. Second, since the polyglutamates of reduced folates generally are better cofactors for their respective enzymes than are the monoglutamates [6–13], polyglutamylation allows the cell to make optimal use of its store of cofactors for the *de novo* synthesis of RNA and DNA nucleotide precursors. Differences in FPGS activity exist among tumors and in normal tissues [35, 36], and are thought to contribute in a major way to the therapeutic selectivity of MTX and other classical antifolates [17–26]. These differences may account for interpatient variability in the initial response to MTX and may also contribute, in part, to acquired MTX resistance [37, 38].

A decade ago, McBurney and Whitmore [39] made the salient discovery that mutant Chinese hamster ovary cells which are auxotrophic for thymidine, hypoxanthine, and glycine also lack the ability to form polyglutamates of reduced folates, suggesting that polyglutamylation may be essential for the survival of dividing mammalian cells. These findings were later confirmed by Taylor and Hanna [40] and have more recently been extended by Foo and Shane [41]. The concept that FPGS is essential for the survival of dividing cells has led to the hypothesis that FPGS inhibition merits consideration as a novel approach to antifolate therapy [42].

We recently reported that replacement of the L-glutamate side-chain in the classical antifolates methotrexate (MTX, **1**) and aminopterin (AMT, **2**) by D,L-homocysteic acid gives analogues (**3**, **4**) with considerable activity as competitive inhibitors of FPGS from mouse liver [43]. The K_i of the γ-sulfonic acids was similar to the K_m of the parent drugs, and it was speculated that tight binding to the enzyme active site may be due to a steric and electronic resemblance to the γ-glutamylphosphate esters **5** and

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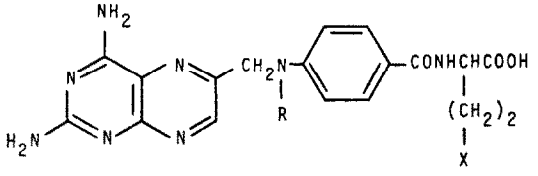

1, 2 : R = Me, H; X = CO_2H

3, 4 : R = Me, H; X = SO_3H

5, 6 : R = Me, H; X = $CO_2PO_3H_2$

7, 8 : R = Me, H; X = PO_3H_2

Fig. 1. Structures of MTX, AMT, and their γ-SO_3H and γ-PO_3H analogues, and of the putative γ-glutamylphosphate intermediates for MTX and AMT polyglutamylation.

6, which are the putative reactive intermediates in the polyglutamylation of **1** and **2** [44]. In addition to being active against FPGS, **3** and **4** were potent inhibitors of dihydrofolate reductase (DHFR) from L1210 murine leukemia cells and displayed significant activity against L1210 leukemia in culture and *in vivo*. The possibility of a dual mechanism of antifolate action was suggested [43, 45], wherein there would be concomitant blockade of tetrahydrofolate synthesis via DHFR inhibition and decreased conversion of tetrahydrofolate cofactors to polyglutamates via FPGS inhibition. An extension of this approach was to investigate MTX and AMT analogues with γ-PO_3H_2 groups in place of γ-CO_2H or γ-SO_3H. The present paper describes the chemical synthesis of the MTX and AMT analogues **7** and **8**, and reports their enzyme-inhibitory activity and effect on cell growth in culture. Since they contain a γ-PO_3H_2 group, these compounds more closely resemble the proposed polyglutamylation intermediates **5** and **6** than do the γ-sulfonates **3** and **4**. The structures of compounds **1–8** are given in Fig. 1.

MATERIALS AND METHODS

D,L-2-Amino-4-phosphonobutyric acid was obtained from Sigma (St. Louis, MO). 4-Amino-4-deoxy-N^{10}-methylpteroic acid (mAPA), 4-amino-4-deoxy-N^{10}-formylpteroic acid (fAPA), and diethyl phosphorocyanidate (DEPC) were prepared as previously described [45]. *N,N*-Dimethylformamide (DMF) used in the coupling reaction was dried over Linde 4A molecular sieves. Rotary evaporation of the DMF from reaction mixtures was achieved with the aid of a vacuum pump. The bath temperature was kept at 35–40°, and the receiving flask was cooled in dry ice/acetone. Thin-layer chromatography (TLC) was carried out on Eastman 13181 silica gel or Eastman 13254 cellulose sheets (fluorescent indicator), and spots were visualized under 254-nm illumination. Ion-exchange chromatography was performed on columns of Whatman DE-52 *N,N*-diethylaminoethylcellulose (DEAE-cellulose). Infrared spectra were obtained on a Perkin–Elmer model 781 double-beam recording spectrophotometer, ultraviolet spectra were determined on a Cary model 210 instrument, and NMR spectra were recorded on a Varian T60A spectrometer with tetramethylsilane as the reference. Microchemical analyses were performed by Galbraith Laboratories, Knoxville, TN, and were within ±0.4% of theoretical values.

Methyl D,L-2-amino-4-phosphonobutyrate. A suspension of D,L-2-amino-4-phosphonobutyric acid (366 mg, 2.0 mmoles) in absolute methanol (10 ml) was cooled in an ice-bath, while thionyl chloride (2 ml) was added dropwise with stirring so that the internal temperature of the mixture did not exceed 12°. When addition was complete, the solution was allowed to come to room temperature and was left overnight. Rotary evaporation afforded a >100% yield of a gummy product; NMR (D_2O) δ 1.6–2.4 (m, 4H, CH_2CH_2), 3.82 (s, 3H, CO_2CH_3), 4.21 (m, 1H, α-CH). The spectrum also showed residual methanol (δ3.32) and dimethyl sulfite (δ3.63). Attempts to remove these contaminants under high vacuum or by repetitive co-evaporation with toluene were only partly successful. The crude ester was dissolved in dry DMF (10 ml), and appropriate aliquots of the solution (0.2 M based on the amount of starting material) were used directly in the coupling step.

N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-D,L-2-amino-4-phosphonobutyric acid (**7**). mAPA (325 mg, 0.9 mmole) was added in small portions to a stirred solution of DEPC (440 mg, 2.7 mmoles) and triethylamine (272 mg, 2.7 mmoles) in dry DMF (30 ml). The clear solution was left to stand at room temperature overnight, at which time activation of the mAPA was found to be complete by TLC (silica gel, 4:1 chloroform–methanol). An aliquot of the 0.2 M stock solution of methyl D,L-2-amino-4-phosphonobutyrate calculated to contain 1.0 mmole of amino ester (see preceding experiment) was dispensed into DMF (5 ml), and this solution was added to the reaction mixture, along with a second portion of triethylamine (505 mg, 5.0 mmoles). Stirring at room temperature was continued for 3 days, and the solvent was removed by rotary evaporation. The residue was washed with ether and then dissolved in 0.1 N NaOH (30 ml). After 30 min at room temperature to allow saponification, the pH was lowered to 8.0 with 1 N HCl, and the solution was applied onto a column of DEAE-cellulose (20 g, HCO_3^- form). The column was eluted initially with a large volume of water to remove inorganic salts, and then with 3% ammonium bicarbonate. Fractions were monitored by TLC (cellulose, pH 7.4 phosphate buffer). The major yellow product (R_f 0.85) was preceded and followed by two minor yellow contaminants (R_f 0.70 and 0.40 respectively). Appropriate tubes were pooled and freeze-dried to obtain pure **7** as a bright-yellow powder (103 mg, 21% yield): m.p. above 300° with decomposition: i.r. (KBr) ν3420 (br), 3200 (sh), 1640, 1610 cm^{-1}; u.v. (pH 7.4) λ_{max} 259 nm (ε22,400), 300 (21,300), 371 (7,400); u.v. (0.1 N HCl) λ_{max} 243 nm (ε17,200), 306 (18,500). Microchemical analysis indicated the product to be a hydrated hemiammonium salt.

Anal. Calc. for $C_{19}H_{23}N_8O_6P \cdot 0.5\,NH_3 \cdot 3H_2O$: C, 41.27; H, 5.56; N, 21.53; P, 5.60. Found: C, 41.18; H, 5.45; N, 21.37; P, 5.60.

N-(4-Amino-4-deoxypteroyl)-D,L-2-amino-4-phosphonobutyric acid (**8**). To a stirred suspension of fAPA (300 mg, 0.8 mmole) in dry DMF (30 ml) were added sequentially triethylamine (707 mg, 7.0 mmoles) and isobutyl chloroformate (104 μl, 109 mg, 0.8 mmole). After 25 min at room temperature, a volume of the methyl D,L-2-amino-4-phosphonobutyrate 0.2 M stock solution calculated to contain 0.8 mmole was dispensed into dry DMF (4 ml) and added to the reaction. TLC (cellulose, pH 7.4 phosphate buffer) showed partial consumption of fAPA (R_f 0.35, blue-fluorescent spot) and formation of a faster-moving product assumed to be the N^{10}-formyl methyl ester of **8** (R_f 0.50, blue-fluorescent spot). A second portion of isobutyl chloroformate (52 μl, 0.4 mmole) was added, followed after 10 min by 2 ml of DMF containing 0.4 mmole of the amino ester. The activation-coupling sequence was repeated twice more on a 0.2 mmole scale, at which point fAPA was barely detectable by TLC. The reaction mixture was concentrated to dryness by rotary evaporation, and the residue was washed with ether and redissolved in 0.1 N NaOH. A few drops of 1 N NaOH were added as necessary, in order to bring the final pH to at least 13. The course of deblocking was followed by TLC (cellulose, pH 7.4 buffer), which showed gradual replacement of the blue-fluorescent spot (R_f 0.50) by a faster-moving u.v.-absorbing spot (R_f 0.70). When deblocking was complete, the pH was adjusted to 8.0 with 1 N HCl and the solution was freeze-dried. The product was chromatographed on a DEAE-cellulose column as described in the preceding experiment, and then purified further by rechromatography with stepwise increases in concentration of the ammonium bicarbonate (1%, 2%, 3%). TLC-homogeneous tubes were pooled and freeze-dried to obtain **8** as a bright-yellow powder (230 mg, 52% yield): m.p. above 300° with decomposition; i.r. (KBr) ν 3380 (br), 3200 (sh), 1640, 1610 cm^{-1}; u.v. (pH 7.4) λ_{max} 260 nm (ε 27,100), 282 (25,700), 371 (8,100); u.v. (0.1 N HCl) λ_{max} 243 nm (ε 17,800), 290 (19,200), 336 sh (10,600). Microanalysis indicated the product to be a hydrated hemiammonium salt.

Anal. Calc. for $C_{18}H_{21}N_8O_6P \cdot 0.5\,NH_3 \cdot 4H_2O$: C, 38.82; H, 5.52; N, 21.38; P, 5.56. Found: C, 38.56; H, 5.60; N, 21.20; P, 5.13.

Dihydrofolate reductase inhibition. Enzyme from L1210/R71 murine leukemia cells was purified by affinity chromatography [46], and the amount of enzyme in the column eluate was assayed by titration with MTX. The rate of dihydrofolate reduction was measured spectrophotometrically at 340 nm, using $\Delta\varepsilon = 12{,}300$ to determine the standard unit of enzyme activity [47]. In the experiments with **7** and **8**, the assay mixture contained NADPH (75 μM) and dihydrofolate (50 μM) in 0.05 M Tris chloride, pH 7.5, while in the experiments with **3** and **4** the assay mixture contained NADPH (100 μM) and dihydrofolate (80 μM) in 0.05 M potassium phosphate buffer, pH 7.0. These minor variations in experimental conditions had no appreciable effect on the IC_{50} or the shape of the inhibition curve in standard experiments with MTX (data not shown). Assays were performed by preincubating the enzyme, NADPH, and test compound in the buffer for 2 min at 22°. Residual enzyme activity was measured after addition of dihydrofolate and was expressed as a percentage of the control with no inhibitor added. Triplicate tubes were used at each inhibitor concentration, and results were averaged. The assay data were normalized for the fact that the amount of enzyme used was not the same in every assay by expressing activity as the ratio $IC_{50}/[E]$, where IC_{50} is the inhibitor concentration needed to decrease the rate of dihydrofolate reduction by 50% and $[E]$ is the DHFR concentration in the assay.

Folylpolyglutamate synthetase inhibition. A partially purified preparation of enzyme from mouse liver cytosol was prepared as described earlier [35] and typically showed a specific activity of 1.2 nmoles·hr^{-1}·(mg protein)$^{-1}$. Activity was measured in an assay mixture containing 10–500 μM folic acid, 1 mM [^{3}H]-L-glutamic acid (4 mCi/mmole, Amersham), 5 mM ATP, 10 mM magnesium chloride, 30 mM potassium chloride, 20 mM 2-mercaptoethanol, and 20 mM Tris, pH 8.6, in a total volume of 0.25 ml [42]. After 1 hr at 37°, [^{3}H]folyloligoglutamates were adsorbed onto activated charcoal and eluted from the charcoal with ethanolic ammonia [48]. The radioactivity of the charcoal-adsorbable fraction was determined in 10 ml of scintillation fluid (RSA-II, Research Products International, Elk Grove Village, IL) in a Beckmann LS3155T counter. Duplicate assays were performed, and data were fitted to a rectangular hyperbola by a standard method of nonlinear regression analysis [42]. K_i values for the inhibitors were derived by replotting the slopes of double-reciprocal plots obtained on a standard computer program [49].

Cell growth inhibition. The ability of **7** and **8** to inhibit the growth of L1210, L1210/R71, and L1210/R81 cells was determined in 24-well cluster dishes at an initial density of 6×10^4 cells/well. The cells were grown in RPMI 1640 medium containing 5% fetal bovine serum (Kansas City Biologicals, Kansas City, MO) and 1% penicillin–streptomycin solution (Grand Island Biological Co., Grand Island, NY). The origin of the MTX-resistant L1210/R71 and L1210/R81 cells has been described elsewhere [50, 51]. The cells were counted after 48 hr, and the IC_{50} (50% inhibitory concentration relative to untreated controls) was determined from the dose–responsive curve. Assays were performed in replicate cultures.

RESULTS AND DISCUSSION

Synthesis of γ-phosphonate analogues of MTX and AMT. The chemical schemes followed in this work were adapted from those we have used previously to obtain side-chain altered MTX and AMT analogues [43, 45, 52, 53]. The carboxyl group in D,L-2-amino-4-phosphonobutyric acid was protected by esterification with methanol and thionyl chloride prior to coupling to mAPA [43] or fAPA [45]. The noncrystalline methyl ester was used without purification, but was characterized by NMR. For the syn-

Table 1. Dihydrofolate reductase inhibition, folylpolyglutamate synthetase inhibition, and cell growth inhibition by the γ-phosphonate and γ-sulfonate analogues of methotrexate and aminopterin

Compound	DHFR inhibition* $IC_{50}/[E]$	FPGS inhibition† K_i (μM)	Cell growth inhibition (IC_{50}, μM)‡ L1210 (1)	L1210 (2)	L1210/R71 (2)	L1210/R81 (2)
MTX	0.50	§	0.012	0.002	19 [9,500]	220 [110,000]
3	0.55	188 ± 56	0.30	ND	ND	ND
7	1.34	185 ± 71	0.19	0.20	42 [210]	76 [380]
AMT	0.50	§	0.0031	0.002	7.9 [4,000]	84 [42,000]
4	0.62	45 ± 6.0	0.037	ND	ND	ND
8	1.26	8.4 ± 2.0	0.035	0.067	220 [3,300]	240 [3,600]

* The DHFR concentration [*E*] was 0.100 μM in the experiments with compounds **3** and **4**, 0.080 μM in the experiments with MTX and AMT, and 0.050 μM in the experiments with compounds **7** and **8**; see Materials and Methods for other details. The data for MTX and AMT are those reported earlier [45].

† Data for compounds **3** and **4** were reported earlier [45] but the K_i for **3** was inadvertently given as 59 ± 28 μM rather than as the correct value presented here.

‡ Data in the L1210 (1) column were obtained at the Dana-Farber Cancer Institute; data in the L1210 (2), L1210/R71 (2), and L1210/R81 (2) columns were obtained at the Medical College of Ohio. Numbers in brackets are normalized with reference to the IC_{50} in the L1210 (2) column for each compound and, therefore, represent fold-resistance; ND = not determined.

§ K_i values for MTX and AMT were not determined since these are FPGS substrates; apparent K_m values of 166 ± 49 μM and 17.6 ± 5.8 μM, respectively, were reported for these compounds earlier [42].

thesis of **7**, mAPA was activated by overnight treatment with DEPC (3 equiv.) at room temperature in DMF containing triethylamine. The amino ester (1.1 equiv.) and additional triethylamine were then added, and the reaction was allowed to proceed for 3 days. In the case of **8**, coupling was via our modified mixed carboxylic-carbonic anhydride procedure [45], with the activation-coupling cycle being repeated a total of four times before all the fAPA was consumed. Removal of the N^{10}-formyl group was achieved quantitatively at room temperature at pH 13. Compounds **7** and **8** were both purified to homogeneity by column chromatography on DEAE-cellulose using ammonium bicarbonate as the eluent. Preliminary washing with distilled water removed the inorganic salts. Freeze-drying afforded products whose elemental analysis indicated them to be hydrated hemiammonium salts, as was observed earlier with the sulfonic acid analogues [45]. The AMT analogue **8** retained four molecules of water, whereas the MTX analogue **7** was a trihydrate. Non-optimized yields of 21% (**7**) and 52% (**8**) were obtained, and were lower than those of the corresponding sulfonic acids [45].

It is of interest to note that, while our synthesis of **7** was in progress, we became aware of work by Sturtz and coworkers [54, 55], who made use of the Taylor pteridine synthesis [56] to obtain the γ-$PO(OEt)_2$ analogue of MTX. The overall yield was extremely low, and there was apparently not enough material to carry out the intended cleavage of the γ-$PO(OEt)_2$ group with trimethylsilyl bromide, which would afford **7**. The authors did succeed, however, in preparing another interesting MTX analogue with PO_3H_2 groups at both the α- and γ-position.

Dihydrofolate reductase inhibition. The γ-phosphonates **7** and **8** were tested as inhibitors of purified DHFR from murine leukemic cells, and the $IC_{50}/[E]$ ratios for these compounds were compared with the ratios for the γ-sulfonate analogues **3** and **4** and for

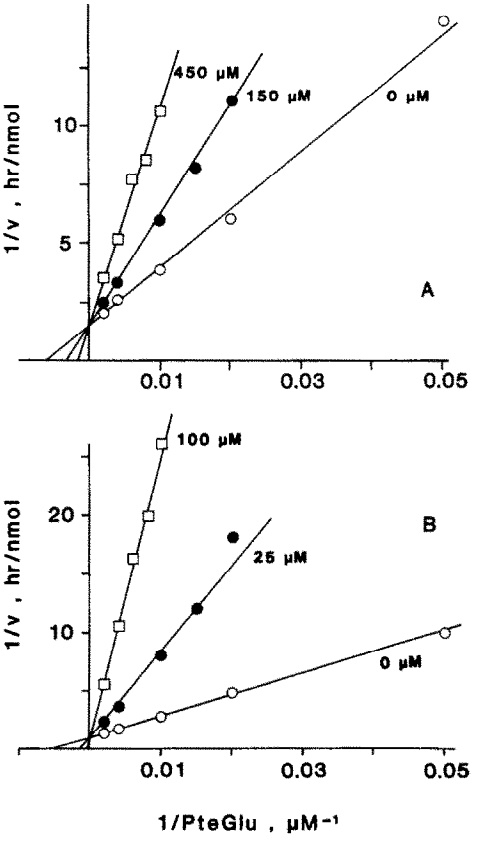

Fig. 2. Inhibition of mouse liver FPGS by *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-D,L-2-amino-4-phosphonobutyric acid (**7**) (panel A) and *N*-(4-amino-4-deoxypteroyl)-D,L-2-amino-4-phosphonobutyric acid (**8**) (panel B). Each point is the mean of two determinations from a representative experiment. The numbers next to each line indicate the concentration of inhibitor used for each saturation curve.

the parent drugs MTX and AMT. As shown in Table 1, the $IC_{50}/[E]$ ratios for **7** and **8** were 1.34 and 1.26, while those for **3** and **4** were 0.55 and 0.62; the ratios we reported earlier for MTX and AMT were both 0.50 [45]. It should be noted that, in contrast to MTX and AMT which are in the L-enantiomeric form, the γ-phosphonates **7** and **8** and γ-sulfonates **3** and **4** are D,L mixtures. Since it is known that D-MTX has <10% of the activity of L-MTX against murine DHFR [57], one would expect that the D,L mixtures would probably be less inhibitory than the pure L-enantiomers; however, in the absence of data for the pure D-enantiomers, it cannot be stated whether this difference would be as great for the γ-sulfonate and γ-phosphonate analogues as it is for MTX.

The DHFR titration curves (not shown) for the γ-phosphonates displayed greater curvilinearity than did those for the γ-COOH and γ-SO_3H analogues, perhaps reflecting the fact that the γ-PO_3H_3 group can form a dianion rather than a monoanion at the pH of the assay. This extra negative charge may result in decreased binding to the enzyme active site. A similar tendency toward curvilinearity and a concomitant increase in $IC_{50}/[E]$ ratio to values >0.5 have been observed with MTX analogues in which the glutamic acid moiety is replaced by cysteic acid [45] or α,ω-diaminoalkanoic acids [53], but not by chain-lengthened α-aminoalkanedioic acids [58]. Deviation from linearity with increasing inhibitor concentration is important insofar as it means that two compounds with nearly the same IC_{50} at a given DHFR concentration may nonetheless be different in potency at the level of DHFR inhibition required to completely suppress thymidylate biosynthesis [59]. Our findings with **7** and **8** indicate that, while an extra minus charge at the γ-position of the inhibitor produces minimal change in the $IC_{50}/[E]$ ratio, there is probably a more profound change in enzyme-binding kinetics than is revealed by these data alone. Detailed kinetic studies on the interaction of the γ-phosphonate and γ-sulfonate analogues with DHFR would be extremely interesting but were beyond the scope of this study.

Folylpolyglutamate synthetase inhibition. Compounds **7** and **8** were evaluated as inhibitors of partially purified FPGS from mouse liver, using folic acid (10–500 μM) and 1 mM L-[^{3}H]glutamic acid as previously described [42]. The reaction was allowed to proceed at 37° for 1 hr at pH 8.6. The K_i was determined from a double-reciprocal plot (Fig. 2). As indicated in Table 1, the K_i of **7** was almost the same as the K_i of the γ-sulfonic acid **3**. Inhibition was competititve with the folyl substrate, and the magnitude of the K_i closely approximated that of the apparent K_m for MTX. It thus appears that the γ-phosphonate analogue **7** binds well to the FPGS active site even though binding is nonproductive for polyglutamylation. It was anticipated, by analogy with the FPGS-binding activity of AMT and **4**, that **8** would have a K_i approximately 4-fold lower than the K_i of **7**. However, as shown in Table 1, the K_i of **8** proved to be 20-fold lower than that of **7**. Moreover, the K_i of **8** was 2-fold lower than the K_m of AMT as a substrate. It appears from these results that AMT may be more sensitive than MTX to structural modifications in the γ-region of the side-chain. It is not immediately clear why there should be such a difference, considering how far removed the γ-region is from N^{10}. A possible explanation is that N^{10} substitution results in a change not only in the torsional angle of the phenyl ring, but also in the orientation of the entire amino acid side-chain in the enzyme-bound form of the drug.

The level of activity displayed by compounds **3**, **4**, **7** and **8** as FPGS inhibitors is noteworthy in the context of recent work on γ-fluoromethotrexate, which is reported to be a potent DHFR inhibitor but a very poor FPGS substrate [60]. In the presence of a 100-fold excess of γ-fluoromethotrexate, MTX polyglutamylation is inhibited by only 20% [60]. It thus appears that merely increasing the acidity of the γ-carboxyl, in this case via the introduction of an electronegative F atom that can decrease the pK_a by as much as two units, is not by itself sufficient to bring about FPGS inhibition. γ-Fluoromethotrexate is in fact a poor inhibitor. Although the original description of the inhibition of FPGS by this compound did not specify the K_i, an approximate K_i of 150–200 μM can be estimated from the published data assuming competitive inhibition. γ-Fluoromethotrexate is therefore about as potent an FPGS inhibitor as our previously described compound **3** but is considerably less active than the compounds **4** or **8**.

Cell growth inhibition. The IC_{50} values of compounds **7** and **8** against MTX-sensitive murine L1210 leukemia cells and against the MTX-resistant sublines L1210/R71 and L1210/R81 [50, 51] are presented in Table 1. Data for MTX, AMT, and the γ-sulfonate analogues **3** and **4** are also given for comparison. The MTX analogue **7** was less inhibitory than MTX against the parental L1210 cells and the AMT analogue **8** was less inhibitory than AMT. The γ-phosphonate and γ-sulfonate analogues **7** and **3** were equipotent; the N^{10}-unsubstituted compounds **8** and **4** likewise displayed comparable activity. The N^{10}-unsubstituted analogues were significantly more potent, however, than their N^{10}-methyl counterparts. It thus appears that a greater degree of growth inhibitory activity characterizes N^{10}-unsubstituted compounds even when these cannot be polyglutamylated. A similar pattern was observed recently with derivatives of MTX and AMT in which the γ-carboxyl is blocked as a sterically hindered and enzymatically stable *tert*-butyl ester [52]. We and others have proposed that the high substrate activity of AMT in comparison with MTX for FPGS contributes to its greater cytotoxicity [25, 28, 42]. The present results suggest that this by itself may not be the sole reason for the high level of activity of AMT, and that AMT ought to be more active than MTX even in the absence of polyglutamylation, e.g. in cells with a polyglutamylation defect [37, 38] or in cells treated with a polyglutamylation inhibitor.

We recently showed that cultured MTX-resistant human [38] and murine [52] tumor cells are only partially cross-resistant to the γ-*tert*-butyl esters of MTX and AMT. It was therefore of interest to examine whether an entirely different type of side-chain modification, one involving a hydrophilic rather than hydrophobic moiety, would also produce incomplete cross-resistance. Compounds **7** and **8**

were tested as inhibitors of the growth of cultured L1210/R71 and L1210/R81 cells, the two lines used earlier with the MTX and AMT γ-esters. As shown in Table 1, the R71 line was 45-fold less resistant to **7** than it was to MTX, whereas with the R81 line this difference in resistance was 290-fold. Indeed, **7** appeared to be three times more potent than MTX against the latter cells. With the γ-*tert*-butyl ester of MTX this increase in potency was 9-fold [52].

A higher degree of cross-resistance was observed between **8** and AMT than between **7** and MTX. Moreover, while **7** was more toxic than MTX against the L1210/R81 cells, the reverse was true for **8** in comparison with AMT. The same trend reversal occurred with L1210/R71 cells. Since it has been found that the DHFR in these cells is kinetically normal [50] and since the IC_{50} values of **7** and **8** toward purified DHFR from R71 cells were nearly the same (Table 1), we think it unlikely that the decreased toxicity of **8** in comparison with **7** in the resistant cells reflects differences in DHFR binding. Since **8** is superior to **7** in its ability to inhibit FPGS, one might expect that **8** would be more effective in interfering with the polyglutamylation of reduced folate cofactors in the cell and that it might therefore show a greater degree of self-potentiating antifolate activity [43]. The fact that this was not the case may be due to an alteration in transport characteristics in the R71 and R81 cells, such that N^{10}-unsubstituted compounds with a *negatively-charged* γ-terminal that cannot undergo polyglutamylation are accumulated less efficiently in the cell than are the corresponding N^{10}-methyl analogues. In contrast to this, the γ-*tert*-butyl esters of AMT and MTX (neutral γ-terminal) are both more potent than the parent acids as inhibitors of R71 and R81 cell growth [52]. MTX and AMT analogues with an ornithine side-chain (positively-charged γ-terminal) have likewise been found to be more active than the parent acids against these cells [53]. The efficiency with which the MTX and AMT γ-phosphonate analogues are accumulated in cells in comparison with the parent drugs is obviously as important as DHFR binding as a determinant of cell growth inhibition. Studies of the transport kinetics of these compounds, including both influx and efflux measurements, would be extremely interesting but were not carried out in this initial work because it was felt that they would best be done with radiolabeled materials, which are not presently available.

In summary, the experiments reported here indicate that replacement of the γ-CO_2H group in MTX and AMT by γ-PO_3H_2 produces compounds that inhibit both DHFR and FPGS, and may therefore have a mode of action unlike that of conventional classical antifolates. With the MTX analogue **7**, binding to DHFR was somewhat weaker than that of the corresponding racemic γ-sulfonate **3**; the inhibitory potency of **7** and **3** toward FPGS, however, was the same, while the ability of **7** to inhibit L1210 cell growth exceeded that of **3**. With the AMT analogue **8**, DHFR inhibition was similar to that of the γ-sulfonate analogue **4**, but potency in the FPGS assay was substantially greater; cell growth inhibition, on the other hand, was not increased. The MTX-resistant L1210/R71 and L1210/R81 cells were less resistant to **7**, but more resistant to **8**, than they were to MTX. These differences were small in comparison with the level of resistance to both compounds shown by the resistant cells. Overall, our findings underscore the idea that structure–activity patterns among N^{10}-methyl and N^{10}-unsubstituted analogues of MTX and AMT, respectively, should not necessarily be expected to be the same, and that in fact these patterns may vary from one test system to another. Other MTX and AMT analogues with dual activity against DHFR and FPGS, as well as their 2-amino-4-oxo counterparts which ought to inhibit only FPGS, are being developed as an extension of this work.

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