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METHOTREXATE ANALOGUES—XVII

ANTITUMOR ACTIVITY OF 4-AMINO-4-DEOXY-N^{10}-METHYLPTEROYL-D,L-HOMOCYSTEIC ACID AND ITS DUAL INHIBITION OF DIHYDROFOLATE REDUCTASE AND FOLYL POLYGLUTAMATE SYNTHETASE

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Abstract—A new analogue of methotrexate was synthesized from 4-amino-4-deoxy-N^{10}-methylpteroic acid and D,L-homocysteic acid. The product (mAPA-HCysA) was bound tightly to L1210 mouse leukemia dihydrofolate reductase (IC_{50} = 1 nM), inhibited L1210 cell proliferation in culture (IC_{50} = 0.3 μM), and prolonged the survival of L1210 leukemic mice (98% increase in lifespan at 120 mg/kg, qdx9). Studies on the interaction of mAPA-HCysA with partially purified mouse liver folyl polyglutamate synthetase revealed that mAPA-HCysA was not a substrate. Hence, the increased dose of mAPA-HCysA required to inhibit tumor growth *in vitro* and *in vivo* relative to methotrexate may reflect, in part, the inability of this compound to form non-effluxing polyglutamates. Folyl polyglutamate synthetase was competitively inhibited by mAPA-HCysA (K_i = 190 ± 70 μM) when folate was the variable substrate. Thus, mAPA-HCysA is the first known compound to inhibit both mammalian dihydrofolate reductase and mammalian folyl polyglutamate synthetase.

The ability of the antitumor agent methotrexate (MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid) to form γ-glutamyl conjugates once it enters cells [1–19] has given rise recently to the concept that MTX polyglutamate synthesis may be an important determinant of therapeutic efficacy and selectivity [20–25] and may influence the outcome of drug combinations involving, for example, MTX and 5-fluorouracil [22].

The enzyme responsible for the conversion of MTX to polyglutamates in cells is folyl polyglutamate synthetase (FPGS), which also converts natural reduced folate cofactors to γ-glutamyl conjugates [26–29]. MTX polyglutamates leave mammalian cells more slowly than MTX [12, 17], presumably due to the specificity of binding to the membrane protein(s) responsible for folate efflux. Just as the polyglutamates of reduced folates are in some instances better substrates for their respective enzymes than the monoglutamates [30–35], polyglutamates of MTX bind at least as well as MTX itself [7, 8, 36] to dihydrofolate reductase (DHFR) and are able to prolong inhibition of DNA synthesis beyond the time required to clear free intracellular MTX from the tumor [17]. Thus, the beneficial effect of MTX in some patients, and the lack of such an effect in others, may be due in part to differences in the ability of various tumors to convert MTX to polyglutamates. When MTX treatment is carried out over long periods of time, as in patients with psoriasis [37], there is also a risk of hepatotoxicity which may stem from the chronic accumulation of MTX polyglutamates in hepatocytes [8, 10, 11, 15]. The known capacity of kidney cells to form polyglutamates [4] may also be expected to contribute to renal toxicity.

In this context we have been interested in designing classical dihydrofolate reductase inhibitors that are not substrates for FPGS. While these compounds might be predicted to have a shorter duration of action than MTX, perhaps even necessitating continuous infusion for optimal effect, their inability to form polyglutamates might also result in decreased chronic toxicity during long term use. Moreover, in the event that such analogues were also inhibitors of FPGS, as opposed to being merely non-substrates, there would be the additional intriguing possibility of a dual inhibition of the *formation* of folyl polyglutamates and of the *reutilization* of the folate cofactors normally catalyzed by DHFR. An analogue of MTX with the ability to simultaneously inhibit both DHFR and FPGS could thus be viewed as a novel type of "self-potentiating antifolate".

This paper reports the synthesis of 4-amino-4-deoxy-N^{10}-methylpteroyl-D,L-homocysteic acid (mAPA-HCysA) (Fig. 1), which differs from MTX only in that it contains a γ-SO_3H group in place of γ-CO_2H at the polyglutamation site. This heretofore undescribed MTX analogue, which was isolated as a water-soluble ammonium salt, bound tightly to DHFR, was cytotoxic in culture, and had antitumor activity *in vivo*. Furthermore, in an *in vitro* assay, mAPA-HCysA showed activity as a competitive inhibitor of mouse liver FPGS, yet was not a substrate for this enzyme.

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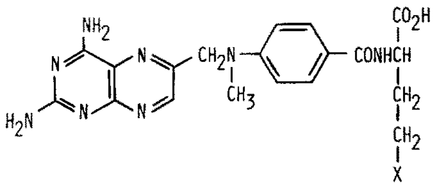

Fig. 1. Structures of MTX (X = CO_2H) and mAPA-HCysA (X = SO_3H).

MATERIALS AND METHODS

Thin-layer chromatography was performed on Eastman 13254 cellulose sheets (with fluorescent indicator), and spots were visualized under room fluorescent light or 254 nm ultraviolet light in a viewing chamber. Column chromatography was carried out on Whatman DE-52 DEAE-cellulose. Microchemical analyses were performed by Galbraith Laboratories, Knoxville, TN, and were within ± 0.4% of calculated C, H, N and S values. Radioactivity measurements were made by means of a Beckman model LS7000 scintillation counter (41% counting efficiency), and spectrophotometric assays of dihydrofolate reductase inhibition were carried out in a Gilford model 240 instrument with a constant-temperature programmable cell-changer assembly. [3′,5′,7-^{3}H]Methotrexate, heareafter called [^{3}H]MTX, was from Amersham, Arlington Heights, IL, and was purified prior to use by chromatography on a DEAE-cellulose column [38]. Partially purified dihydrofolate reductase from MTX-resistant *Lactobacillus casei* (*L. casei*/MTX) was a gift from the New England Enzyme Center, Boston, MA. Rigid polystyrene microtiter plates (8 × 12 wells) for the competitive [^{3}H]MTX-binding assay were from Dynatech, Alexandria, VA. 4-Amino-4-deoxy-*N*^{10}-methylpteroic acid dihydrate was prepared as previously described [39], and methotrexate was from the National Cancer Institute. Diethyl phosphorocyanidate for the coupling reaction was synthesized from cyanogen bromide and triethyl phosphite [40, 41]. Dihydrofolate, NADPH, bovine serum albumin type V (BSA), neutral activated charcoal (Norite), dextran (M_r 161,000), and D,L-homocysteic acid were from the Sigma Chemical Co., St. Louis, MO. Biofluor scintillation fluid was from the New England Nuclear Corp., Boston, MA. The Norite suspension used in the competitive [^{3}H]MTX-binding assay (see below) consisted of 5 g of neutral activated charcoal (thoroughly prewashed with distilled water to remove the "fines"), 1.25 g of BSA, and 0.05 g of dextran in 100 ml of deionized water. Mice for the *in vivo* antitumor assays were from Jackson Laboratories, Bar Harbor, ME, and were fed standard laboratory chow.

*Synthesis of 4-amino-4-deoxy-*N^{10}*-methylpteroyl-D,L-homocysteic acid (mAPA-HCysA) ammonium salt.* A suspension of D,L-homocysteic acid (915 mg, 5 mmoles) in dry benzene (25 ml) was treated with triethylamine 2 g, 20 mmoles) and trimethylchlorosilane (2.5 ml, 2.16 g, 20 mmoles). After being stirred at room temperature for 42 hr, the mixture was filtered to remove the triethylamine hydrochloride, and the filtrate was evaporated under reduced pressure to leave a pale amber-colored oil or a soft semisolid (1.93 g, 90% yield). This silylated material was kept protected from atmospheric moisture and used without further purification in the next step.

4-Amino-4-deoxy-*N*^{10}-methylpteroic acid dihydrate (720 mg, 2.0 mmoles) [39] was added in small portions to a stirred solution of diethylphosphorocyanidate (915 mg, 5 mmoles) and triethylamine (500 mg, 5 mmoles) in *N*,*N*-dimethylformamide (75 ml) previously dried over Linde 4A molecular sieves. The solution was left at room temperature overnight, whereupon TLC (silica gel, 4:1 chloroform–methanol) indicated that the formation of the activated intermediate was complete. The silylated D,L-homocysteic acid triethylammonium salt (1.8 g, 4 mmoles) was then added, and the reaction mixture was left at room temperature for 3 days. After addition of a few milliliters of water, the solvent was removed with the aid of a rotary evaporator, and the residue was dissolved in 3% ammonium bicarbonate, with a few drops of concentrated ammonia being added as needed. TLC (cellulose, pH 7.4 phosphate buffer) revealed two spots (R_f 0.4 and 0.8) of unequal size, the faster-moving one being larger. The solution was placed on a DEAE-cellulose column that had been pre-equilibrated with 3% ammonium bicarbonate. Elution with distilled water removed the product with $R_f = 0.4$, and subsequent elution with 3% ammonium bicarbonate yielded the desired product ($R_f = 0.8$). The by-product that eluted with water had an ultraviolet absorption consistent with a 2,4-diaminopteridine structure, but appeared to have lost all acidic groups since it was insoluble in concentrated ammonia or 0.1 N sodium hydroxide. Its structure was not investigated further. The material that eluted from the column in 3% ammonium bicarbonate was homogeneous by thin-layer chromatography and, on freeze-drying of appropriately pooled eluates, a yellow solid was obtained (850 mg, 78% yield); m.p. above 300°, with decomposition. The product was very soluble in water and, in contrast to methotrexate, did not precipitate from basic solution on adjustment to pH 6. By elemental analysis, the product was a hydrated monoammonium salt, presumably of the γ-sulfonic acid group.

Anal. Calcd. for $C_{19}H_{26}N_9O_6 \cdot 2.25H_2O$: C, 41.58; H, 5.56; N, 22.98; S, 5.83. Found: C, 41.38; H, 5.31; N, 22.75; S, 6.21.

Dihydrofolate reductase binding. The affinity of mAPA-HCysA for L1210 dihydrofolate reductase was measured by a slight modification of the standard assays for competitive [^{3}H]MTX binding [42, 43]. Dihydrofolate reductase, partially purified from L1210 cells by the procedure of Arons *et al.* [43], was diluted in 0.1 M potassium dihydrogen phosphate buffer (pH 6.2) containing 0.1% BSA and 1 mM NADPH, and the solution was divided into 5-ml aliquots that were kept frozen until needed. Prior to each experiment, thawed enzyme was diluted 8-fold with buffer, fresh NADPH was added to a final concentration of 1 mM, and the mixture was kept on ice. A 100-μl aliquot of phosphate buffer–BSA solution containing *ca.* 10^4 cpm of [^{3}H]MTX was added to each of eight wells of a 96-

well microtiter plate, 25 μl of a 6 M stock solution of mAPA-HCysA was added to the first well, and serial 5-fold dilutions were made through the next six wells. The eighth well (control) contained no drug. A 50 μl aliquot of the ice-cold preincubated enzyme was added to each well, followed after 5 min by 50 μl of Norite suspension. The plate was centrifuged (0°, 7 min, 640 *g*), 100 μl of the supernatant fraction was transferred from wells to scintillation vials containing 5 ml of Biofluor, and radioactivity was counted. The IC_{50}, i.e., the concentration of mAPA-HCysA required to decrease enzyme-bound radioactivity in the supernatant fraction by 50%, was determined from a dose–response curve. A methotrexate positive control was also run for comparison in each experiment. The same assay was also utilized to measure the binding affinity of mAPA-HCysA and MTX to dihydrofolate reductase from *L. casei*/MTX.

A spectrophotometric assay of the abilities of mAPA-HCysA and MTX to compete with dihydrofolate for the active site of the enzyme from *L. casei*/MTX was also performed [44]. The enzyme was made up and stored in 0.5-ml aliquots in pH 6.2 buffer with 0.1% BSA and 1 mM NADPH as described above. Fresh NADPH and dihydrofolate solutions were prepared daily in distilled water and were kept cold during use. Prior to each experiment the enzyme was diluted with fresh buffer, with the volume being adjusted so that a final volume of 50 μl in the cuvette would give an uninhibited ΔA_{340} of *ca.* 0.8 absorbance units/min at 37°. In a typical run, 450 μl of 0.2 mM NADPH and 450 μl of 0.2 mM dihydrofolate were placed in a 1-ml cuvette, and the reaction was started by adding 50 μl of enzyme solution that had been preincubated on ice for 5 min. Changes in absorbance at 340 nm were followed at 37° for 5 min, appropriate background corrections were subtracted, and the results were plotted on a logit-log scale. The IC_{50}, defined as the concentration of mAPA-HCysA or MTX required to produce a 50% decrease in absorbance relative to the uninhibited control, was determined from the logit-log plot.

Inhibition of folyl polyglutamate synthetase. Mouse liver cytosol fractions were treated with 30% ammonium sulfate, and the precipitated protein was dissolved in 50 mM Tris buffer, pH 8.6, containing 50 mM 2-mercaptoethanol and was desalted on a Sephadex G-25 column (0.9 × 35 cm) equilibrated with the same buffer. The FPGS activity of this fraction was determined. The assay mixture contained 10–500 μM folate, 1 mM [^{3}H]glutamic acid (4 mCi/mmole), 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. The mixture was incubated at 37° for 60 min, and the [^{3}H]folyl oligoglutamates were adsorbed onto activated charcoal. After extensive washing of the charcoal to remove unreacted [^{3}H]glutamic acid, the [^{3}H]folyl oligoglutamates were eluted with ethanolic ammonia. The product obtained by this method was characterized by chromatography in three systems. The characteristics of this assay will be described in greater detail separately (R. G. Moran and P. D. Colman, manuscript in preparation). Preliminary experiments indicated that rapid consumption of substrate at concentrations below the K_m was a problem for any of the tetrahydrofolate compounds, which had approximate K_m values in the range of 2–8 μM. This complication was circumvented by using folic acid (K_m 160 μM) as a substrate for these experiments. The K_i for the inhibition of an enzyme that utilizes several alternative substrates should be independent of the test substrate used. In the experiments reported here, the FPGS typically had a specific activity of 1.2 nmoles of product formed per hr per mg of protein, and there were 3.6 cpm/pmole of product. Negligible reaction was observed in the absence of folate, ATP, or magnesium chloride. Duplicate assays were performed, and all experiments were done at least twice. Data were analyzed by weighted nonlinear regression fitting to a rectangular hyperbola according to a standard statistical procedure for enzyme data [45].

Cytotoxicity. L1210 mouse leukemia cells were grown in suspension culture in Eagle's minimal essential medium supplemented with 15% fetal bovine serum, streptomycin (100 μg/ml), penicillin (100 units/ml), and 0.05 mM 2-mercaptoethanol. Exponentially growing cells (5×10^5/ml) were exposed to regularly spaced doses of drug over a four-log range of concentrations (10^{-8}–10^{-4} M) and were incubated at 37° in a 5% CO_2 humidified atmosphere. At the end of 48 hr the cells were counted with the aid of a Coulter Counter (model F), a dose–response curve was plotted, and the IC_{50} was determined. Experiments were performed in triplicate, and cell counts were averaged.

In vivo *antitumor activity.* A standard protocol [46] was used to measure the ability of mAPA-HCysA to prolong the lifespan of L1210 leukemic mice. Male B6D2F$_1$J mice were inoculated intraperitoneally with 10^5 L1210 cells on day 0, and, after being randomly assigned to cages in groups of five, were treated with various doses of mAPA-HCysA by intraperitoneal injection in sterile 0.9% sodium chloride, starting on day 1. One group of animals was treated on an intermittent schedule (q4d×3), a second was treated daily (qd×9), and a third was treated twice daily (bid×10) except that single doses were given on days 5 and 6. Methotrexate was used as a positive control in each experiment. Animals were weighed on days 1 and 7, and weight changes were recorded as an index of toxicity. The day of death of each animal was recorded, and the increase in mean survival was expressed as a percent of the untreated controls.

RESULTS AND DISCUSSION

Activity of mAPA-HCysA as an inhibitor of DHFR. To determine whether replacement of the γ-carboxyl group in MTX by a γ-SO_3H group would cause a decrease in affinity for DHFR, mAPA-HCysA was tested as an inhibitor of [^{3}H]MTX binding to DHFR from L1210 cells and also as an inhibitor of the enzymatic activity of *L. casei*/MTX DHFR. As indicated in Table 1, the concentration of mAPA-HCysA needed to inhibit [^{3}H]MTX binding to DHFR from L1210 cells by 50%, i.e. the IC_{50}, was essentially the same as the IC_{50} for MTX. In the

Table 1. Dihydrofolate reductase (DHFR) binding and cytotoxicity in culture of 4-amino-4-deoxy-N^{10}-methylpteroyl-D,L-homocysteic acid (mAPA-HCysA)

	Assay system		
Compound	L1210 DHFR* IC$_{50}$ (nM)	*L. casei* DHFR† IC$_{50}$ (nM)	L1210 cells IC$_{50}$ (μM)
mAPA-HCysA	1.0	17	0.30
MTX	0.95	10	0.01
Ratio (mAPA-HCysA/MTX)	1.1	1.7	30

* Competitive [^{3}H]MTX-binding assay (see Materials and Methods).

† Spectrophotometric assay at 340 nm (see Materials and Methods).

Table 2. Activity of mAPA-HCysA as a substrate for mouse liver FPGS*

	FPGS activity (nmoles/hr)	
Substrate	Experiment 1	Experiment 2
(A) MTX controls		
Pte Glu	1.40 ± 0.002	
MTX	1.42 ± 0.13	
(B) mAPA-HCysA		
Pte Glu	1.11 ± 0.004	0.65 ± 0.03
mAPA-HCysA	< 0.001	< 0.001
Pte Glu + mAPA-HCysA	0.61 ± 0.05 (45% inhibition)	0.37 ± 0.01 (43% inhibition)

* Each compound was incubated with mouse liver FPGS at 500 μM as indicated in the text. For incubations containing Pte Glu and mAPA-HCysA, each compound was present at 500 μM. Values are the mean ± S.D. of duplicate assays.

spectrophotometric assay using DHFR from *L. casei*/MTX, the ratio of the potency of inhibition by MTX to that by mAPA-HCysA was 1.7. The 10-fold difference in IC$_{50}$ in these assays was consistent with our previous experience with a number of structurally related compounds [41]. It must be noted that IC$_{50}$ data obtained spectrophotometrically are a measure of the ability of mAPA-HCysA or MTX to compete with dihydrofolate in a functional assay, whereas data obtained by competitive [^{3}H]MTX binding represent a measure of the interaction between the inhibitor and the enzyme in the absence of substrate. Nevertheless, the main conclusion to be drawn from Table 1 is that replacement of the glutamate side-chain in MTX by homocysteic acid does not appreciably diminish the affinity of the molecule for the binding site of either mammalian or bacterial DHFR.

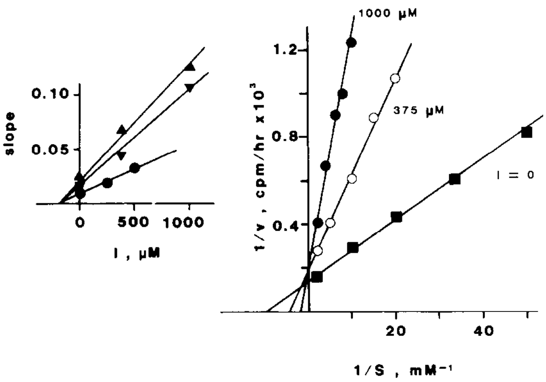

Fig. 2. Inhibition of mouse liver FPGS by mAPA-HCysA. FPGS was incubated with various concentrations of PteGlu in the presence or absence of mAPA-HCysA for 1 hr, and the product was isolated by charcoal adsorption. The numbers indicate the concentrations of inhibitor. Each point represents the mean of two determinations. Inset: Replot of the slope of double-reciprocal plots versus inhibitor concentration. The slope is expressed in units of μM·(cpm/hr)$^{-1}$. Each symbol represents a separate experiment.

Activity of mAPA-HCysA as a substrate and as an inhibitor of FPGS. mAPA-HCysA was tested as a substrate for FPGS in an *in vitro* assay utilizing partially purified enzyme from mouse liver [26]. In these experiments folic acid was used as a positive control to test the FPGS activity of each enzyme preparation. The maximum velocity of FPGS is similar using folic acid or any of the monoglutamyl folate cofactors as substrate [26]. As shown in Table 2, activity of mAPA-HCysA as a substrate for mouse liver FPGS was not detectable (< 0.1% of the activity of folic acid), while MTX and folate were found to have equivalent activity when tested at 500 μM. In the course of these experiments it was noted that the presence of equimolar mAPA-HCysA partially interfered with the FPGS reaction using folic acid as the substrate (Table 2).

A more detailed examination of this effect indicated that mAPA-HCysA inhibited mouse liver FPGS with a K_i of 190 ± 70 μM (mean of three separate experiments). From the double-reciprocal data of Fig. 2, inhibition can best be described as competitive, though it should be noted that, for each experiment, computer analysis indicated that inhibited and non-inhibited lines intersected slightly to the left of the ordinate and did not cross the ordinate at a common point.

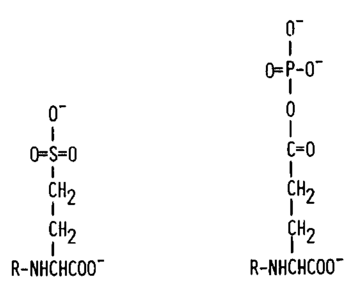

Fig. 3. Structural analogy between mAPA-HCysA and the putative phosphorylated intermediate in MTX and folate polyglutamation.

The reason that mAPA-HCysA was an inhibitor of FPGS is not established at this time. It is reasonable to suggest that mAPA-HCysA and MTX both bind to the enzyme, since a structural analogy exists between these compounds. This postulate is supported by a close agreement between the K_i for mAPA-HCys (190 ± 70 μM) and the apparent K_m for MTX (163 ± 31 μM), which we have determined using this preparation of mouse liver FPGS in related studies to be described elsewhere. However, since the covalent radius of sulfur is larger than that of carbon, the overall length of the side-chain is somewhat greater in mAPA-HCysA than in MTX. Furthermore, the γ-CO_2H carbon in MTX is sp_2-hybridized and trigonal, whereas the hydridization state of the γ-SO_3H sulfur in mAPA-HCysA is of a higher order as a result of *d*-orbital resonance and has a tetrahedral geometry. The hydridization state of the γ-SO_3H sulfur also may be compared with that of the similarly tetrahedral phosphorus in phosphate groups, where *d*-orbital resonance can occur as well. These considerations are relevant to the mechanism of action of mAPA-HCysA in that they suggest that a structural analogy, more important than the one between mAPA-HCysA and MTX, may be the one shown in Fig. 3, where mAPA-HCysA is compared to the phosphorylated species postulated by Coward [47] to be a transient intermediate in the polyglutamation reaction.

Cytotoxicity of mAPA-HCysA to leukemic cells in culture. The activity of mAPA-HCysA as an inhibitor of the growth of mouse L1210 cells *in vitro* was compared to that of MTX. As shown in Table 1, proliferation of L1210 cells in culture was inhibited by 50% after 48 hr of continuous exposure to a concentration of mAPA-HCysA 30 times greater than the equiactive dose of MTX. Since mAPA-HCysA was about as potent as MTX as a DHFR inhibitor (Table 1), this difference in cytotoxicity may well reflect the fact that only the MTX molecule can form polyglutamates. It is relevant in this regard that a good correlation has been reported between the ability of cultured tumor cells to form MTX polyglutamates and the toxicity of MTX as measured by recovery of DNA synthesis following resuspension of treated cells in MTX-free medium [17]. Another possibility that cannot be ruled out at this time is that the difference between the cytotoxicities of mAPA-HCysA and MTX reflects differences in membrane transport kinetics of the two drugs [48]. Since FPGS inhibition by mAPA-HCysA occurred only at concentrations > 10^4 times higher than those that inhibit DHFR, it is unlikely that this factor contributed much to the effect of mAPA-HCysA at the doses that inhibited cell growth. However, it may well be possible in the future to develop analogues of this prototype compound that will inhibit DHFR and FPGS at comparable doses, thus allowing concomitant depletion of intracellular tetrafolate pools via two distinct mechanisms.

Antitumor activity of mAPA-HCysA. The *in vitro* enzymological and cytotoxicity data in Tables 1 and 2 and Fig. 2 suggested that the *in vivo* antitumor activity of mAPA-HCysA might approach that of MTX but that any facet of drug action attributable to polyglutamation in the case of MTX might not be operative with mAPA-HCysA. To test this prediction we compared the effects of three different schedules on the ability of mAPA-HCysA to prolong the survival of L1210 leukemic mice. As shown in Table 3, treatment with mAPA-HCysA at 240 mg/kg (q4d×3) was non-toxic (<10% weight loss at 7 days), and there was a moderate increase in lifespan (ILS) of 42%. On this intermittent regimen MTX was more effective than mAPA-HCysA, giving a +115% ILS at the maximum tolerated dose of 60 mg/kg. It is very possible that longer survivals would have been achieved with higher doses of mAPA-HCysA. To conserve material, however, we chose not to exceed 240 mg/kg and instead gave the drug daily (qd×9). At 120 mg/kg of mAPA-HCysA the ILS was +98%, as compared with +119% for MTX at 2 mg/kg. At 180 mg/kg (qd×9) the ILS of mAPA-HCysA decreased to +75%, and there was a 10% weight loss indicative of some toxicity. A similar weight loss was observed with MTX at 4 mg/kg (data not show). When the frequency of drug administration was increased further, to twice-daily injection with an 8-hr interval between doses, a marked reduction in therapeutic dose was observed for mAPA-HCysA, but less so for MTX, in comparison with once-daily treatment. Whereas a +75% ILS required a total dose of mAPA-HCysA of 1080 mg/kg on the qd×9 schedule, the same lifespan increase could be achieved with less than one-third the total dose when the drug was given bid×10. These findings suggest that, in contrast to MTX, the therapeutic efficacy of mAPA-HCysA is enhanced by administering the compound more often. Since it cannot form polyglutamates inside the cell, one would expect that as the drug is excreted and circulating levels in the plasma decline, intracellular mAPA-HCysA in excess of the DHFR level would also fall rapidly to a concentration below that required for sustained DNA synthesis inhibition [17]. If this assumption is correct, the optimal therapeutic utilization of mAPA-HCysA may prove to be on a q3h schedule or by continuous infusion.

Two additional points relating to our *in vitro* and *in vivo* results deserve comment. One is that there is a rather good correlation between the 30-fold difference in the cytotoxicities of mAPA-HCysA and MTX *in vitro* and the 30- to 60-fold difference in dose to achieve either an arbitrary therapeutic endpoint (e.g. a doubling of survival) or a toxic level *in vivo*. This suggests that the factors responsible for

Table 3. Antitumor activity of 4-amino-4-deoxy-N^{10}-methylpteroyl-D,L-homocysteic acid (mAPA-HCysA) against L1210 leukemia in mice*

Schedule	Compound	Dose (mg/kg)	No. of mice	7-Day wt change (%)	Mean day of death	ILS (%)
q4d×3	None		11	+15	8.5	
	mAPA-HCysA	120	5	+12	10.2	+20
		180	5	+8	11.2	+32
		240	5	+15	12.0	+42
	MTX	60	5	−1	18.2	+115
qd×9	None		11	+8	8.6	
	mAPA-HCysA	60	5	0	13.6	+59
		120	5	−7	17.0	+98
		180	5	−10	15.0	+75
	MTX	2	5	−2	18.8	+119
bid×10	None		31	+15	9.4	
	mAPA-HCysA	8	5	+1	13.6	+45
		16	5	0	17.0	+81
		24	5	0	18.8	+100
		32	5	−1	17.6	+87
	MTX	1	5	+4	16.8	+79

* Groups of male B6D2F$_1$J mice were inoculated i.p. with 10^5 L1210 cells on day 0, and treatment was begun on day 1. Drugs were dissolved in water and administered i.p. In the experiment using the bid×10 schedule, the animals received only a single dose (doubled) on days 5 and 6. The doses of MTX listed are the highest that gave a 7-day weight loss of less than 10%.

this difference at the cellular level are probably also the basis for this difference in the intact animal, and that gross differences in pharmacokinetics and distribution between mAPA-HCysA and MTX are not likely. The second point is that all the data reported here are for the D,L- rather than the L-homocysteic acid adduct of 4-amino-4-deoxy-N^{10}-methylpteroic acid. It has been shown previously that the D-enantiomer of MTX binds almost as tightly to DHFR as the L-enantiomer, but that it is much less potent *in vivo* [49]. The low cytotoxicity of D-MTX in culture has been ascribed to poor uptake, which we believe reflects, in part, the fact that it has less than 2% of the substrate activity of L-MTX for FPGS (R. G. Moran, P. D. Colman, A. Rosowsky, R. A. Forsch and K. K. Chan, manuscript in preparation). In agreement with this, we have found that approximately 30 times more D- than L-MTX must be given on a qd×9 schedule to achieve a comparable 50–60% ILS in L1210 leukemic mice (A. Rosowsky, R. A. Forsch, and M. Wick, unpublished results). On the other hand, since neither the D- nor the *L*-enantiomer of mAPA-HCys would be expected to form polyglutamates, antitumor activity differences between these enantiomers should be much smaller than those between D- and L-MTX. Moreover, the activity of the D,L mixture should be similar to that of the individual mAPA-HCysA enantiomers.

In summary, mAPA-HCysA is a new structural analogue of MTX that has the interesting property of being a classical antifolate while lacking the ability to form polyglutamates. Reflecting this, mAPA-HCysA had a high DHFR affinity, comparable to that of MTX itself, but had a lower molar potency than MTX, both as an inhibitor of cell proliferation in culture and as an antitumor agent in mice. However, when the frequency of drug administration was increased, the molar potency of mAPA-HCysA rose markedly and the drug was well tolerated. This is in contrast to MTX, whose efficacy was not improved, on this type of schedule. In addition to its ability to block DHFR, mAPA-HCysA also appears to be a competitive inhibitor of FPGS, and this offers the attractive possibility of a dual mechanism of action involving simultaneous blockade of tetrahydrofolate synthesis and decreased conversion of various reduced folates to their polyglutamates. Further chemical and pharmacological studies with this compound, and others related to it, might result in a new class of self-potentiating antifolates with a spectrum of toxicity different from that of MTX.

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