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New folate analogs of the 10-deaza-aminopterin series

Basis for structural design and biochemical and pharmacologic properties

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Summary. *Structural modification of the N^{10} position of 4-amino folates affects mediated membrane transport in mammalian cells but has little or no effect on target enzyme (dihydrofolate reductase) inhibition. Some of these modifications have been associated with differential effects on transport in various cell types in a manner which favored greater accumulation and persistence of drug in reponsive tumor cells than in normal proliferative tissue. With the aim of identifying new structures with greater potential for differential mediated accumulation, we have studied three new 10-alkyl analogs of 10-deaza-aminopterin. Two of these analogs showed therapeutic efficacy substantially greater than 10-deaza-aminopterin, an analog with antitumor properties superior to methotrexate. These analogs, the 10-methyl, 10-ethyl, and 10,10-dimethyl derivatives, were equivalent to the parent compound, 10-deaza-aminopterin, and aminopterin, and slightly more potent than methotrexate, as inhibitors of L1210 cell dihydrofolate reductase. The three new analogs, 10-deaza-aminopterin, and aminopterin exhibited similar transport properties in L1210, Ehrlich, and S180 cells. Efflux and influx V_{max} were similar to those of methotrexate, but influx K_m was 4- to 14-fold lower than for methotrexate. That is, substitution at N^{10}, but not at C^{10}, reduced influx potential in these tumor cells. These differences in transport properties among this group of analogs which determine net accumulation were reflected in the individual values for growth-inhibitory potency. In contrast to that seen in tumor cells, alkylation at both N^{10} and C^{10} reduced influx potential (increased K_m) in isolated intestinal epithelial cells from mouse small intestine. Influx was in the order aminopterin > 10-deaza-aminopterin with further reduction in each series showing a magnitude in proportion to the size of the 10 substituent. Otherwise, influx V_{max} and efflux were similar for the group. Accumulation of polyglutamates in small intestine was greater following aminopterin administration than following administration of other analogs (10-ethyl, 10-deaza-aminopterin < methotrexate < 10-deaza-aminopterin). Polyglutamate accumulation for all the analogs was greater in tumor cells, but accumulation of each varied between the two tumors (L1210 and S180) examined. Differences among the analogs were not as great in L1210 as in S180 cells, and their metabolism was not in the same relative order. Plasma pharmacokinetics for 10-methyl and 10-ethyl derivatives were similar to those for 10-deaza-aminopterin and methotrexate. The 10,10-dimethyl analog was cleared more rapidly.*

As in our prior reports, a greater selective action of some of the new analogs was associated with increased persistence of the analog in tumor versus small intestine. The greatest differential in persistence was found with analogs which had a lower value for influx K_m in tumor but a higher value in intestinal epithelium. Two analogs (10-deaza-aminopterin and 10-ethyl, 10-deaza-aminopterin) which exhibited identical transport properties but different extents of polyglutamylation had pharmacokinetics in tumor that were indistinguishable. However, the analog that was more rapidly polyglutamylated (10-ethyl, 10-deaza-aminopterin) was therapeutically more effective.

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Introduction

The importance of mediated transport as a determinant of cytotoxic action of folate analogs in murine and human systems has been documented in numerous reports from our own laboratory and elsewhere [for reviews see 7, 8, 13, 18, 19]. We have also provided evidence [3, 14–16, 20] which strongly suggests that differences in the ability of tumor cells versus normal proliferative tissue to transport these agents accounts, to at least a major extent, for the selectivity of antitumor action observed. In studies involving a number of folate compounds with various structural modifications we examined by kinetic analysis the specificity of membrane transport vis-à-vis target enzyme (dihydrofolate reductase) inhibition. The results of these studies [3, 7, 13] showed that modification of a variety of positions on the folate molecule affected transport, with little or no effect on target enzyme inhibition. Moreover, modification at the N^{10} position in particular was associated with differential effects on transport in different cell types in a manner which often favored greater accumulation and persistence of drug in responsive tumor cells than in normal proliferative tissue. On the basis of these results, a new analog, 10-deaza-aminopterin, was synthesized [5] and subsequently shown [21] to be more effective than methotrexate against a group of murine tumors in vivo. Since the superiority of 10-deaza-aminopterin in these models was predictable by the results of [22] of our biochemical and pharmacologic studies, analogs of this compound were synthesized [6] for study. The results of therapy experiments given in the companion paper to this report [23] showed further improvement in the antitumor efficacy of some of these analogs against both ascitic and solid murine tumors. We now report on relevant biochemical and pharmacologic properties of these analogs and also provide data on their relative cytotoxic potency against murine tumor

cell lines in culture. Our results continue to be consistent with the notion that greater therapeutic efficacy of some of these analogs is determined to a major extent at the level of mediated transport. In addition, some of our results documenting differences in the extent of polyglutamylation of these analogs indicate a possible role for this metabolism also.

Materials and methods

Most of the methodology employed in these studies has already been described [13] in detail in a previous report. Maintenance of the various ascites tumors and their harvesting from the peritoneal cavity of female $BD2F_1$ mice (Sprague-Dawley, Inc., Madison, WI) has already been described [13–15]. Epithelial cell suspensions were prepared [3, 13] by hyaluronidase treatment of mucosa in everted small intestine. This provides metabolically active, transport-competent cells. During the isolation of a 2- to 3-fold purification of crypt cells occurs so that the final suspension consists [3] of approximately equal amounts of crypt and mature absorptive (columnar) cells.

During transport experiments with tumor cell and epithelial cell suspensions, data on initial influx, net accumulation, and efflux are obtained during measurements of flux at 37° C in buffered salts medium [13, 16] plus 7 m*M* glucose (pH 7.4). A double reciprocal plot of the influx data (v/[drug]) obtained at different external drug concentrations was constructed to obtain values for maximum velocity V_{max}^{37} and apparent Michaelis constant (K_m). Values for influx K_i were derived from similar data measuring influx of [^{3}H] methotrexate in the presence and absence of competing analog and using the following calculation ($K_i = [I]/K_p/K_m$), where K_p is the apparent K_m in the presence of inhibitor. Efflux of drug was expressed as value for the first-order rate constant ($K^{37°C} = min^{-1}$) derived [13, 16] during measurements of initial efflux. In the case of both fluxes, precautions [13, 16] were taken to ensure measurement of unidirectional passage of substrate through the membrane.

During pharmacokinetic experiments blood was obtained from the orbital sinus, tumor cells were harvested from the peritoneal cavity, and small intestine was removed and washed as already described [13–15] at various times after the SC administration of drug to tumor-bearing animals (2–4 days after IP implantation of 10^6 cells). During transport and pharmacokinetic experiments levels of tritiated samples of drug were measured [13] following extraction by scintillation spectrophotometry. Otherwise drug levels were measured directly by titration inhibition assay [13] using a microbial dihydrofolate reductase [13]. The dihydrofolate content of various tissue was determined [13] by the same titration inhibiton assay on tissue extracts. Inhibition of dihydrofolate reductase by various folate analogs was also carried out by a titration inhibition assay and the data analyzed by the procedure of Henderson [9] to derive values for K_i applicable to tight-binding inhibitors.

All folate analogs [see companion report, 23, for structural formulas] were prepared as the sodium salt in aqueous solution. The synthesis of 10-deaza-aminopterin and the 10-alkyl analogs has been described elsewhere [23]. All samples were > 95% pure as determined [23] by UV spectroscopy and high-performance liquid chromatography. Samples of methotrexate and aminopterin used during these studies were provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. [^{3}H]Methotrexate (specific activity = 20 Ci/mmol), [^{3}H]aminopterin (specific activity = 12 Ci/m*M*), and [^{3}H]10-deaza-aminopterin (specific activity = 12 Ci/m*M*) were purchased from Moravek Biochemicals, City of Industry, CA; they were purified prior to use and stored at −70° C. Radiochemical purity was > 98%. All other chemicals were reagent grade.

Cell culture procedures have been described in detail in one of our earlier [4] reports. For growth inhibition studies RPMI medium was supplemented with 12% horse serum, 2.2 μ*M* folic acid, 2 m*M* glutamine, 1 m*M* sodium pyruvate, and 0.05 m*M* 2-mercaptoethanol.

Analysis for polyglutamates of methotrexate and other folate analogs was carried out [2] by reverse-phase high-performance liquid chromatography. The basic unit was supplied by Waters Associates (Milford, MA) and included a systems controller, data module and automatic (WISP) injector, two pumps, and variable-wavelength CV detector. Samples were extracted [13] and filtered and injected onto a Waters μBondapak C_{18} reverse-phase column. The solvent system consisted of a linear acetonitrile gradient (5%–15%) in 0.1 *M* sodium acetate buffer, pH 5.1. The flow rate was maintained at 1 ml and fractions collected at 30-s intervals for 20 min. For radioactive analogs the content of individual fractions was determined by scintillation spectrophotometry. For nonradioactive analogs the content of individual fractions was assayed by titration inhibition assay with dihydrofolate reductase [13]. Standards were collected and monitored by UV detection (313 n*M*).

Results

Membrane transport and inhibition of dihydrofolate reductase

The identity of the N^{10} position on the 4-amino folate molecule as relevant to selective action of this category of agent was originally derived from studies [3, 18, 19] which evaluated structural modifications at this site in terms of two biochemical parameters, membrane transport, and dihydrofolate reductase. Both these parameters are major determinants of the cytotoxicity [7, 8, 13, 18, 19] of these agents. However, structural modifications which had little or no effect on inhibitory potency [18, 19] of these agents for the enzyme target were found [13, 19] to affect transport and related pharmacokinetic parameters in tumor cells and normal proliferating tissues in a manner which correlated with relative responsiveness in a variety of murine tumor models. On this basis, the parent compound of this new series, 10-deaza-aminopterin, was synthesized [5] and similarly evaluated [19, 22]. The increased therapeutic potential predicted from the results of this evaluation was in fact demonstrated [21] by the appropriate trials in animals. In a similar fashion we have evaluated three new 10-alkyl analogs of this series, 10-methyl, 10-ethyl and 10,10-dimethyl derivatives of 10-deaza-aminopterin, and have attempted to interpret the results in terms of relative inhibitory potency for growth of tumor cells in culture.

A summary of biochemical studies carried out with L1210 cells is shown in Table 1. As inhibitors of dihydrofolate reductase at a pH in the neutral range (pH 7.3), all of the new analogs were slightly better than methotrexate but approximately equivalent to aminopterin. This extends our earlier results [18, 19, 22] showing minimal effects of either alkylation

Table 1. Biochemical properties of aminopterin, methotrexate, and 10-deaza-aminopterin analogs in L1210 cells

Analog	Dihydrofolate reductase inhibition[a] K_i ($n = 3-5 \pm$ SE) (pM)	Mediated transport ($n = 3-5 \pm$ SE)			
		Influx		Efflux $K^{37°C}$ (min^{-1})	Net $[drug]_{int}$ (μM)
		K_m (μM)	37° C V_{max}[b]		
Aminopterin	2.3 ± 0.2	1.18 ± 0.2	7.83 ± 1.1	0.21 ± 0.02	6.4 ± 0.7
Methotrexate	3.8 ± 0.3	3.84 ± 0.2	7.77 ± 0.9	0.19 ± 0.02	1.8 ± 0.2
10-deaza-aminopterin	2.1 ± 0.1	0.93 ± 0.1	8.13 ± 0.7	0.23 ± 0.03	6.8 ± 0.6
10-CH_3,10-deaza-AM	2.4 ± 0.2	0.97 ± 0.2	8.02 ± 0.6	0.23 ± 0.01	6.6 ± 0.8
10-C_2H_5,10-deaza-AM	2.8 ± 0.3	0.89 ± 0.1	7.80 ± 1.1	0.18 ± 0.02	6.5 ± 0.6
10-$(CH_3)_2$,10-deaza-AM	2.6 ± 0.3	0.95 ± 0.9	8.31 ± 0.9	0.24 ± 0.03	6.7 ± 0.7

[a] Done at pH 7.3, and data analyzed according to the method of Henderson [9]
[b] nmol/min per g dry wt
[c] $[drug]_{ext} = 0.2\ \mu M$. Total intracellular drug less drug bound to dihydrofolate reductase (2.92 ± 0.3 nmol/g dry wt) as determined from measurements [Ref. 3] of nonexchangeable drug

of N^{10} or a C-for-N substitution at this position on this enzyme inhibition. Alkylation of C^{10} apparently has no effect on inhibitory potency. We have also noted (data not shown) a pH dependence for inhibition for the C^{10} alkyl analogs similar to that we reported earlier for 10-deaza-aminopterin and we [22] and others [for review see 2] reported for the two N^{10} analogs. All the analogs are more potent inhibitors at pH 6.2 and less potent at pH 8.0.

A summary of the data from experiments measuring mediated membrane transport of this group of compounds is also shown in Table 1. In these experiments influx for all of the analogs was saturable and efflux was first order (nonsaturable). Since net intracellular accumulation of folate analogs reflects [7, 8, 13, 18, 19] values for three kinetic parameters (influx V_{max} and K_m and the efflux rate constant), all three were derived for each analog in these experiments. Values for both influx V_{max} and the efflux rate constant were essentially identical among this group of compounds. However, values for influx K_m for aminopterin and all the 10-deaza analogs were 3- to 4-fold lower. Thus, in contrast to that seen for alkylation at the N^{10} position, alkylation at C^{10} was without effect on influx. In competition experiments measuring the effects of each analog on [^{3}H]methotrexate transport, values for influx K_i were derived (Table 2) that were essentially indistinguishable from the values for influx K_m. We conclude from all these data that aminopterin and all the new analogs are better substrates for transport inward by the same system which has 5-methyltetrahydrofolate as its natural substrate [7, 8, 13, 18, 19]. In view of the results showing no difference among these compounds during efflux, the same conclusion can also be derived from data on net accumulation of drug (Table 1) at external concentrations in the limiting range ($< K_m$). Exchangeable levels (unbound to dihydrofolate reductase) at steady state during incubation at a concentration of 0.2 μM aminopterin and the 10-deaza analogs were 3- to 4-fold higher than for methotrexate.

In other competition experiments, we obtained similar data (Table 2) signifying relative influx potential among these analogs in Ehrlich and S180 cells. Values for K_i (versus [^{3}H]methotrexate) for aminopterin and all the 10-deaza-aminopterin analogs derived with these two cell types were essentially the same as values derived with L1210 cells. However, corresponding values for methotrexate were 2- to 3-fold (Ehrlich) and 3- to 4-fold (S180) greater than that derived with L1210 cells. These results reaffirm the reduced potential for mediated influx of methotrexate and the similar potential for mediated influx of aminopterin shown for these two tumors compared with L1210 cells in our earlier [22] studies. However, as in the case of L1210 cells, whereas the effect of alkylation at N^{10} was to reduce influx, alkylation at C^{10} had no effect at all on influx in these cell types. In light of other results (footnote to Table 2) which document minimal differences in values for influx V_{max} or efflux among these analogs in each cell type, these data suggest larger differences in net accumulation for all these analogs compared with methotrexate in Ehrlich (8- to 10-fold) and S180 (12- to 14-fold) cells compared with L1210 (3- to 4-fold) cells. Actual measurements of net accumulation for some of these analogs (data not shown) showed this to be the case.

Table 2. Summary of data on influx properties of folate analogs in L1210, S180, and Ehrlich cells

Analog	Influx K_i ($n = 3-5 \pm$ SE)		
	L1210[b] (μM)	Ehrlich[b] (μM)	S180[b] (μM)
Aminopterin	1.08 ± 0.1	1.43 ± 0.2	1.46 ± 0.2
Methotrexate	3.57 ± 0.4	8.42 ± 1.3	11.80 ± 1.5
10-deaza-aminopterin	0.86 ± 0.1	0.89 ± 0.1	0.97 ± 0.2
10-CH_3,10-deaza-AM	1.07 ± 0.2	0.93 ± 0.2	0.93 ± 0.2
10-C_2H_5,10-deaza-AM	0.99 ± 0.1	0.86 ± 0.1	0.84 ± 0.1
10-$(CH_3)_2$,10-deaza-AM	0.86 ± 0.1	0.97 ± 0.1	0.87 ± 0.1

[a] Derived by experiments measuring competition of mediated influx of [^{3}H] methotrexate [3]
[b] Values for influx V_{max} (μM) for [^{3}H] methotrexate, [^{3}H] aminopterin, and [^{3}H] deaza-aminopterin were 7.48 ± 0.9 (L1210), 4.91 ± 0.7 (Ehrlich), and 8.23 ± 1.1 (S180). Values for efflux rate constant (min^{-1}) were 0.213 ± 0.2) (L1210), 0.208 ± 2 (S180), and 0.169 ± 0.0 (Ehrlich)

Some validation of the predictiveness of this biochemical data (Tables 1 and 2) for relative antitumor potency was derived from studies of growth inhibition in cell culture. This is shown in Table 3. Inhibitory potency for aminopterin and the 10-deaza analogs were equivalent, but greater than methotrexate in all three cell lines, as predicted by the biochemical data. Also, the increased potency of these compounds compared with methotrexate were in the same relative order, i.e., 3- to 4-fold (L1210), 8- to 9-fold (Ehrlich), and 10- to 12-fold (S180).

In contrast to the results of transport studies carried out with these murine tumor cells, data derived in similar studies with isolated intestinal epithelial cells showed substantial differences among the new analogs. These results are summarized in Table 4. Mediated transport in these isolated cell suspensions was similar [3] to that derived with tumor cells. That is, influx is a saturable process and efflux is nonsaturable. Also, influx exhibits lower saturability than is seen in tumors. A reduced influx of methotrexate compared with aminopterin in these cells, as indicated by the large difference in the value for influx K_m, has already been reported [3] from this laboratory. A value for 10-deaza-aminopterin was also reported [19, 22] by us, which was intermediate between that derived for the two N^{10} analogs. The results in Table 4 show that alkylation of C^{10}, like alkylation of N^{10}, markedly increased the value for influx K_m in these cells. The increase was in the order dimethyl $\cong$ ethyl $<$ methyl. Since no differences were observed among the entire group of compounds in any other kinetic parameter (influx V_{max} and efflux constant), these differences in value for influx K_m alone determine the relative net accumulation of these analogs in intracellular water. Limiting toxicity in this rodent species is associated [10] with antifolate effects in crypt cell epithelium of small intestine rather than proliferative elements in bone marrow. In the case of methotrexate this seems related [3, 12, 17] more to transport than to polyglutamylation of drug (polyglutamates of methotrexate accumulate to a 3-fold higher level in bone marrow than in small intestine). From an examination of data on toxic potency of these compounds given in the companion report [23], it can be seen that the analogs exhibiting lower values for influx K_m are more potent and the rank order is the same when based on values for the reciprocal of the influx K_m or toxic potency (the extremely low toxicity of the 10,10-dimethyl analog reported by us [23] can also be attributed to more rapid plasma clearance; see below). Similarly, the relationship between mediated entry (influx K_m) in intestinal epithelial cells versus tumor cells for this group of analogs is significant for relative therapeutic efficacy. The normal/tumor ratios of values for influx K_m are summarized in Table 4 (column 4). Analogs showing the smallest ratio are least effective [13, 15, 23] against these tumors in vivo. Excluding the 10,10-dimethyl derivative, which is a special case, the order of ranking in therapeutic efficacy is approximately the same as that shown in Table 4 (column 4).

Table 3. Summary of data on inhibition of growth of L1210, Ehrlich, and S180 cells in culture by folate analogs

Analog	Growth inhibition[a] (IC_{50})		
	L1210[b] (μM)	Ehrlich[b] (μM)	S180[b] (μM)
Aminopterin	0.81 ± 0.2	0.86 ± 0.1	0.92 ± 0.2
Methotrexate	2.92 ± 0.3	8.31 ± 1.7	9.84 ± 1.9
10-deaza-aminopterin	0.79 ± 0.1	1.05 ± 0.3	0.75 ± 0.1
10-CH_3,10-deaza-AM	0.71 ± 0.2	0.96 ± 0.2	0.82 ± 0.3
10-C_2H_5,10-deaza-AM	0.65 ± 0.1	0.91 ± 0.3	0.92 ± 0.2
10-$(CH_3)_2$,10-deaza-AM	0.88 ± 0.3	0.99 ± 0.2	1.12 ± 0.1

[a] $n = 3-5 \pm$ SE

Table 4. Summary of data on mediated membrane transport of folate analogs by isolated murine intestinal epithelial cells

Analog	Mediated transport ($n = 3-5 \pm$ SE)			
	Influx		Efflux K (min^{-1})	Ratio K_m (epithelial)/ K_m (tumor)[a]
	K_m (μM	V_{max} (mol/min per g dry wt)		
Aminopterin	18 ± 3	3.21 ± 0.4	0.089 ± 0.01	12– 17×
Methotrexate	239 ± 37	2.88 ± 0.3	0.076 ± 0.01	25– 80×
10-deaza-aminopterin	82 ± 11	2.87 ± 0.5	0.091 ± 0.02	85– 96×
10-CH_3,10-deaza-AM	268 ± 42	3.04 ± 0.4	0.083 ± 0.01	250–290×
10-C_2H_5,10-deaza-AM	398 ± 47	2.92 ± 0.2	0.079 ± 0.01	395–470×
10-$(CH_3)_2$,10-deaza-AM	406 ± 34	3.12 ± 0.3	0.084 ± 0.02	420–470×

[a] Data from Table 2

Table 5. Plasma clearance of folate analogs in mice given 12 mg/kg SC

Analog	Plasma concentration (ng/ml) t (h)							
	0.25	0.5	1	2	3	7	16	24
Methotrexate	5,803	18,122	6,345	850	56	28	14	10
10-deaza-aminopterin	4,990	20,050	7,560	910	49	29	13	9
10-CH_2,10-deaza-AM	5,330	15,750	6,540	–	46	28	15	10
10-C_2H_5,10-deaza-AM	4,406	20,781	5,850	–	55	38	21	13
10-$(CH_3)_2$,10-deaza-AM	5,250	3,300	265	62	23	12	5	3

Experimental details are given in the text. Each value shown is an average of individual determination done on two to four mice on at least 2 different days (SE ± 21%)

Plasma and tissue pharmacokinetics

All of the analogs in the 10-deaza-aminopterin series were examined for their plasma clearance kinetics in mice. These results are summarized in Table 5. All but one, the 10,10-dimethyl, 10-deaza-aminopterin analog were cleared in a manner identical to methotrexate. The dimethyl analog was cleared more rapidly. From data shown in Fig. 1, it can be seen that the difference occurred during the early phase of clearance associated [1] with biliary and renal secretion. The approximate values for $t_{1/2}$ derived for this phase by 'exponential peeling' were 26 min for methotrexate, but only 5 min for the 10,10-dimethyl derivative. At the onset of the 'reabsorptive phase' [16], at approximately 7 h, levels of this alkyl analog were approximately 5-fold lower than for methotrexate. The rate of clearance ($t_{1/2}$ = 8 h) of the new analog during this phase was identical with that of methotrexate. More rapid clearance of the 10,10-dimethyl derivative accounts to a large measure for the markedly lower toxic protency of this analog than of methotrexate, 10-deaza-aminopterin, and the other derivatives of 10-deaza-aminopterin.

A positive correlation between the extent of persistence of exchangeable levels of folate analogs in various murine proliferative tissues (normal and tumorous) and their cytotoxicity for these tissues has been documented [for review see 3] in numerous reports from this laboratory. Greater persistence of methotrexate at pharmacologically effective levels in cells of responsive tumors than in normal proliferative tissues appeared to account [13–15] for the selectivity of effects against tumor. In the current studies evidence was obtained for an even larger differential in persistence of 10-deaza-aminopterin and the 10-alkyl derivatives in cells from tumors that showed a greater response to these agents than to methotrexate. A representative experiment is described in detail in Fig. 2. Methotrexate or 10-deaza-aminopterin was given SC to L1210-bearing mice at a dose of 12 mg/kg. In small intestine, the time-course for persistence of each drug was similar. In both cases, intracellular levels approached the equivalence of the nonexchangeable level in approximately 6 h. In L1210 cells the individual time-courses were different. 10-Deaza-aminopterin persisted at levels higher than methotrexate during the 24 h period over which measurements were done. Administration of a small dose of methotrexate (0.6 mg/kg) in these experiments made it possible to differentiate the nonexchangeable fraction of drug from the exchangeable fraction existing following the administration of a higher dose of either antifolate. At 6 h the ratio of exchangeable level to plasma level was approximately 9 for the new analog and only 4–5 for methotrexate. In S180 cells exchangeable levels with time for 10-deaza-aminopterin were similar (Fig. 1) to that shown for L1210 cells (exchangeable intracellular/plasma level = 8–9). Levels after the same dose of methotrexate were much lower in S180 cells than in L1210 cells. Intracellular levels approached enzyme level by 6 h and the nonexchangeable level could be defined from the time-course at this dosage. At 6 h the ratio of intracellular exchangeable methotrexate to plasma level was approximately 1. A similar experiment (data shown for L1210 cells and small intestine) was carried out with 10-ethyl, 10-deaza-aminopterin (12 mg/kg SC) with the same result (Fig. 1) as that shown for 10-deaza-aminopterin. Since higher doses of the 10-methyl- or 10-ethyl analogs of 10-deaza-aminopterin are tolerated [23] in these animals, the net persistence of these analogs in tumor cells compared at equitoxic doses to 10-deaza-aminopterin and methotrexate would be expected to be somewhat greater than that shown for equimolar dosages in vivo.

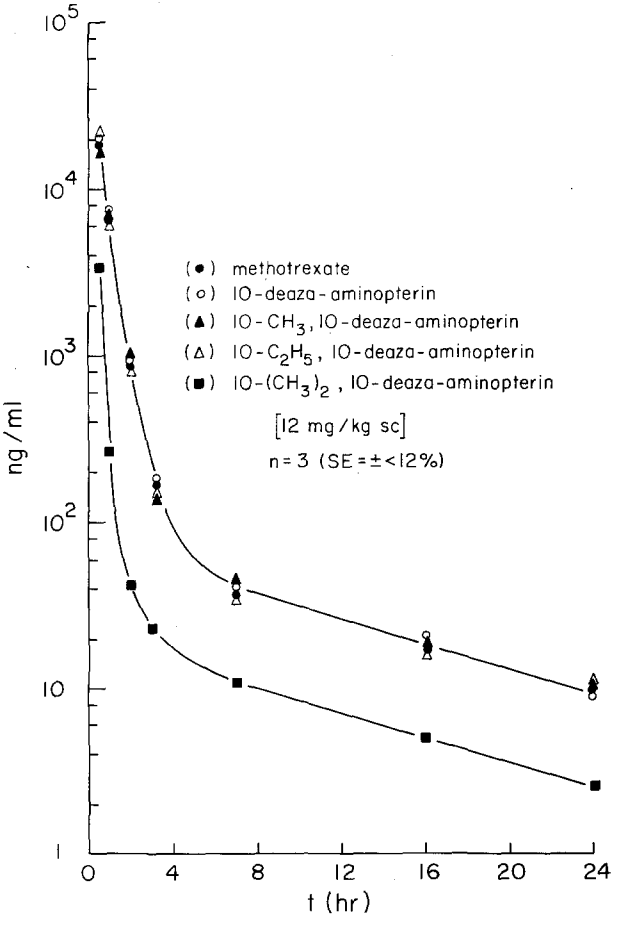

Fig. 1. Plasma pharmacokinetics for various folate analogs in mice after SC administration

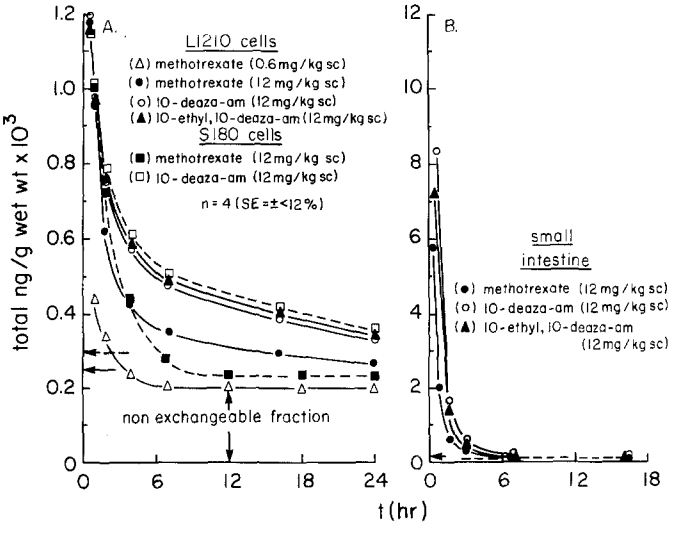

Fig. 2. Pharmacokinetics of methotrexate and 10-ethyl, 10-deaza-aminopterin in small intestine and tumor cells after SC administration to tumor-bearing mice. Initial dihydrofolate reductase-binding equivalence is indicated on the vertical axis for L1210 cells (*solid arrow, A*), S180 cells (*broken arrow, A*) and small intestine (*arrow, B*)

Polyglutamylation of folate analogs

Although a reasonably good correlation can be shown for these analogs with respect to mediated transport in intestinal epithelial cells and their lethal potency, the biochemical data appear to suggest a lower lethal potency for aminopterin and a higher potency for 10-deaza-aminopterin than that shown [23] for the other analogs in this group. Similarly, the increased therapeutic index of the 10-ethyl derivative of 10-deaza-aminopterin compared with the parent compound cannot be explained by the modest difference in the tolerated dosage [23]. The two agents show identical transport properties and inhibition of dihydrofolate reductase at least in the tumors examined. For this reason, some of these analogs were studied for their net accumulation in tissue as a polyglutamylated form [12] following administration in vivo. There is considerable interest at present [for reviews see 12, 13] in 4-amino folylpolyglutamylation. Although a role for this metabolism in the pharmacologic action of this category of agents remains to be established, tissue-specific differences in the extent to which it occurs have been shown [12, 13]. Furthermore, it is conceivable that other differences could also occur in the extent to which polyglutamates of various analogs accumulate in these normal and tumor tissues. In these studies we examined both small intestine and tumor cells for polyglutamates 4 h after administration to tumor-bearing mice of aminopterin (3 mg/kg), methotrexate, (12 mg/kg), 10-deaza-aminopterin (12 mg/kg), or 10-ethyl, 10-deaza-aminopterin (12 mg/kg). The 4- to 6-fold lower dose of aminopterin compared with the other analogs was used to maintain approximate equivalence [15] in intracellular concentration. Although authentic standards were available only for polyglutamates of methotrexate (di-, tri-, and heptaglutamates) and aminopterin (diglutamate), polyglutamates of the other analogs were identified by means of their relative retention times, inhibition of dihydrofolate reductase, and sensitivity to carboxypeptidase. Our results are given in Table 6. In L1210 cells and S180 cells the accumulation of polyglutamates was higher than in small intestine after the administration of each of the analogs. Also, there were considerable differences among these tissues in the metabolism of the group of analogs. In small intestine the extent of polyglutamylation was in the order aminopterin > 10-ethyl, 10-deaza-aminopterin > methotrexate > 10-deaza-aminopterin (2.8 : 0.9 : 0.5 : 0.3). The same relative order of polyglutamylation was found in L1210 cells, but the differences were not as great. In S180 cells 10-ethyl, 10-deaza-aminopterin was polyglutamylated most rapidly and the relative order was 10-ethyl, 10-deaza-aminopterin > aminopterin > methotrexate > 10-deaza-aminopterin (5 : 4.3 : 1.9 : 1). Polyglutamate accumulation in L1210 and S180 cells was primarily in the form of the tri- and heptaglutamates for all four analogs. In small intestine accumulation occurred primarily as the triglutamate with approximately equal but lower levels of the di- and heptaglutamates.

Table 6. Recovery of folate analogs as polyglutamates in murine tissues 4 h after in vivo administration (SC)

Analog[a]	% as polyglutamate ($n = 2-3 \pm$ SE)		
	Small intestine	L1210	S180
Aminopterin	28.0 ± 5	79.0 ± 9	35.3 ± 5
Methotrexate	5.0 ± 2	51.0 ± 6	19.1 ± 4
10-deaza-aminopterin	3.2 ± 0.6	39.6 ± 6	10.5 ± 2
10-Ethyl,10-deaza-AM	8.7 ± 3	65.0 ± 8	42.8 ± 6

[a] Dosages: aminopterin, 3 mg/kg, methotrexate and 10-deaza-aminopterin, 12 mg/kg, and 10-ethyl, 10-deaza-aminopterin, 18 mg/kg

Discussion

Some of the 10-alkyl derivatives of 10-deaza-aminopterin exhibit [23] substantially increased therapeutic activity in murine tumor models compared with methotrexate and the parent compound of this new series, 10-deaza-aminopterin. From the results obtained in the present study, it appears that this increase is largely related to a greater differential effect of alkylation at C^{10} rather than at N^{10} on mediated transport in tumor versus normal proliferative tissue. Otherwise this group of new analogs, with the exception of the 10,10-dimethyl derivative, has properties similar to those of the N^{10} analogs. Although the 10-deaza-aminopterin analogs as a group are slightly better inhibitors of dihydrofolate reductase than methotrexate, they show approximate equivalence to aminopterin. Similarly, with one exception their plasma pharmacokinetics are indistinguishable from those observed [17, 22] for the N^{10} analogs. The more rapid clearance shown for the 10,10-dimethyl derivative has also been shown [11] for some N^{10} analogs with bulky substituents at the 10 position. The basis for these differences, other than that they appear to relate to increased biliary and (or) renal excretion, is not known. It is of interest to note that in some of our earlier [11] studies the presence of some alkyl substituents at N^{10} also reduced inhibition of dihydrofolate reductase.

From the current studies, it can be seen that the parent compounds in both the N^{10} and C^{10} series had essentially the same transport properties in tumor cells. Alkylation of either compound at the 10 position had no effect on efflux. However, alkylation of N^{10} reduced the potential for influx 3- to 10-fold (increased K_m) depending upon the tumor cell type, while alkylation at C^{10} had no effect on this flux. In intestinal epithelial cells, analogs of both the N^{10} and the C^{10} series exhibited identical efflux. However, the influx potentials of the parent compounds were different. Influx K_m for aminopterin was substantially smaller than for 10-deaza-aminopterin. Moreover, in contrast to that seen in tumor cells, alkylation at both N^{10} and C^{10} increased the value in influx K_m. The net effect of these differences in alkylation seen for th C^{10} series was a larger differential in mediated entry into tumor cells versus normal tissue compared with the N^{10} analogs. Further analysis of the transport data with respect to pharmacologic implications, i.e., beyond the correlation derived between values for influx K_m (tumor versus intestinal epithelial cells) and relative therapeutic efficacy [23] is not possible at this time. Data derived in these in vitro studies do not take into consideration differences in flux capacities which probably occur in vivo as against in vitro, particularly in the case of the isolated intestinal epithelial cells in which the proliferative cell component represents only a portion of the total cell suspension.

In addition to the biochemical parameters examined in the current study, these new analogs were also examined elsewhere [6] for their inhibition of thymidylate synthetase. In this earlier study employing a microbial enzyme, 10-deaza-aminopterin and its alkyl analogs were found to be equivalent to

methotrexate as inhibitors of this enzyme, with values for K_i varying in the range of $1-5 \times 10^{-6} M$. In view of the high inhibitory potency of these compounds against tumor cells in culture (Table 3), it is unlikely that direct inhibition of these analogs at this enzymic site plays a role in determining their increased potency relative to methotrexate. It would seem that their relative inhibitory potency in this in vitro growth inhibition assay against the three murine cell types examined can best be explained by their transport properties and the potential these determine for net accumulation of drug in intracellular water. It should be pointed out, however, that in the case of S180 and Ehrlich cells, we are assuming effects against dihydrofolate reductase which are equivalent to that seen for the L1210 cell enzyme, since only selected analogs were actually examined against these other two tumors. We are also assuming the same relative inhibitory potency of these new analogs against mammalian thymidylate synthetase which has been shown [6] for the microbial enzyme.

The results of in vivo experiments measuring net accumulation in tissue of four of these analogs as a polyglutamate are of interest. All the analogs examined appear to be substrates for folypolyglutamyl synthetase, but net accumulation of polyglutamates of each analog was consistently lower in small intestine than in tumor tissue, a result originally obtained [12] in our earlier studies with methotrexate. However, the N^{10}-substituted derivative accumulated to a lesser extent as a polyglutamate than the unsubstituted N^{10} analog. The opposite was true for 10-deaza-aminopterin and its C^{10} analog. In tumor cells, the overall level of accumulation was 4- to 5-fold greater 4 h after administration than in small intestine and differences among the analogs were similar, but varied in magnitude. The extent to which these differences relate to varying rates of synthesis mediated by folypolyglutamyl synthetase and/or hydrolysis by carboxypeptidase will require further study. But it is clear that analog specificity is a characteristic of net accumulation.

The interpretation of these results on polyglutamylation of these analogs in terms of their relative cytotoxic effects observed in each tissue following in vivo administration is difficult. The overall level of accumulation of these metabolites was lower in small intestine than in tumor cells on a weight basis. However, it should be noted that although the major fraction of this organ is epithelial tissue, the crypt cell compartment (site of drug-limiting toxicity) represents only a small fraction. If polyglutamylation of these analogs is limited to this proliferative compartment, but accumulation of each occurs in the total epithelial cell fraction, gross underestimation of the extent of this metabolism in the crypt cell compartment will result. Even so, it is of interest to note that the greatest accumulation of polyglutamates in small intestine occurred in the case of aminopterin, the most toxic analog [10, 13]. Also, the least accumulation occurred in the case of 10-deaza-aminopterin, an analog with a lower lethal potency than expected in view of the transport data. On the other hand, the least toxic analog (10-ethyl, 10-deaza-aminopterin) showed the second highest accumulation as a polyglutamate in this tissue.

Although accumulation of polyglutamates of three of these folate analogs was greater in L1210 cells than S180 cells, this should be viewed in light of our earlier [12] studies, which showed no consistent correlation between the extent of polyglutamylation of methotrexate and responsiveness among these two tumors and Ehrlich carcinoma. Yet the results showing differences among the four analogs are intriguing. In particular, the fact that 10-ethyl, 10-deaza-aminopterin was more extensively polyglutamated in tumor than the parent compound, while the reverse was true for small intestine, might in some way account for the greater than expected therapeutic effect of the 10-alkyl derivative and the lower than expected toxicity of the parent compound in light of their transport properties. The fact that the pharmacokinetics for these two analogs in both tumor cells and small intestine were indistinguishable argues against the notion that the difference was related solely to the production of a retentive form [see 21 for a discussion of this concept]. However, further work will be required for full assessment of a possible role for polyglutamylation in determining net cytotoxicity of these folate analogs at a pharmacologic level.

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