ABSTRACT

Four folate analogues, methotrexate, aminopterin, 10-deazaaminopterin, and 10-ethyl-10-deazaaminopterin were assessed for their ability to be metabolized to poly-γ-glutamyl derivatives in three tumor lines which vary in their sensitivity to these agents. Cytotoxicity of the four analogues against the murine L1210 leukemia and the human Manca B cell leukemia, as determined by a 3-h clonogenic assay, showed aminopterin and the two 10-deazaaminopterin compounds to be approximately equivalent for each cell type and were 3- to 10- (L1210) and 7- to 14-fold (Manca) more potent than methotrexate. In murine Sarcoma 180 cells, 10-ethyl-10-deazaaminopterin and aminopterin were similarly potent but were 5- to 10-fold more potent than 10-deazaaminopterin and 40- to 80-fold more potent than methotrexate. These results could be explained in part by the differences in transport properties and substrate activities for polyglutamyl derivatives of methotrexate and the two 10-deazaaminopterin compounds. In L1210 and Sarcoma 180 cells, the relative rates of polyglutamylation were in the order aminopterin > 10-ethyl-10-deazaaminopterin > methotrexate > 10-deazaaminopterin. In contrast, the relative rates of polyglutamylation in Manca cells were in the order 10-ethyl-10-deazaaminopterin = aminopterin > 10-deazaaminopterin > methotrexate, suggesting that folypolyglutamyl synthetase may have varying substrate preferences in different cell types. The maximum relative extents of total polyglutamate accumulation in L1210 cells were 85 to 95% of the total drug at 24 h. In Manca cells, the maximum polyglutamate accumulation was also 85 to 95%, but this was obtained by 6 h. However, in Sarcoma 180 cells, only aminopterin polyglutamates reached a similar maximum percentage of accumulation, while lower relative polyglutamate levels were achieved with the other analogues. Accumulation of individual polyglutamates in each cell line was similar for all analogues except aminopterin. For methotrexate and the two 10-deazaaminopterins, accumulation occurred mainly as the tetraglutamate or as higher polyglutamates. Aminopterin was accumulated mainly as the diglutamate, particularly in Manca cells where 70% of total drug was in the diglutamate form within the first 3 h and remained the predominant form for 24 h. Total intracellular drug continued to accumulate in all cell types during the 24-h period of drug exposure. This appeared to be accounted for by retention of longer-chain length polyglutamates. In L1210 cells incubated for 3 h with drug, efflux of methotrexate and aminopterin polyglutamates was found to be slower than the parent drug and decreased with the addition of polyglutamyl residues. In contrast, the efflux of methotrexate and aminopterin analogues from cells preloaded with these derivatives and the parent compounds was the same, and efflux overall was markedly more rapid.

INTRODUCTION

Baugh et al. (5) first demonstrated that methotrexate, like the naturally occurring folate compounds (4, 8), can be metabolized to poly-γ-glutamyl peptide derivatives. It is now well established (3, 12, 13, 15, 17, 18, 22, 23, 27, 34, 35) that polyglutamylation of folate analogues occurs in a variety of mammalian tissues including tumor tissue biopsied from patients treated with high dose methotrexate (25). These anabolites appear to be equivalent to the parent drug as inhibitors of DHFR (14, 16). Also, evidence for the "retentive" nature of at least the polyglutamates of methotrexate has been derived (2, 11, 12, 14, 18, 24) in the case of several tumor cell types in vitro, and these derivatives appear to be substantially more potent as inhibitors of the folate dependent enzymes, thymidylate synthase and aminopterin polyglutamates was found to be slower than the parent drug and decreased with the addition of polyglutamyl residues. In contrast, the efflux of methotrexate and aminopterin diglutamates from cells preloaded with these derivatives and the parent compounds was the same, and efflux overall was markedly more rapid.
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MATERIALS AND METHODS

Materials. [3',5',9-3H]Methotrexate and [3',5',9-3H]aminopterin with a specific activity of 10 to 20 Ci/mmol were obtained from Moravek Biochemicals (Brea, CA). [3',5',9-3H]-10-Deazaaminopterin (specific activity, 9 Ci/mmol) and [3',5',9-3H]-10-ethyl-10-deazaaminopterin (specific activity, 7 Ci/mmol) were purchased by special order from Moravek Biochemicals. These samples were purified by HPLC using a C18-reverse-phase column (Waters Associates, Menlo Park, CA) and eluted at 1 ml/min using a 15-min gradient from 5 to 25% methanol in water. Authentic standards of methotrexate polyglutamates (+G1, +G2, and +G3) and aminopterin diglutamate (+G2) were supplied by Dr. John A. Montgomery (Southern Research Institute, Birmingham, AL). Nonradioactive methotrexate and aminopterin were provided by the Drug Synthesis Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 10-Deazaaminopterin and its 10-ethyl analogue were synthesized (9) at SRI International, Menlo Park, CA. Thymidine and inosine were purchased from Sigma Chemical Co., St. Louis, MO, and glutamine was obtained from Grand Island Biological Co., Grand Island, NY. Aquasol liquid scintillation cocktail was purchased from New England Nuclear, Boston, MA. Aconitnitrile, purchased from Waters Associates, was of HPLC grade. All other chemicals were of reagent grade.

Cells and Incubation Techniques. L1210 murine leukemia, murine Sarcoma 180, and Manca human B-cell leukemia were maintained at 37°C in RPMI 1640 medium supplemented with 10% horse serum, 1% sodium pyruvate, and 0.05 mM mercaptoethanol in a 5% CO2:95% air atmosphere. Cell culture procedures have been described previously (30) as were procedures (28) for transplanting and harvesting L1210 cells from the CS7BL × DBA/2 F1 mice used in these studies. Cells in log phase of growth were used in all experiments. In experiments measuring polyglutamate synthesis, culture media was supplemented with 10 μM thymidine and 100 μM inosine (to protect cells from drug toxicity) and 2 mM glutamine. Cells were incubated up to 24 h with a concentration of radiolabeled drug which approximated the Km for influx for each analogue. The concentration of methotrexate used in cultures of L1210, Sarcoma 180, and Manca was 10, 5, and 5 μM, respectively. For the other analogues, a concentration of 2 μM was used. After the appropriate incubation period, cell aliquots were collected and added to 10 volumes of ice-cold PBS. After the second wash with PBS, cells were resuspended in 1 ml PBS, and a cell number determination was made. The cell suspension was boiled for 15 min. The cell debris was pelleted, and the supernatant was frozen until analysis by HPLC.

Cytotoxicity Assays. Growth inhibition assays were performed as described previously (30). Briefly, cells were exposed to a range of drug concentrations for 72 h and the IC50 was determined. A soft-agar cloning assay (7) was used to establish the extent of growth inhibition on the clonogenicity of the 3 cell lines studied. Cells were exposed to a range of drug concentrations for 3 h. After washing the cells with ice-cold PBS, 50 cells, in 2 ml of culture media, were added to 3 ml of a soft-agar solution and incubated at 37°C. After 7 to 10 days, the number of colonies formed were counted and plotted as a percentage of untreated control cells versus drug concentration, and an IC50 concentration was obtained.

Measurement of Intracellular Drug and Their Respective Polyglutamyl Derivatives. Intracellular concentrations of methotrexate, aminopterin, 10-deazaaminopterin, and 10-ethyl-10-deazaaminopterin and their respective polyglutamyl metabolites were determined using reverse-phase HPLC. A Waters Model 720 gradient controller equipped with 2 pumps, a U6K injector, and a Model 730 integrator were used for drug analysis. Samples were injected into a C18-reverse-phase column (Waters Associates) and eluted at 1 ml/min using a 20-min linear gradient from 5 to 15% acetonitrile in 0.1 mM sodium acetate, pH 5.1, a procedure modified from Cashmore et al. (6). For 10-ethyl-10-deazaaminopterin samples, a 30-min linear gradient from 5 to 25% acetonitrile was used. Authentic standards were injected along with samples, and eluent was monitored at 313 nm using a Waters 480 variable wavelength UV detector. Authentic standards were not available for polyglutamyl metabolites of aminopterin above +G1. In order to verify radioactive peaks which were thought to be polyglutamyl derivatives, the peaks were assayed for their ability to inhibit DHFR and incubated in fetal calf serum (22) to determine their lability to hydrolase activity. An elution profile for the folate analogues and their respective polyglutamyl derivatives is shown in Chart 1.

Efflux Studies. For determination of folate analogue efflux, L1210 cells were incubated with 2 μM [3H]aminopterin or [3H]methotrexate for 3 h at 37°C in culture medium supplemented with 2 mM glutamine, 10 μM thymidine, and 100 μM inosine. The cells were harvested at 0°C, washed twice with PBS, and resuspended in transport buffer (107 mM NaCl:20 mM Tris-HCl:26.2 mM NaHCO3:5.3 mM KCl:1.9 mM CaCl2:7 mM glucose, pH 7.4). After incubation at 37°C, aliquots were
removed at various times, washed twice with cold PBS, and resuspended in 1 ml PBS. Cell counts, which were obtained with a Model ZB1 Counter, were determined prior to boiling samples for 15 min. After centrifugation, the supernatants were collected and frozen until analysis by HPLC. For efflux measurements following preloading of L1210 cells in suspension with methotrexate, aminopterin, and their +G4 derivatives, cells were incubated with drug for 5 min in transport buffer. Cells were then cooled to 0°C, washed twice, and resuspended in transport buffer. After incubation at 37°C, aliquots were removed and processed as described above. Samples were assayed by dihydrofolate reductase titration as described previously (28). No significant intracellular hydrolysis of the methotrexate +G4 or aminopterin +G4 occurred during a loading period as long as 15 min.

RESULTS

Growth-inhibitory Properties and Cytotoxicity of Folate Analogues Against Tumor Cells in Culture. L1210, Sarcoma 180, and Manca cells were exposed in culture to various concentrations of one of the 4 antifolates used in this study, methotrexate, aminopterin, 10-deazaaminopterin, and 10-ethyl-10-deazaaminopterin. Cells were exposed for either 3 h or continuously. After pulse (3 h) exposure, cells were diluted in fresh medium without drug and plated in semisolid agar medium. The results expressed as IC50 concentration for growth inhibition or colony formation are given in Table 1. Substantial differences were observed in the sensitivities of these cell types to each of the analogues used. Methotrexate was the least effective analogue overall, but it was a better inhibitor of L1210 cell growth compared to Manca cells (3-fold) and Sarcoma 180 cells (4-fold). Aminopterin and the two 10-deazaaminopterin analogues were substantially more potent than was methotrexate as inhibitors of growth in the case of all 3 cell lines. Values for IC50 among the 3 analogues were approximately equivalent for each cell type and 6- to 8- (L1210), 8- to 12- (Manca) or 17- to 40- (Sarcoma 180) fold lower than for methotrexate.

Results obtained with the clonogenic assay (see Table 1) were extensively different and variable compared to those derived during measurement of growth inhibition. Overall, levels of drug required for cytotoxicity in this system were as much as 1 to 2 log order greater, but again, methotrexate was the least effective analogue. Activity of this analogue against L1210 and Manca cells were 8- to 10-fold greater than against Sarcoma 180 cells. However, in the case of aminopterin, overall potency was similar against all 3 cell types, but compared to methotrexate, this was no less than 12- to 15-fold greater for L1210 and Manca cells but as much as 80-fold greater for Sarcoma 180 cells. Overall, potency for 10-ethyl-10-deazaaminopterin was similar to aminopterin, but individual values for IC50 were about 2-fold greater, and the differential when compared to methotrexate was not quite as large. In contrast to these results, potencies for 10-deazaaminopterin varied substantially between these cell lines. It was 2- and 4-fold less potent than was aminopterin against Manca and L1210 cells but 12-fold less potent against Sarcoma 180 cells.

Initial Rate of Drug Entry and Polyglutamate Formation in Tumor Cells. Measurements of polyglutamate formation that are a valid representation of initial rates of formation and accumulation of these metabolites in each cell type were obtained by using concentrations for each analogue in the external medium which would result in a comparable rate of entry and level of accumulation. These concentrations were derived from the kinetic data available (20, 28) from our previous studies on membrane transport of these analogues in each cell type.

Time courses for accumulation of the various analogues in L1210 cells are shown in Chart 2. Cells were exposed to one of the following: 2 μM [3H]aminopterin; 10 μM [3H]methotrexate; 2μM [3H]-10-deazaaminopterin; or 2 μM [3H]-10-ethyl-10-deazaaminopterin, during growth at 37°C. Initial rates of intracellular accumulation were similar for each of these agents, varying only from 1.8 to 2.2 nmol/min/g dry weight. Intracellular drug levels exceeded the binding equivalence for DHFR within 5 to 10 min, at which time polyglutamates of each analogue began to appear. Rates of accumulation of these anabolites were constant by 20 min and remained so for the next 40 min during which measurements were made. Rates of accumulation of polyglutamates were 7- to 18-fold lower than rates of drug entry into these cells. These rates varied among analogues in the order [3H]aminopterin > [3H]-10-ethyl-10-deazaaminopterin > [3H]methotrexate > [3H]-10-deazaaminopterin. Similar experiments were carried out with Sarcoma 180 and Manca cells (data not shown). The results from studies with all 3 cell types are summarized in Table 2. From the data, it can be seen that the same relative order of substrate activity for these analogues in L1210 cells was also seen in Sarcoma 180 cells. However, the ranking was different for Manca cells. The best substrate overall for conversion to a polyglutamate was [3H]aminopterin, while [3H]-10-ethyl-10-deazaaminopterin was least effective as a substrate except in Manca cells. However, substantial differences were observed in the substrate activity of these 4 analogues among the 3 cell types. Although [3H]aminopterin conversion was highest of the 4 analogues in

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Growth-inhibitory and cytotoxic effects of various folate analogues against murine and human tumor cells in culture</td>
</tr>
<tr>
<td>In the GIA, a cells were exposed to drug for 72 h following which a cell concentration was determined and an IC50 was established. In the CA, cells were exposed to drug for 3 h and then plated on soft agar as described in &quot;Materials and Methods.&quot; Colony formation was assessed 7 to 10 days later, and an IC50 determination was made.</td>
</tr>
</tbody>
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<table>
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<tr>
<th>Table 2</th>
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<tbody>
<tr>
<td>Half-maximal concentration (nM)</td>
</tr>
<tr>
<td>Analogue</td>
</tr>
<tr>
<td>Methotrexate</td>
</tr>
<tr>
<td>Aminopterin</td>
</tr>
<tr>
<td>10-Deazaaminopterin</td>
</tr>
<tr>
<td>10-Ethyl-10-deazaaminopterin</td>
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a GIA, growth inhibition assay; CA, clonogenic assay. 
 b IC50 for reduction in colony formation in the CA or inhibition of growth after 72-h incubation in GIA. 
 c Mean ± SE of 4 different experiments. 

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Chart 2. Time course for accumulation of total drug and polyglutamates in L1210 cells following incubation in either 2 μM aminopterin (Am), 2 μM 10-deazaaminopterin (10dAm), and 2 μM 10-ethyl-10-deazaaminopterin (10EdAm) or 10 μM methotrexate (Mtx) as radiolabeled drug. □, △, O, ☐, total 4-aminofolylpolyglutamate levels; □, △, O, ☐, total drug levels; bars, ± SE; FAH2, dihydrofolate; g dw, g dry weight.

Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>L1210</th>
<th>Sarcoma 180</th>
<th>Manca</th>
</tr>
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<tbody>
<tr>
<td>Methotrexate</td>
<td>0.200 ± 0.02*</td>
<td>0.040 ± 0.005</td>
<td>0.423 ± 0.03</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>0.315 ± 0.04</td>
<td>0.315 ± 0.04</td>
<td>1.099 ± 0.10</td>
</tr>
<tr>
<td>10-Deazaaminopterin</td>
<td>0.100 ± 0.02</td>
<td>0.013 ± 0.002</td>
<td>0.505 ± 0.05</td>
</tr>
<tr>
<td>10-Ethyl-10-deazaaminopterin</td>
<td>0.237 ± 0.05</td>
<td>0.079 ± 0.007</td>
<td>1.017 ± 0.06</td>
</tr>
</tbody>
</table>

* Mean ± SE.

L1210 and Sarcoma 180 cells, its rate of conversion in Manca cells was similar to that of [3H]-10-ethyl-10-deazaaminopterin. The lowest level of conversion was observed with [3H]-10-deazaaminopterin in Sarcoma 180 cells. Also, the differential in substrate activity of these analogues was greatest in these tumor cells.

Kinetics of Accumulation of Polyglutamates in Tumor Cells.

In Chart 3, we show the extended time courses for relative accumulation of polyglutamates of each of the 4 analogues. Differences in the initial accumulation of polyglutamates that are observed reflect the data on absolute rates of accumulation shown in Table 2. Time courses for total accumulation of polyglutamates after prolonged incubation with each analogue also varied substantially. In L1210 cells, despite the initial differences observed between conversion of the different analogues, relative extents of accumulation of polyglutamates after 6 h of incubation were similar for the 4 analogues. The accumulation approached 85 to 95% of total drug in 24 h. Similar results were obtained in Manca cells, but a maximum accumulation of 85 to 95% was obtained by 6 h. In Sarcoma 180 cells, the results were substantially different. A maximum in percentage accumulation occurred only in the case of [3H]aminopterin. Lower extents of accumulation of polyglutamates are seen for the other analogues. These results are in agreement with the appreciably lower substrate activity of the other analogues in this cell type.

Data on the absolute accumulation of the various polyglutamates from these analogues are shown in Charts 4 to 6. For all of the analogues except [3H]aminopterin, the accumulation of individual polyglutamates in each cell type followed the same pattern. Polyglutamates of 1, 2, and 3 or more additional glutamyl residues appeared in sequential order during incubation with [3H]methotrexate, [3H]-10-deazaaminopterin, or [3H]-10-ethyl-10-deazaaminopterin. By 24 h, derivatives containing 3 or more glutamyl residues represented the predominant form of each analogue. In the case of [3H]aminopterin, the pattern of accumulation in L1210 cells was somewhat similar. However, substantially higher levels of aminopterin +G2 accumulated and were sustained during the 24-h period of incubation. Similar results (Chart 5) for [3H]aminopterin +G2 accumulation were obtained in Sarcoma 180 cells. However, in the case of Manca cells (Chart 6) the accumulation of [3H]aminopterin +G2 was dramatic. Within the first 3 h of incubation, it was virtually the only polyglutamate
POLYGLUTAMYLLATION OF FOLATE ANALOGUES

Chart 4. Accumulation of individual polyglutamates in L1210 cells. Measurements were made following incubation in either 2 μM [3H]-aminopterin (Am), 2 μM [3H]-10-deazaaminopterin (10dAm), 2 μM [3H]-10-ethyl-10-deazaaminopterin (10EdAm), or 10 μM [3H]-methotrexate (Mtx). At the indicated times, intracellular metabolites were determined by HPLC as described in "Materials and Methods." O, total drug; C, parent drug; gdw, g dry weight.

Chart 5. Accumulation of individual polyglutamates in Sarcoma 180 (S180) cells. Experimental conditions were the same as for Chart 4, except that the [3H]-methotrexate (Mtx) concentration was 5 μM. Am, [3H]-aminopterin; 10dAm, [3H]-10-deazaaminopterin; 10EdAm, [3H]-10-ethyl-10-deazaaminopterin; gdw, g dry weight.

found (approximately, 70% of total intracellular drug) and remained the predominant form (50% of total intracellular drug) during the 24-h period of incubation.

Efflux Properties of [3H]Methotrexate and [3H]Aminopterin and Their Polyglutamates in L1210 Cells. Previous work done by others in cell culture systems (2, 12, 14, 18, 24) or with resting cell suspensions provided evidence for the retentive nature of the various polyglutamated derivatives of methotrexate. In the present studies, the continuing intracellular accumulation with time of these analogues implied the same property of retentiveness of these metabolites derived from each analogue under similar situations. This was confirmed for methotrexate polyglutamates in L1210 cells by the results of efflux experiments shown in Chart 7. After incubation with [3H]methotrexate for 3 h, cells were harvested at 0° C, washed, and resuspended in transport buffer with 7 mM D-glucose. The suspension was then incubated at 37° C for the times shown, and aliquots were harvested at various times for analysis. From these results (Chart 7), it can be seen that compared to [3H]methotrexate itself, the polyglutamates of [3H]methotrexate showed lower efflux. Also, efflux decreased with the increase in number of additional glutamyl residues. In view of the unusual pattern of accumulation observed with [3H]aminopterin polyglutamates, particularly in the case of [3H]aminopterin +G1, we also carried out similar efflux experiments with L1210 cells grown in the presence of this analogue. From the results shown in Chart 8, we find that the relative efflux of the parent drug and the various polyglutamates were essentially the same as that seen with the corresponding forms of [3H]methotrexate.

Earlier studies reported from our laboratory (21, 22) documented a similarity in efflux of methotrexate and the lower polyglutamates (+G1 and +G2) of methotrexate from in vivo derived L1210 cells. In view of the results described here, we sought a straightforward comparison of methotrexate-polyglutamate efflux done in parallel with the same L1210 cell subline derived both in vivo and in vitro. For these experiments, we loaded L1210/C1 cells derived from each source with methotrexate and methotrexate +G1. The efflux time courses for each derivative obtained following washing and resuspending in vivo-derived cells in drug-free transport buffer are given in Chart 9.
**Chart 6.** Accumulation of individual polyglutamates in Manca cells. Experimental conditions were the same as Chart 4, except that the [3H]methotrexate (Mtx) concentration was 5 μM. Am, [3H]aminopterin; 10dAm, [3H]-10-deazaaminopterin; 10EdAm, [3H]-10-ethyl-10-deazaaminopterin; gdw, g dry weight.

**Chart 7.** Efflux of methotrexate (Mtx) and methotrexate polyglutamates from L1210 cells after a 3-h incubation in vitro in 2 μM [3H]methotrexate. After incubation, cells were removed at various times and assayed as described in “ Materials and Methods.” [drug]int, intracellular drug; bars, SE; gdw, g dry weight.

From the data presented in Chart 9, it can be seen that efflux of methotrexate and methotrexate +G1 was the same in these L1210 cells. Similar results were found in L1210 cells derived in culture (data not shown). Likewise, the efflux of aminopterin and aminopterin +G1 from in vivo-derived L1210 cells was the same (Chart 10). These results are in contrast to the data in Charts 7 and 8 showing slower efflux of methotrexate +G1 and aminopterin +G1 than do the parent compounds. Also, from a comparison of the data given in each chart, it is apparent that efflux from cells preloaded as resting cell suspensions (Charts 9 and 10) was much more rapid than from cells (Charts 7 and 8) which had accumulated methotrexate and its polyglutamates during growth in cell culture.

**DISCUSSION**

Tumors and normal proliferative tissues (12, 13, 16-18, 22, 23, 27, 34, 35) appear to exhibit different extents of 4-aminofo late polyglutamylation. For tumors at least, this would appear to reflect to a large extent differences in specificity for various folate analogues rather than the inherent capacity for polyglutamate synthesis in each tissue. This conclusion is derived from the striking and unexpected results of the present studies which show substantial differences in the ability of various 4-amino folates to serve as substrates for this metabolic conversion in each cell type. These results document individual rates of polyglutamylation in situ under conditions in which transport inward for
the various analogues studies was not rate limiting. Variable substrate activities were also reflected in the rapidity with which the various polyglutamates accumulate during prolonged incubation of these cells in the presence of individual analogues.

When carefully considered in the light of other parameters of antifolate action such as the extent of membrane transport and DHFR inhibition (reviewed in Refs. 28 and 31), our results also appear to establish a correlation between the accumulation of polyglutamates of the various 4-aminofolyl analogues and their cytotoxicity as determined by a clonogenic assay. In the case of methotrexate and aminopterin, the higher potencies of the former analogue in the 3 tumor cell types reflect both higher-mediated influx (28, 29, 31) and polyglutamylation of each. Likewise, variable potency of each analogue among the 3 cell types is determined by differences in both biochemical parameters. Comparisons between aminopterin and the two 10-deazaminopterin analogues is even more straightforward. For these 3 analogues, membrane transport as well as binding affinity for DHFR is very similar (31, 32) among all of the cell types studied, and relative differences in cytotoxic potency among them is accounted for by differences in polyglutamylation alone. In view of these results, it was of some interest to note that a similar correlation with the extent of polyglutamylation could not be demonstrated for growth inhibition during prolonged exposure of the various tumor cell types to these analogues.

Another result of the present studies which was also unexpected was the vast difference in the pattern of accumulation of polyglutamates derived from aminopterin compared to those accumulating which were derived from the other analogues. Preferential accumulation of aminopterin as the +Gi in L1210, Sarcoma 180 and, in particular, Manca cells is in sharp contrast to that seen for the accumulation of the +Gi derivative derived from the other analogues. Since polyglutamates of aminopterin and methotrexate, including +Gi, showed similar reduction in efflux with variation in chain length, at least for L1210 cells, it would appear that this difference pertaining to aminopterin +Gi reflects its reduced substrate activity compared to the +Gi derivatives of the other analogues. The biochemical or pharmacological significance of this finding is not immediately apparent. Since earlier studies showed little difference in inhibition of DHFR among methotrexate polyglutamates and that the parent compounds of the 4 analogues have similar affinities for DHFR, few differences in DHFR-binding affinities are expected among polyglutamates of the other 3 folate analogues. However, it is of some interest to note that the +Gi derivative of aminopterin is the predominant polyglutamate following the 3-h pulse exposure used in the clonogenic assay. In this assay, aminopterin was found to be the most potent analogue overall.

The results of our studies examining the relative efflux of the various 4-aminofolyl polyglutamates under different conditions are of interest from the point of view of earlier studies from our laboratory (21, 22) and elsewhere (2, 12, 14, 18, 24) examining the same question. The results of current studies in cell culture with [3H]methotrexate and [3H]aminopterin confirm those reported by others (2, 12, 14, 18, 24) using cultured tumor cells and resting tumor cell suspensions which had been incubated for prolonged periods of time in the presence of [3H]methotrexate. Efflux of the polyglutamates under these conditions was reduced compared to the parent derivative beginning with +Gi and decreasing with an increase in the glutamyl chain length.

In contrast to these results, we found that efflux of methotrexate +Gi and aminopterin +Gi and their parental derivatives were the same when resting L1210 cell suspensions were loaded with these compounds during a brief period of exposure in transport medium. Moreover, the same result was obtained when cells were derived either from animals or in cell culture. Although these data basically confirm the results of our earlier studies with in vivo-derived L1210 cells (21, 22), the reason for this marked discrepancy pertaining to retentiveness of the polyglutamate derivatives under these varying experimental conditions is not apparent. One experimental difference is that efflux of the derivative was measured in the absence of longer-chain length polyglutamates which may either compete with the diglutamate or be hydrolyzed to the diglutamate and alter its concentration, thus changing the apparent rate of methotrexate +Gi efflux. We also noted that efflux of these parent compounds themselves from preloaded resting cells was an order of magnitude greater than from cells accumulating these compounds during prolonged exposure in cell culture. The rapid exit of drug shown in these experiments is characteristic of mediated efflux of folate analogues by these cells under similar conditions reported in our...
earlier studies (28–30, 32). Since the meaning of these observations, if any, remain undetermined, additional studies will be required which focus on these questions.

The possible significance of our findings on the apparent difference in substrate requirements for polyglutamylation in various cell types may be 2-fold. If the differences observed are in fact due to varying properties of the folylpolyglutamyl synthetase in each cell type, then it might be inappropriate to extrapolate findings obtained with this enzyme from one tissue to that found in others. However, the validity of this conclusion will first require some confirmation at an enzymic level. Also, these observed differences in structural specificity might be highly exploitable toward the design of new analogues with greater antitumor selectivity if they extend to synthetases derived from normal drug-limiting tissues. In work from our own laboratory (22, 32) and subsequently elsewhere (11), some information from in vivo experiments in mice has been obtained that documents differences in polyglutamylation of folate analogues between some tumors and normal proliferative tissue in small intestine which favor greater accumulation of these anabolics and perhaps cytotoxicity in these tumors. In an in vitro system, Fabre et al. (10) found greater accumulation of methotrexate polyglutamates in tumor cell types than in murine bone marrow granulocyte progenitor cells. Since crypt cell epithelium in small intestine and bone marrow progenitor cells are drug-limiting renewal sites in animals and humans, any increased differential derived for the extent of polyglutamylation in these tissues versus some tumor cells could, along with a differential at the level of membrane transport in vivo (28, 29, 31), contribute to improved antitumor selectivity. In this regard, it is of interest to note that position 10 of the folate molecule appears to be specified for both membrane transport inward and polyglutamylation (29, 32).

REFERENCES

Similar Differential for Total Polyglutamylation and Cytotoxicity among Various Folate Analogues in Human and Murine Tumor Cells in Vitro

Lawrence L. Samuels, Donna M. Moccio and Francis M. Sirotnak


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