Enhanced Polyglutamylation of Aminopterin Relative to Methotrexate in the Ehrlich Ascites Tumor Cell in Vitro

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ABSTRACT

The polyglutamylation of aminopterin and methotrexate (N¹⁰-methylaminopterin) was compared in the Ehrlich ascites tumor in vitro. Three poly-γ-glutamyl conjugates of methotrexate and aminopterin were detected, although at an equal (1 μM) extracellular drug concentration, the net accumulation of aminopterin polyglutamates exceeded that for the methotrexate polyglutamyl derivatives by a factor of 9. When compensation was made for transport differences between these compounds by adjusting the extracellular drug concentrations to achieve equivalent intracellular monoglutamyl substrate levels, the polyglutamylation of aminopterin was still 2.8-fold greater than that for methotrexate, suggesting that aminopterin is a better substrate for the folypolyglutamate synthetase as well as the transport carrier. An additional metabolite of aminopterin was detected within seconds following drug exposure. This derivative did not bind tightly to dihydrofolate reductase, yet it was rapidly converted to a polyglutamate. The formation of both aminopterin polyglutamates and these novel derivatives was enhanced by increases in the free intracellular level of aminopterin. Aminopterin polyglutamates were bound tightly to dihydrofolate reductase and were retained intracellularly relative to unaltered aminopterin when Ehrlich cells containing these forms were suspended in drug-free medium. These findings support a role for the polyglutamylation of aminopterin as a critical element in drug action and as a factor in addition to membrane transport in the disparate antifolate potencies of aminopterin and methotrexate.

INTRODUCTION

AMT was the first antifolate introduced into the clinic and the agent first documented to produce remissions in children with acute lymphatic leukemia (6). Despite its greater antitumor activity (14), AMT was subsequently replaced by MTX in clinical use because the considerable host toxicity accompanying its use compromised its therapeutic efficacy (14), and toxicity with methotrexate was more predictable possibly due to its greater purity and stability (13).

The pharmacological basis for the increased cytotoxicity of AMT has been attributed to its more efficient membrane transport relative to MTX, resulting in higher intracellular levels of drug at low extracellular concentrations (3, 16, 30, 37, 38). However, it is now recognized that an additional important element in the pharmacology of the antifolates is the intracellular conversion of these drugs to polyglutamyl derivatives, analogous to that described for the natural folates and catalyzed by the folypolyglutamate synthetase (18). This metabolism has now been characterized for MTX in a variety of cell types (5, 9, 10, 12, 24, 31, 42). The pharmacological significance of the polyglutamylation of MTX lies in the greater intracellular retention of these compounds, in general, relative to the unmetabolized drug (9, 11, 12, 24, 33) and their equivalent binding affinity compared to undervatized MTX for DHFR (20, 23, 35). In addition, recent studies suggest that the reduced extent of formation of polyglutamyl derivatives of MTX in the drug-sensitive host tissues, the intestinal epithelium (7) and bone marrow (4), relative to tumor cells may be an important basis for the selectivity of this agent.

AMT has also been reported to form PGs in tumor cells (34, 39) or in cell-free systems (27, 29), suggesting that this metabolism may also contribute to the pharmacological action of this drug. However, to date there has been no detailed investigation of this conversion. The present report compares the metabolism of AMT to that of MTX in the Ehrlich ascites tumor including the relative rate of polyglutamylation and the intracellular retention and binding of the derivatives of AMT.

MATERIALS AND METHODS

Chemicals. [7,9-³H]AMT was obtained from Amersham Corp. (Arlington Heights, IL) and purified by reversed-phase HPLC as described below. The purity was confirmed by periodic rechromatography using the 2 methods described below. 2,4-Diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine (metoprine) and unlabeled AMT were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD. The unlabeled AMT was purified on DEAE-cellulose (17). DHFR was purified from the Ehrlich tumor as described previously (25). 7-Hydroxymethylaminopterin was prepared as described elsewhere (22), and 10-formylaminopterin was synthesized using a procedure identical to that for the preparation of 10-formylfolic acid (19). Bio-Gel P-6 (200 to 400 mesh) and Bio-Gel P-60 (100 to 200 mesh) were purchased from Bio-Rad Laboratories (Richmond, CA). Other chemicals were obtained from commercial sources.

Cells, Media, and Transport Methodology. Ehrlich ascites tumor cells were grown in male CF-1 mice and passed weekly by i.p. inoculation of 0.2 ml of undiluted ascitic fluid. The cells were harvested after 7 to 10 days and washed twice with 0.85% NaCl solution (saline). Transport experiments were performed at 37° in specially designed flasks in a buffer composed of 125 mM NaCl, 4.4 mM KCl, 16 mM NaHCO₃, 1.1 mM KH₂PO₄, 1 mM MgCl₂, 1.9 mM CaCl₂, and 5 mM glutamine. The pH was maintained at 7.4 by passing warm and humidified 95% O₂:5% CO₂ over the cell suspension.

Transport fluxes were terminated by injection of portions of the cell
POLYGLUTAMYLATION OF AMT

RESULTS

Cellular Accumulation and Metabolism of AMT. As reported previously for other cell types (3, 30, 37, 38), Ehrlich cells readily accumulated intracellular AMT when exposed to low extracellular concentrations of drug. Chart 1 compares net cellular uptake of radiolabel over a 2-hr interval at equal (1 μM) extracellular AMT and MTX concentrations. For cells exposed to MTX, intracellular 3H achieved a steady state by 20 min, exceeding the DHFR binding capacity (approximately, 2.1 nmol/g dry weight), whereas the cells exposed to AMT continuously accumulated radiolabel throughout the course of the incubation. After 2 hr, the intracellular 3H in cells exposed to MTX was 3.13 ± 0.20 (S.D.) nmol/g dry weight (n = 4), while that for the AMT-treated cells was 9.92 ± 1.78 nmol/g dry weight (n = 4).

This difference in the intracellular levels of radiolabel achieved for these drugs was attributable, in part, to the greater transport efficacy of AMT, since this drug has an affinity for the transport carrier approximately 3 times greater than does MTX (3, 16, 28, 37). Moreover, HPLC analysis of the intracellular drug forms after 2 hr of exposure of the antifolates revealed that there was also a marked difference in the metabolism of these closely related compounds. There was only limited metabolism of MTX to form 3 derivatives previously identified (9) as MTX PCs (24.47 ± 1.55% of the total intracellular drug). Three derivatives of AMT were also detected (PG, to PG3, Chart 2, top) comprising 70.14 ± 1.94 of the cellular radiolabel. When a sample containing these AMT derivatives was incubated with a conjugase preparation from chicken pancreas as described in "Materials and Methods," these compounds quantitatively reverted to the parent compound, establishing their identities as polyglutamate derivatives (Chart 2, bottom).

In contrast to the metabolism of MTX in Ehrlich cells, not all of the derivatives formed from AMT were AMT PGs. Using these chromatographic conditions, 2 additional compounds were also detected, eluting prior to the parent compound, a position inconsistent with their identities as polyglutamyl conjugates of AMT. In addition, conjugase treatment did not convert these derivatives to AMT but resulted rather in the quantitative disappearance of the radioactive associated with one of these compounds (Chart 2, A-PG, bottom) and a corresponding increase in the radioactivity associated with the other (Chart 2, A bottom). The characteristics of these additional metabolites of AMT are further considered below.

Comparison of Rates of Polyglutamation of AMT and MTX at Comparable Intracellular Levels of Monoglutamyl Substrate. The 9-fold greater polyglutamation of AMT relative to MTX seen above is probably partially a consequence of the higher free intracellular drug levels achieved for the former compound at equivalent extracellular drug concentrations. The increased level of intracellular AMT substrate should alone increase the extent of polyglutamylation. To exclude the contribution of the membrane transport system as a factor in the much greater extent of AMT PG formation relative to MTX, cells were incubated with a concentration of AMT (1 μM) lower than that of MTX (5 μM) in order to achieve equivalent intracellular monoglutamate levels. As depicted in Chart 3, under these conditions, the underderivatized AMT concentration was in fact comparable to that of MTX. However, the rate of AMT polyglutamylation still exceeded that for MTX by a factor of 2.8.
POLYGLUTAMYLLATION OF AMT

Chart 1. Net accumulation of total intracellular MTX and AMT. Cells were incubated with 1 μM [3H]MTX or 1 μM [3H]AMT at 37°. At the indicated times, total intracellular ³H was determined as described in "Materials and Methods."

Chart 2. HPLC analysis of AMT metabolites. Cells were incubated for 2 hr with 1 μM [3H]AMT. The intracellular radiolabel was analyzed by ion-pairing HPLC as described in "Materials and Methods" before (top) and after (bottom) treatment with a preparation of chicken pancreas conjugase.

Chart 3. Comparison of polyglutamylation of AMT and MTX at equal intracellular substrate levels. Cells were incubated with 5 μM [3H]MTX and 1 μM [3H]AMT. At the indicated times, the intracellular derivatives were quantitated as described in "Materials and Methods" for unmetabolized drug and PGs.

Time Course of Formation and Binding of Intracellular Derivatives of AMT. Chart 4A illustrates the time course of accumulation of intracellular AMT and its metabolites during a 2-h exposure to 0.5 μM drug. As seen above in Chart 1, there was an initial rapid uptake of ³H largely associated with unaltered AMT followed by a slower constant accumulation of total radioactivity. Only when drug levels sufficient to saturate DHFR were achieved in the intracellular compartment could any AMT polyglutamylation derivatives be detected. Beyond this point, the level of the PGs rapidly exceeded that for unaltered drug resulting in a displacement of the latter from the target enzyme (Chart 4B). By 40 min, uptake of total ³H essentially paralleled the accumulation of PG derivatives. Derivatives A and A-PG were detected at the earliest sampling times (Charts 4A and 5), prior to saturation of intracellular DHFR. However, these derivatives did not appear to bind tightly to DHFR since no complex could be isolated by gel filtration (Chart 4B).

The kinetics of formation of these various drug forms are depicted in Chart 5. Derivative A formed rapidly (within 30 sec, inset) and achieved a peak level at about 2 min. It then declined to a lower steady state level associated with its rapid exit from the cells (see below) and its conversion to a polyglutamyl form, A-PG. The latter was also detected by 30 sec (inset); however, with the appearance of free AMT in the intracellular compartment which likely competes with A for polyglutamylation, the rate of A-PG formation slowed markedly even though this derivative continued to accumulate over the remaining interval of observation. After 2 hr, A and A-PG constituted approximately 4 and 14%, respectively, of the total intracellular radioactivity. Of the AMT PGs, PG1 appeared at 6 min followed by the appearance of PG2 at 10 min. As indicated above, PG3 was also detected in some experiments. However, this compound was generally undetectable at an extracellular AMT concentration of 0.5 μM. At the end of the 2-hr interval of observation, PG2 was the predominant intracellular component, constituting 43% of the total cellular drug whereas PG1 represented 30% of the total derivatives formed from AMT.

Binding of AMT Metabolites to Purified Ehrlich Tumor DHFR. The data presented in Chart 4B suggest that the polyglutamyl forms of AMT readily bind to intracellular DHFR in contrast to the unmodified drug.
excess (not shown). Moreover, the similarities of the relative levels of the bound AMT monoglutamate and PG derivatives to bind appreciably to the purified enzyme when present in A-PG, isolated from the other AMT derivatives by HPLC, failed or purified DHFR occurs under these conditions. Similarly, A and the early forming derivatives A and A-PG which show no apparent tendency to associate with this intracellular locus. These binding interactions were further evaluated by the use of a purified DHFR preparation from Ehrlich cells. Cells were exposed to 0.5 μM [3H]AMT, and at the indicated times, the cellular radiolabeled drug components were measured as described in "Materials and Methods." The derivatives are designated as in Chart 2 including PG1, PG2, A, and A-PG.

Table 1

<table>
<thead>
<tr>
<th>Total cellular</th>
<th>Bound in cell</th>
<th>Total extract</th>
<th>Bound to DHFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(nmol/g dry wt of cells)</td>
<td>(nmol/dry wt of cells)</td>
<td>(pmol)</td>
<td>(pmol)</td>
</tr>
<tr>
<td>Total [3H]</td>
<td>5.54</td>
<td>2.26</td>
<td>38.31</td>
</tr>
<tr>
<td>AMT</td>
<td>1.44</td>
<td>0.81</td>
<td>9.62</td>
</tr>
<tr>
<td>A</td>
<td>0.22</td>
<td>0</td>
<td>1.49</td>
</tr>
<tr>
<td>A-PG</td>
<td>0.77</td>
<td>0</td>
<td>5.34</td>
</tr>
<tr>
<td>PG1</td>
<td>2.21</td>
<td>1.02</td>
<td>15.00</td>
</tr>
<tr>
<td>PG2</td>
<td>0.89</td>
<td>0.41</td>
<td>6.67</td>
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</tbody>
</table>

Chart 5. Kinetics of formation of the derivatives of AMT. Ehrlich cells were exposed to 0.5 μM [3H]AMT, and at the indicated times, the cellular radiolabeled drug components were measured as described in "Materials and Methods." The derivatives are those used in Chart 2 including PG1, PG2, A, and A-PG.

Intracellular Retention of AMT and Its Derivatives. Chart 6 (top) illustrates the extent to which the various forms of AMT were retained in the Ehrlich tumor after loading the cells for 90 min in the presence of 0.5 μM of radiolabeled AMT. After this preexposure, greater than 65% of the intracellular drug was present as the higher conjugates of AMT whereas approximately 18% was present as A and A-PG (not shown in Chart 6, top). Following resuspension of the cells into drug-free medium, the major portion (greater than 90%) of the intracellular AMT exited the cells within 60 min. The large decline in the level of PG1 was attributable mainly to its conversion to the longer chain length form, PG2 (Chart 6, bottom) resulting in a slower decrease in the level of AMT PGs during this interval (18% of the total PGs). Some PG1 and PG2 could be detected in the extracellular medium, consistent with the slow exit of intact PGs from the cells. While a loss of the intracellular A and A-PG was observed (approximately 90 and 75%, respectively; Chart 6, bottom), very little of the latter relative to A was detected in the medium, suggesting its hydrolysis by intracellular and/or extracellular conjugases. Of particular importance in the experiment depicted in Chart 6, the PG derivatives of AMT, potent DHFR inhibitors as described above, remained at levels exceeding the intracellular concentration of the target enzyme throughout the interval of observation.

Nature of Compound A and Its Polyglutamyl Derivative. The experiments described above show clearly that the compound designated A rapidly accumulated in Ehrlich cells exposed to radiolabeled AMT and is converted to a polyglutamyl derivative. The use of highly purified preparations of AMT in these studies would seem to exclude an origin for this compound as a contaminant of the commercial radiolabeled AMT. Similarly, no series of their relative abundances in the cell or the cell-free extract as seen in Table 1 is consistent with a similar binding affinity of these compounds for this enzyme.
treatments of the AMT (exposure to light, heat, or acid) resulted in the formation of a compound with a retention time on HPLC of derivative A or A-PG. Finally, pretreatment of Ehrlich cells with the lipophilic antifolate 2,4-diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine (250 µM) so as to effectively abolish binding of AMT to DHFR (less than 10% of the untreated binding level) elevated the free intracellular AMT level (approximately 165%) at early exposure times. This increase in the free AMT monoglutamate substrate resulted in greater polyglutamylation (Table 2). Additionally, there was an approximately 2-fold elevation in the intracellular peak level of compound A achieved, consistent with its formation in a metabolic step from AMT within the cell. The amount of A-PG formed also increased under these conditions. A metabolism of AMT in addition to its polyglutamylation has been described, including its hydroxylation (21), and formylation (2). However, derivative A did not have the same chromatographic properties of either 7-hydroxymethotrexate, synthesized by a rabbit liver homogenate from AMT, or 10-formylaminopterin, prepared by a procedure for the corresponding folic acid derivative.

DISCUSSION

AMT is distinguished from MTX only by the absence of a methyl group (at position N-10), a change which profoundly influences the antifolate potency (14). Since this structural alteration only minimally affects the binding of the drug to DHFR (36), the increased cytotoxic activity of AMT has been attributed generally to the higher intracellular levels achieved compared to MTX following drug exposure, arising from its more efficient transport into cells (3, 16, 28, 30, 37, 38).

The present study describes an additional difference between these 2 closely related antifolates, namely the more rapid polyglutamylation of AMT relative to MTX in the Ehrlich ascites tumor. This increased metabolism was clearly attributable in part to the higher transport of AMT than of MTX, resulting in increased levels of intracellular substrate for polyglutamylation. However, even when the extracellular AMT concentration was reduced relative to MTX so as to achieve comparable intracellular levels of the unmetabolized drugs, thereby compensating for the difference in net drug uptake, AMT was still polyglutamylated approximately 2.8-fold faster than was MTX. This suggests that AMT is a better substrate than is MTX for the folyl-PG synthetase. While the structural differences at the level of the folyl-PG synthetase. While the structural differences between AMT and MTX allow for their target enzyme is of potential pharmacological importance, the demonstration of a determinant of the cytotoxic potency of this antifolate as well. However, the increased cytotoxicity of AMT relative to MTX could also relate to other factors not evaluated in this study, including an inhibition of alternate cellular loci.

Of particular interest was the demonstration of the formation of 2 intracellular derivatives of AMT in addition to the AMT PGs. These derivatives clearly represent metabolites of AMT since (a) they were derived from highly purified preparations of radiolabeled drug, (b) they could not be generated from AMT in the absence of cells by degradative conditions, and (c) their intracellular accumulation increased following treatment with an agent which elevated free intracellular AMT available as a substrate for metabolic conversion. The metabolites A and A-PG were observed to form rapidly, prior to saturation of the intracellular DHFR. Hence, the formation of A is fast relative to AMT binding to intracellular DHFR. The early formation of the PG form, A-PG, moreover, probably derives from the failure of unconjugated A to bind to DHFR, since the corresponding AMT PGs were undetectable until the enzyme was saturated and a free drug component appeared in the intracellular space. With the accumulation of free AMT and AMT PGs, A-PG formation slowed, presumably due to a competition for binding between AMT and A at the level of the foly-PG synthetase. While the structural elucidation of these novel metabolites derived from AMT in Ehrlich tumor cells has not been established in the present study and is currently under investigation, the demonstration of a mechanism in tumor cells for diminishing the affinity of antifolates for their target enzyme is of potential pharmacological importance and represents an additional element in the differential sensitivity of cells to these antineoplastic agents.

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REFERENCES

POLYGLUTAMYLLATION OF AMT


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