A Case for the Use of Aminopterin in Treatment of Patients with Leukemia Based on Metabolic Studies of Blasts in Vitro

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INTRODUCTION

A significant amount of laboratory investigation and clinical experience during the past three to four decades (e.g., there are more than 19,000 citations for MTX since 1966) has resulted in the testing of numerous schedules, doses, and routes of delivery of MTX in patients with cancer (reviewed in Refs. 3 and 4). Biochemical parameters, such as mutations or amplification of DHFR, decreased transport or metabolism of drug to a polyglutamyl form, and/or increased catabolism have been associated with resistance to MTX (reviewed in Ref. 4). With special regard to the treatment of children with ALL, Whitehead et al. (5) have shown that MTX accumulation (uptake and metabolism to a polyglutamyl form) is of prognostic significance. If blasts accumulated ≥0.5 pmol MTX polyglutamates/10⁶ cells during a 24-h incubation with 1 μM ³H-MTX in vitro, the statistical likelihood of a cure was greater compared with that in children whose blasts accumulated less. Having a biochemical or pharmacological end point as a prognostic indicator, in addition to an immunological or karyotypic marker, is a potentially important marker of why a patient will relapse rather than just who will relapse. It may afford the clinician an opportunity to alter therapy based on drug metabolism; i.e., there is a defined pharmacodynamic value required for therapy to succeed. Currently, the Pediatric Oncology Group is studying MTX metabolism in children, as assessed by monitoring RBC folate and drugs (6), and a small subset of the group is obtaining data to confirm and extend the initial observations of Whitehead et al. (5). As part of this study, we have studied not only ³H-MTX but also the metabolism of the physiological (plasma) folate 5-methyl-³H-tetrahydrofolic acid (7) and/or

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3 The abbreviations used are: ALL, acute lymphoblastic leukemia; AMT, aminopterin; MTX, methotrexate (amethopterin); DHFR, dihydrofolate reductase; AML, acute myelogenous leukemia; HPLC, high-performance liquid chromatography.
3H-AMT. We reasoned that if accumulation of a “stoichiometric inhibitor” of dihydrofolate reductase by leukemic blasts was of prognostic importance, then whether it was either AMT or MTX polyglutamate may be relevant only with respect to the amount of the drug (mg/m²) needed. Maybe the potency of AMT can be harnessed by applying knowledge obtained during the 40 years of empiric and laboratory studies of antifolates. The results of our study of AMT and MTX metabolism by blasts in vitro obtained from children with leukemia constitute the subject of this report.

MATERIALS AND METHODS

Patients. In the last 18 months, we had the opportunity to study blast cells from 63 children diagnosed with leukemia. Of these, 40 samples provided sufficient numbers of blasts to do a comparative study of AMT and MTX metabolism. Twenty-four patients had B-cell-lineage ALL, 10 had T-cell-lineage ALL, and 6 had primary or secondary AML.

Media and Radiolabel. Cells were incubated in RPMI 1640 with 25 mM HEPES, 10% FCS (GIBCO 26140-020), and GAT (5 mg glycine, 1 mg adenine, and 1 mg thymidine/ml). 3H-MTX and 3H-AMT (Moravek MT701 and MT506; 15–30 Ci/mmol) were added to the media with the unlabeled drug to a concentration of 1 μM (final specific activity, 10 μCi/ml). Unlabeled polyglutamate standards of MTX-Glu₂₋₆ were purchased from Dr. B. Schircks (Jona, Switzerland). AMT-Glu₂ was supplied very kindly by Dr. J. Piper (Southern Research Institute, Birmingham, AL). By convention, the parent drug is Glu₁, i.e., the monoglutamate. The addition of one glutamate residue results in a diglutamate, the addition of a second is a triglutamate, etc.

Tissue Culture Conditions. Fresh lymphoblasts were isolated from bone marrow or blood through Ficoll-Hypaque (Phar-macia 17-0840-02) by standard methods as detailed by Whitehead et al. (5), washed with HBSS (without phenol red; GIBCO 310-4025AJ), counted on a Coulter counter, and incubated in 35-mm dishes (Falcon 3001) at a concentration of 5 × 10⁶/2 ml media containing the drug of interest for 24 h at 37°C in a humidified atmosphere in 5% CO₂. Cells were then collected, washed three times in 5 ml HBSS at room temperature, suspended in 2 ml HBSS, counted again, subjected to viability determination by trypan blue exclusion, and boiled for 5 min. An aliquot was counted on a scintillation counter to determine the total radioactive uptake.

HPLC. An aliquot of the cell extract containing approximately 4000 cpm was filtered, “spiked” with known polyglutamate standards, and then injected onto a C18 column (Waters WAT086684) connected to a Flo-OneBeta (Radiomatic) radioactive flow detector. The parent drug and polyglutamates were eluted with a gradient of 5 mM tetrabutylammonium phosphate (PicA) (Waters WAT085101) and 5 mM PicA with 40% acetonitrile (Baxter 015-4; Ref. 8). Standards were monitored at a wavelength of 280 nm.

Statistical Analysis. A two-tailed Wilcoxon signed rank test and Mann-Whitney tests were done using a commercially available software package (INSTAT; GraphPad, Inc., San Diego, California).

RESULTS

A comparison of MTX and AMT accumulation by blasts from newly diagnosed patients with B-cell-lineage ALL incubated overnight with 1 μM radiolabeled drug in vitro is presented in Fig. 1. The mean uptakes of MTX and AMT were 0.7 ± 0.7 and 1.47 ± 0.9 pmol drug/10⁶ cells, respectively. Thus, AMT uptake is twice the uptake of MTX. The median values for MTX and AMT uptake were 0.63 and 1.19 pmol/10⁶ cells, respectively. The Wilcoxon two-tailed rank test revealed these populations to be significantly different (P = 0.0006). The Mann-Whitney statistic confirmed that these two populations are different (P = 0.007). In only one of the 24 cases was uptake of the two drugs equivalent. This was in a sample in which MTX uptake was very large (>3.0 pmol/10⁶ cells). A more important determinant of cytotoxicity may be the amount of drug accumulated as a polyglutamate. These species have a long intracellular half-life and are inhibitors of enzymes in the de novo synthesis of purines, in addition to the inhibition of dihydrofolate reductase (9). The product of the total uptake times the percentage of metabolism yields a mean MTX-Glu₅ value of 0.65 pmol/10⁶ cells and an AMT-Glu₅ value of 1.43 pmol/10⁶ cells. Although the percentages of drugs metabolized to a polyglutamate were similar, 92 and 97% for MTX and AMT, respectively, the interpatient variability was significantly greater for MTX (±26.5%) than for AMT (±5.2%). Whether this reflects a higher efficiency of uptake and/or substrate preference of polyglutamate synthetase for AMT cannot be ascertained completely. In a cell-free system, it has been established that AMT has a lower Kₘ and greater Vₘₐₓ than MTX (10). Because 59% of the patients accumulated >0.5 pmol MTX to a larger polyglutamate and thus had drug uptakes consistent with a favorable outcome (5), we did a separate comparison of the patients who would be considered poor metabolizers of MTX and, therefore, at increased risk for relapse. MTX metabolism to
MTX and 95.2 ± 7.9% for AMT. pmol and for AMT was 0.61 ± 0.4 pmol. This 3-fold increase is because the percentage of metabolism in this population was only 85 ± 17% for MTX and 95.2 ± 7.9% for AMT.

In the 10 samples in which the MTX uptake was less than 0.5 pmol/10^6 cells, the amount associated with successful therapy was only 0.20 ± 0.11 mol MTX polyglutamates/10^6 cells, which translates to only 0.20 ± 0.11 pmol/10^6 cells, whereas AMT metabolism was still near complete (95.2 ± 7.9%), yielding a net accumulation of 0.61 ± 0.4 pmol AMT polyglutamates/10^6 cells (Fig. 2). These results allow the prediction that 5 of 10 of these patients would be considered good risks (i.e., ≥0.5 pmol drug/10^6 cells) if AMT were used. This suggests that there may be a therapeutic benefit to using AMT versus MTX in this select group of patients, whether the pharmacological reason is either initial transport or subsequent metabolism.

To address issues of transport and metabolism further, in addition to the matched incubations done using 1.0 μM drug, we also had enough sample to analyze 3H-AMT uptake in 11 samples incubated in only 0.1 μM drug. Uptake at only 0.1 μM AMT was 0.57 ± 0.6 pmol/10^6 cells, and 5 of 11 samples had uptakes greater than 0.5 pmol/10^6 cells, the amount associated with successful therapy. Only one sample was not metabolized to a polyglutamate completely (i.e., ≥Glu₂). That the uptake of AMT increased only 2.4-fold with a 10-fold increase in the extracellular drug also suggests that this is a saturable process. In contrast, 3H-MTX uptake at 0.1 μM was generally so small that meaningful data with the number of cells usually acquired cannot be obtained routinely. This finding supports the work of others and our own studies of human leukemia cells in vitro, i.e., that AMT is the preferred substrate for transport and/or metabolism compared with MTX (11–13), and that there is a saturable process for AMT uptake and metabolism in the 1–5 μM range, which is only the range of the K_m for MTX transport.

In all the samples, we also had the opportunity to study some T-lineage and AML cells, because these patients often present with markedly increased WBC counts. This comparison is also of interest, because these two types of leukemia generally carry worse prognoses than B-cell-lineage ALL, and antifolates traditionally have not been considered to be important in initial therapy. The results are presented in Table 1. Accumulation of AMT by T-cell-lineage leukemia is similar to B-cell-lineage leukemia and is significantly greater than the uptake of MTX. Four of six AML cell samples accumulated ≥0.15 pmol of MTX, and one accumulated more than 3 pmol, whereas in six of six samples, AMT uptake was ≥0.5 (range, 0.7–2.33) pmol/10^6 cells.

To make more definitive comments on differences between the metabolism of MTX and AMT (i.e., polyglutamate chain length) we would need to obtain a more complete set of AMT standards as well as long-term outcome data. Fig. 3, however, shows the increased metabolism of AMT compared with MTX in a patient who would be a “MTX failure” but an “AMT success.” The total accumulation of MTX was 0.3 pmol/10^6 cells, and the total amount of polyglutamates was 0.15 pmol/10^6 cells (Fig. 3C). Total and polyglutamate uptakes of AMT uptake were 0.75 and 0.7 pmol/10^6 cells, respectively (Fig. 3B). Because we had only authentic AMT and AMT-Glu₂, we confirmed that the peaks seen in the regions of standard MTX-Glu₃–₆ were “polyglutamated” inhibitors of DHFR in two ways. First, these peaks all disappeared, and the radiolabel coeluted with an AMT standard when treated with plasma conjugase (γ-glutamyl carboxypeptidase), and, second, the tritium was >90% bound by DHFR in the presence of NADPH using established methodologies for radioligand-binding assays before or after digestion with conjugase (14).

**DISCUSSION**

There are compelling reasons for considering a second look at the clinical usefulness of AMT. Few if any useful new drugs have emerged in the past two decades for the treatment of children with ALL. Looking at antifolates specifically, a lipophilic inhibitor of dihydrofolate reductase, trimetrexate, approved recently for use in the treatment of patients with *Pneumocystis carinii* pneumonia, did not prove useful in the treatment of patients with recurrent leukemia (15, 16). Trimetrexate is not a substrate for polyglutamate synthetase. Its potential superiority to MTX is in its transport characteristics (17). Edatrexate, a MTX analogue with good efficacy in preclinical trials (reviewed in Ref. 18) has had mixed results in clinical trials in adults with solid tumors, but there are as yet no published reports documenting activity in children with ALL with or without prior exposure to MTX. Other new antifolates that are inhibitors of either thymidylate synthetase (e.g., Tomudex) or the de novo purine pathway (e.g., Lometrexol) do not yet have a record in therapy for children with ALL. Because MTX or a metabolite inhibits both thymidine and purine synthesis, it will be interesting to know whether drugs that are selective for one or the other pathway have activity, especially after patients suffer a relapse while receiving MTX.

**Table 1 Accumulation of AMT and MTX in non-B-lineage cells**

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>MTX (pmol/10^6 cells)</th>
<th>AMT (pmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cell-lineage</td>
<td>10</td>
<td>0.75 ± 0.52</td>
<td>1.59 ± 1.02</td>
</tr>
<tr>
<td>AML</td>
<td>6</td>
<td>0.89 ± 1.33</td>
<td>1.66 ± 1.19</td>
</tr>
</tbody>
</table>

Fig. 2 Increased accumulation of AMT compared with that of MTX in the 10 samples in which the MTX uptake was less than 0.5 pmol/10^6 cells as shown in Fig. 1 is detailed more completely here. One-half the samples had sufficient increases in AMT uptake to be considered more likely successes. The value of polyglutamates for MTX was 0.2 ± 0.11 pmol and for AMT was 0.61 ± 0.4 pmol. This 3-fold increase is because the percentage of metabolism in this population was only 85 ± 17% for MTX and 95.2 ± 7.9% for AMT.
Although the explosion of knowledge and techniques in molecular biology and genetics has given us tremendous insights into the biology of leukemia and who will most likely relapse, exploitable reasons for why a relapse occurs have not emerged. Clinical trials continue to show the importance of antimetabolites and especially the advantage of prolonged patient exposure (e.g., Ref. 19). Recent studies of 6-mercaptopurine and MTX metabolism both in vitro and in vivo have begun to shed light on potential reasons for the empiric success and the failure of these two agents in the treatment of children with ALL (20–23; reviewed in Ref. 24). The studies of AMT and MTX metabolism reported here, coupled with the likely significance of antifolate accumulation by blasts as a prognostic indicator (5), support the idea that AMT may be a useful drug in those patients deemed to be at increased risk for relapse.

Because there has been little progress made by way of introducing new classes of drugs into the armamentarium, finding a safe dose and schedule for a more potent analogue successfully based on pharmacological end points should be a worthwhile endeavor. Currently, we are developing plans for studying AMT in patients. Increased and new knowledge of folate and antifolate metabolism and homeostasis (e.g., Refs. 7 and 25–28), as well the development of assays that allow an assessment of pharmacodynamic parameters, should allow a safe and constructive reassessment of AMT in the clinical arena. The early preclinical and clinical literature suggested that AMT is 10–20 times more potent than MTX (2, 29–32). A basis for the difference in potency has been explored in an animal model (33, 34). There are little, if any, significant head-to-head comparisons in clinical trials. A clinical trial of “high dose” AMT, which included some pharmacokinetic data in adults, suggests that AMT has a longer $t_{1/2}$ than MTX (35). This information will be incorporated into a planned Phase I trial of AMT in children with cancer. Three other points regarding the use of AMT are important. First, AMT free of significant amounts of folate can be prepared. Second, we have a better understanding of acute neurological and renal toxicity, the latter being noted especially for AMT by Glode et al. (35), which may be mediated by adenosine and ameliorated by aminophylline (36). Third, as noted above, radioligand-binding assays and HPLC detection will allow detailed pharmacodynamics and kinetics to be done on biopsies, plasma, RBC, etc., in a timely fashion. It may be that the algorithm for the treatment of patients with ALL will include not only age, WBC, DNA index, and karyotype considerations, but also a biochemical phenotype with respect to drug metabolism.

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