Biochemical and Biological Studies on 2-Desamino-2-methylaminopterin, an Antifolate the Polyglutamates of Which Are More Potent Than the Monoglutamate against Three Key Enzymes of Folate Metabolism


ABSTRACT

Biochemical and biological studies have been carried out with 2-desamino-2-methylaminopterin (dmAMT), which inhibits tumor cell growth in culture but is only a weak inhibitor of dihydrofolate reductase (DHFR). Since it was possible that the species responsible for growth inhibition are polyglutamylated metabolites, the di-, tri-, and tetraglutamates of dmAMT were synthesized and tested as inhibitors of purified recombinant human DHFR, murine L1210 leukemia thymidylate synthase (TS), chicken liver glycaminide ribonucleotide formyltransferase (GARFT), and murine L1210 leukemia aminomimidazolecarboxamidribonucleotide formyltransferase (AICARFT). The compounds with three and four γ-glutamyl residues were found to bind two orders of magnitude better than dmAMT itself to DHFR, TS, and AICARFT, with 50% inhibitory concentration values in the 200 to 300 nm range against all three enzymes. In contrast, at a concentration of 10 μM, dmAMT polyglutamates had no appreciable effect on GARFT activity. These findings support the hypothesis that dmAMT requires intracellular polyglutamylation for activity and indicate that replacement of the 2-amino group by 2-methyl is as acceptable a structural modification in antifolates targeted against DHFR as it is in antifolates targeted against TS. In growth assays against methotrexate (MTX)-sensitive H35 rat hepatoma cells and MTX-resistant H35 sublines with a transport defect, dmAMT was highly cross-resistant with MTX, but not with the TS inhibitors N9-propargyl-5,8-dideazafolic acid and N9-[5-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl)]-N9-methylaminomethyl[thenoyl-[γ-glutamic acid, implicating DHFR rather than TS as the principal target for dmAMT polyglutamates in intact cells. On the other hand, an H35 subline resistant to 2′-deoxy-5-fluorouridine by virtue of increased TS activity was highly cross-resistant to N9-propargyl-5,8-dideazafolic acid and not cross-resistant to MTX, but showed partial cross-resistance to dmAMT. Both thymidine and hypoxanthine were required to protect H35 cells treated with concentrations of dmAMT and MTX that inhibited growth by >90% relative to unprotected controls. In contrast, N9-propargyl-5,8-dideazafolic acid and N9-[5-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl)]-N9-methylaminomethyl[thenoyl-[γ-glutamic acid required only thymidine for protection. Like MTX, therefore, dmAMT appears to inhibit purine as well as pyrimidine de novo synthesis, and its effect on cell growth probably reflects the ability of dmAMT polyglutamates to not only block dihydrofolate reduction but also interfere with other steps of folate metabolism, either directly or indirectly via alteration of reduced folate pools. A similar protection pattern was obtained with mouse L1210 leukemia cells as with H35 cells, in that both thymidine and hypoxanthine were required for normal growth in the presence of dmAMT. Although folinic acid alone afforded full protection, 5-aminomimidazole-4-carboxamidine could not be used instead of hypoxanthine, suggesting that de novo purine synthesis inhibition by dmAMT probably occurs at the level of AICARFT rather than GARFT. In antitumor assays against L1210 leukemia in mice, comparable lifespan increases were achieved with dmAMT and MTX, but more dmAMT than MTX had to be used to produce the same therapeutic effect. The results of this study suggest that dmAMT may be a promising lead for the development of other, more potent, 2-desamino analogues of classical 2,4-diamino antifolates.

INTRODUCTION

2,4-Diamino analogues of folic acid have been important in cancer chemotherapy ever since the discovery of the potent antileukemic activity of AMT1 (1) and MTX (2) in the late 1940s (for an historical account, see Ref. 3). Although their biochemical mode of action is complex and not fully understood, the underlying basis of cell growth inhibition by these compounds is their ability to block de novo synthesis of the purine and pyrimidine nucleotide precursors of DNA (4). An important aspect of the biochemical pharmacology of all classical antifolates with a glutamate side chain, not recognized until some years after their original discovery, is intracellular “activation” by the ubiquitous enzyme FPGS, the normal function of which is to convert endogenous reduced folate cofactors to polyglutamylated metabolites (for reviews of this topic, see Refs. 5–7). Reduced folyl polyglutamates are more efficiently utilized than the monoglutamates in purine and pyrimidine nucleotide biosynthesis because they are better substrates for most of the enzymes of the folate pathway and also because their polyanionic character prevents exit from cells. Polyglutamates of classical antifolates also accumulate efficiently in many types of animal (8–12) and human (13–17) cells even though as a rule their FPGS substrate activity is less than that of natural reduced folate cofactors (18–21). Nonetheless, the polyglutamates of antifolates are pharmacologically important in that, like the polyglutamates of natural reduced folates, they do not efflux from cells and may bind more tightly than the monoglutamates to key enzymes of folate metabolism. For example, MTX itself has relatively low affinity for TS, GARFT, and AICARFT, whereas the longer polyglutamates of MTX have been found to bind more strongly to these enzymes (22–24), leading to the suggestion that MTX, and by implication other classical antifolates, should be viewed as prodrugs (25).

1 The abbreviations used are: AMT, aminopterin, 4-amino-4-deoxypteroyl-l-glutamic acid; MTX, methotrexate, 4-amino-4-deoxy-N9-methylhytrol-l-glutamic acid; dmAMT, 2-desamino-2-methylaminopterin; FPGS, folyl polylglutamate synthetase; TS, thymidylate synthase (EC 2.2.2.45); GARFT, glycinamide ribonucleotide formyltransferase (EC 2.1.2.1); AICARFT, 5-aminomimidazole-4-carboxamidine ribonucleotide formyltransferase (EC 2.1.2.2); DHFR, dihydrofolate reductase (EC 1.5.1.3); PDDF, N9-propargyl-5,8-dideazafolic acid (CB3717); IC1 D1694, N9-[5-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl)]-N9-methylaminomethyl[thenoyl-[γ-glutamic acid; IC50, 50% inhibitory concentration; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridylate; T/C, treated/control ratio. The subscript n in dmAMT+Glu4 indicates the number of γ-glutamyl residues.

Received 1/15/91; accepted 2/10/92.

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2 To whom requests for reprints should be addressed, at Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115.
A complicating factor regarding the concept of MTX as prodrug is that the binding of MTX polyglutamates to DHFR, the primary target for MTX, is only slightly greater than that of the parent drug when nonpolyglutamylated dihydrofolate is used as the substrate and is increased at most 10-fold with dihydropteroyl pentaglutamate as the substrate (26–28). Thus, while the prodrug character of MTX is very pronounced with respect to secondary targets like TS, GARFT, or AICARFT, this is less clearly the case where DHFR is concerned. Thus, an MTX analogue that inhibited DHFR only after polyglutamation was of interest, as it would be more truly an antifolate prodrug. Moreover, in terms of therapeutic selectivity, it was reasonable to think that differences in FPGS activity between tumor and normal tissues might be exploited better with a DHFR inhibitor that becomes cytotoxic only after polyglutamylation than with one which is already very active as the monogluta-mate. An opportunity to test this possibility came when AMT and MTX analogues lacking a 2-amino group were synthesized and found to be 100- to 1000-fold more active against tumor cells in culture than could be accounted for by their inhibition of purified DHFR (29, 30). Because these 2-desamino compounds were good FPGS substrates, with K\text{m} values comparable to that of AMT itself, it was proposed that they were probably being metabolized to polyglutamates intracellularly and that the polyglutamates, rather than the monoglutamates, were the species responsible for growth inhibition. Possible scenarios were (a) that the polyglutamates were more tightly bound to DHFR; (b) that the polyglutamates were not better inhibitors of DHFR but were inhibitors of other folate pathway enzymes such as TS, GARFT, or AICARFT; and (c) that the polyglutamates were better inhibitors of DHFR as well as one or more of the other enzymes. One way to address these possibilities was to synthesize the polyglutamates and test them against each of the various purified enzymes. This paper describes such studies, which were carried out with the recently synthesized polyglutamates of dmAMT (31) (Fig. 1). In addition, we present the results of cross-resistance studies with dmAMT and other antifolates in cultured H35 rat hepatoma and L1210 murine leukemia cell lines and report in vivo antitumor data for dmAMT against L1210 leukemia in mice.

MATERIALS AND METHODS

Materials. Swim's Medium S-77, horse serum, and fetal calf serum were obtained from Gibco (Grand Island, NY), and plastic tissue culture plates were from Falcon/Becton Dickinson (Lincoln Park, NJ). 7,8-Dihydrofolate was prepared by chemical reduction according to Blakley (32), and (6R,6S)-N\text{~}N\text{~}O\text{~}methylene tetrahydrofolate, by reaction of formaldehyde with (6R,6S)-tetrohydrofolate according to the method of Osborn et al. (33). (6R,6S)-N\text{~}N\text{~}O\text{~}formyltetrahydrofolate was prepared from (6R,6S)-5-formyltetrahydrofolate as described by Mueller and Benkovic (34). [5-\text{H}]UMP was obtained from Moravek Biochemicals (Brea, CA), and NADPH, 2-mercaptoethanol, and other reagents used for tissue culture and enzyme inhibition assays were purchased from Sigma Chemical Co. (St. Louis, MO). Other common chemicals were of the highest purity commercially available. MTX was a gift of Lederle Laboratories (Plymouth, NY). PDDF and ICI D1694 from ICI Pharmaceuticals (Merside, Macclesfield, Cheshire, England) were kindly provided by Dr. Ann Jackman (Institute of Cancer Research, Sutton, Surrey, England). DDATHF was synthesized as described earlier (35). C57BL/6 x DBA/2 F\text{~} (hereafter called B6D2F\text{~}) mice used for the in vivo antitumor assays were purchased from the Jackson Laboratory (Bar Harbor, ME).

Synthesis of dmAMT Polyglutamates. dmAMT+Glu\text{~} (n = 0 to 4) were prepared as previously reported (31). Briefly, tert-butyl-\text{~}gluta-\text{~}mate or tert-butyl esters of di- through pentaglutamate residues in each glutamate residue; e.g., "Glu\text{~}n" corresponds to dmAMT+Glu\text{~}n. NH\text{~}4OAc, ammonium acetate; MeCN, acetonitrile; aufs, absorbance units, full scale.

Fig. 2. HPLC separation of 2-desamino-2-methylaminopterin polyglutamates. Glu\text{~}n indicates the total number of glutamate residues in each species; e.g., "Glu\text{~}n" corresponds to dmAMT+Glu\text{~}n. NH\text{~}4OAc, ammonium acetate; MeCN, acetonitrile; aufs, absorbance units, full scale.

Fig. 1. Structure of 2-desamino-2-methylaminopterin and its polyglutamates. A and B represent the A-ring and B-ring, respectively.
concentration of TS, as determined by titration with FdUMP, was 0.2 µM.

GARFT inhibition was measured spectrophotometrically at 295 nm by a modification of the method of Young et al. (39). The enzyme was prepared from chicken liver and was homogeneous according to sodium dodecyl sulfate-gel electrophoresis. The assay mixture contained 50 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.5, 10 µM dmAMT+Glu4, 10 µM N5-formyl-5,8-dideazafolate, 250 µM glycaminide ribonucleotide (α/β-anomer mixture), 25% glycerol, and 0.5 µg of GARFT in a final volume of 0.5 ml. DDATHF was used in place of dmAMT+Glu4 as a positive control. The reaction was carried out at room temperature, and the change in absorption at 295 nm was followed spectrophotometrically.

AICARFT was purified by a modification of the method of Mueller and Benkovic (34), using enzyme isolated from human leukemic lymphoblasts (CCRF-CEM cells) and purified to electrophoretic homogeneity by affinity chromatography on AICAR-Sepharose. Purification also included an HPLC step using a BioGel HTP column. The kinetic constants of the human AICARFT were in good agreement with those reported for the enzyme from chicken liver (34). The standard reaction mixture for the inhibition assays contained 100 µM each of AICAR and (6R,6S)-N5-formyltetrahydrofolate in Tris-Cl, pH 7.4, containing 5 mM 2-mercaptoethanol and 25 mM potassium chloride. The concentration of enzyme was 0.6 nM, equivalent to 4 x 10^{-4} µmol/min of activity. AICARFT activity was monitored spectrophotometrically by following the appearance of tetrahydrofolate at 295 nm.

Cell Growth Inhibition. Mycoplasma-free H35 rat hepatoma cells (10) and their resistant sublines H35Rα3 (selected in 0.3 µM MTX; resistant because of reduced transport) (10), H35Rα5 (selected in 10 µM MTX; resistant because of decreased transport and increased DHFR activity) (40), and H35SFF (sensitive to MTX but resistant to TS inhibitors because of a 100-fold increase in TS activity after selection with FdUrd (40), and H35FF (sensitive to MTX but resistant to TS inhibitors because of reduced transport) (10), H35R10 (selected in 10 µM MTX; resistant because of reduced transport and increased DHFR activity) (40), and H35SFF were implanted i.p. with 1 x 10^6 L1210 leukemia cells on Day 0 and treated with a dose of 200 µg/ml of GARFT in a final volume of 0.5 ml. DDATHF was used in place of dmAMT+Glu4 as a positive control. The reaction was carried out at room temperature, and the change in absorption at 295 nm was followed spectrophotometrically.

RESULTS

Enzyme Inhibition by dmAMT+Glu4. The ability of dmAMT and its di-, tri-, and tetraglutamates to inhibit DHFR, TS, GARFT, and AICARFT was determined with the aim of assessing the effect of chain length on enzyme binding. As shown in Table 1, the IC50 values for dmAMT+Glu4 against purified human DHFR ranged from >50 µM (n = 0) to 0.25 µM (n = 4), those against human TS ranged from 29 µM (n = 0) to 0.20 µM (n = 3), and those against AICARFT ranged from 71 µM (n = 0) to 0.25 µM (n = 4). The Ki for AICARFT inhibition by dmAMT+Glu4 with (6R,6S)-N5-formyltetrahydrofolate as the variable substrate was 0.09 µM (Table 1), and inhibition was strictly competitive over a range of inhibitor concentrations from 0.05 to 0.25 µM (data not shown). None of the dmAMT+Glu4 species inhibited the GARFT-catalyzed reaction of 10 µM N5-formyl-5,8-dideazafolate and 250 µM glycaminide ribonucleotide by more than 15% at inhibitor concentrations of 10 µM, whereas the same concentration of DDATHF inhibited enzyme activity by 92% (data not shown). The highest degree of DHFR and AICARFT inhibition was observed with dmAMT+Glu4, with a marked increase in going from dmAMT+Glu1 to dmAMT+Glu4. In the case of TS, maximal inhibition was likewise achieved with dmAMT+Glu4, but nearly maximal increase in binding occurred with dmAMT+Glu3. Thus, addition of four glutamates to dmAMT led to a >200-fold increase in DHFR binding, an approximate 150-fold increase in TS binding, and an approximate 200-fold increase in AICARFT binding. No quantitative estimate of GARFT binding as a function of polyglutamate chain length was possible, since even the longest compound tested (dmAMT+Glu4) had no appreciable effect on GARFT activity.

Cell Growth Inhibition and Cross-Resistance. To extend our previous findings that dmAMT inhibits the growth of cultured tumor cells (29, 30), the IC50 values of the desamino compound against the parental H35 rat hepatoma line, the MTX-resistant

| Table 1 Enzyme inhibition by dmAMT+Glu4 |
|-------------------------------|-----------------|------------------|-------------------|
| Compound                      | DHFR IC50 (µM) | TS IC50 (µM)    | GARFT IC50 (µM)  |
| dmAMT+Glu1                    | >50 (1<)        | >10 (1<)         | >10 (1<)          |
| dmAMT+Glu2                    | >16 (1<)        | 0.53             | 0.53              |
| dmAMT+Glu3                    | >16 (1<)        | 0.53             | 0.53              |
| dmAMT+Glu4                    | >25 (1<)        | 0.53             | 0.53              |

Data shown are the means of two or more replicate experiments and have variability limits of <±15%.

* Spectrophotometric assay (340 nm) using 0.05 µM human enzyme purified by affinity chromatography.

* Tritium release assay using [5-3H]dUMP and 0.2 µM murine enzyme purified by affinity chromatography.

* Spectrophotometric assay (295 nm) using purified chicken liver GARFT (1 µM/ml) with N5-formyl-5,8-dideazafolate (10 µM) and glycaminide ribotide (250 µM) as the forimin donor. Less than 15% inhibition of activity relative to controls was observed at a dmAMT or dmAMT+Glu4 concentration of 10 µM.

* Spectrophotometric assay (295 nm) using purified AICARFT from CCRF-CEM human lymphoblasts and (6R,6S)-N5-formyltetrahydrofolate (100 µM) as the forimin donor. The IC50 values listed were determined directly from titration curves of enzyme activity as a function of inhibitor concentration. Ki values calculated from the equation IC50 = Ki(1 + S/Km) were found to be: dmAMT, 23.4; dmAMT+Glu4, 5.3; dmAMT+Glu3, 1.2; dmAMT+Glu2, 0.10; and dmAMT+Glu4, 0.082 µM. The values used for Km, the Michaelis-Menten constant for (6R,6S)-N5-formyltetrahydrofolate, and S, the concentration of (6R,6S)-N5-formyltetrahydrofolate substrate, were 46 and 94 µM, respectively. The Ki for AICARFT, determined by the standard double-reciprocal plot method was found to be 0.09 µM, in good agreement with the value estimated from the IC50 data.

* Numbers in parentheses after the IC50 values for dmAMT+Glu4 and dmAMT+Glu for DHFR, TS, and AICARFT columns are normalized relative to the IC50 of dmAMT (= 1.0) against each enzyme.

G. P. Beardsley and O. Russello, unpublished manuscript.
the MTX-resistant H35R0 cells were only slightly cross-resistant to PDDF (IC50 = 13 μM) but 180-fold cross-resistant to ICI D1694. Thus, dmAMT resembled MTX and ICI D1694 with regard to transport resistance (H35R0). However, cells with increased DHFR activity (H35R0) exhibited increased resistance to dmAMT and MTX, but not to the two TS inhibitors, PDDF and ICI D1694.

When the cross-resistance patterns among dmAMT, PDDF, and ICI D1694 were compared against H35FF cells, dmAMT (IC50 = 4 μM) was found to be only 36-fold resistant, whereas PDDF and ICI D1694 were >360-fold and >5000-fold resistant. In contrast, the H35FF cells showed no cross-resistance to MTX. Thus, dmAMT was closer to MTX than to the TS inhibitors when cross-resistance was examined in cells resistant toFdUrd by virtue of increased TS activity.

L1210 cells cultured for 72 h in the presence of 0.003 to 1.0 μM dmAMT were either not protected or only sparingly protected by dThd, HX, or AICA alone (Table 4). However, as with the rat hepatoma cells (see above) and other cell lines previously studied (29, 30), normal growth was observed in the presence of dThd and HX. Normal growth was likewise observed in cultures of CCRF-CEM human leukemic lymphoblasts treated with dmAMT and folic acid, and the degree of protection was dose related (data not shown); however, these cells differed in being intrinsically somewhat resistant to dmAMT, with an IC50 of 1.5 μM as compared with previously reported values of 0.042 μM against L1210 cells and 0.028 μM against WI-L2 cells (29, 30).

In Vivo Antitumor Activity. To confirm that dmAMT was active in vivo as well as in vitro, the drug was administered to mice with L1210 leukemia. As shown in Table 5, dmAMT doses of 6, 12, and 24 mg/kg on the once daily 9-day treatment schedule had no significant activity (T/C < 125%), whereas MTX at the optimally tolerated dose of 2 mg/kg gave a T/C of 250%. On the other hand, when dmAMT was administered on the twice daily 10-day treatment schedule at doses of 12, 24, and 48 mg/kg/day (6, 12, and 24 mg/kg/injection), T/C values of 242, 257, and 228% were obtained. Where T/C values for dmAMT on the once daily 9-day and twice daily 10-day treatment schedules could be compared at the same total daily dose (12 and 24 mg/kg), split dosing was clearly superior. Treatment with MTX at 4 mg/kg/day (2 mg/kg/injection) on the twice daily 10-day treatment schedule gave a T/C value of 200%. Thus, dmAMT was at least as effective as MTX in prolonging survival in the in vivo L1210 model (i.p. tumor/i.p. drug), provided that it was administered twice daily and at a higher dose than that of MTX.

Table 2 Activity of dmAMT against cultured H35 rat hepatoma cell lines and resistant sublines

Cells were incubated for 72 h in the presence of drugs; see “Materials and Methods.” Data shown are the means of two or more replicate experiments and have a limit of variability of ±11%. dmAMT was only tested once at a concentration of 5000 μM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>H35</th>
<th>H35R3</th>
<th>H35R9a</th>
<th>H35FF</th>
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<tbody>
<tr>
<td>dmAMT</td>
<td>0.11 (1.0) + 100 (910)</td>
<td>&gt;5.000 (&gt;45.000)</td>
<td>4 (36)</td>
<td></td>
</tr>
<tr>
<td>MTX</td>
<td>0.01 (1.0) + 1 (100)</td>
<td>100 (10,000)</td>
<td>0.01 (1)</td>
<td></td>
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<tr>
<td>PDDF</td>
<td>3.9 (1.0) + 8 (2.1)</td>
<td>13 (3.3)</td>
<td>&gt;1.400 (&gt;360)</td>
<td></td>
</tr>
<tr>
<td>ICI D1694</td>
<td>0.02 (1.0) + 3.4 (170)</td>
<td>3.6 (180)</td>
<td>&gt;100 (&gt;5,000)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, fold resistance for each compound relative to the H35 parental line (1.0).

Table 3 Protection of cultured H35 rat hepatoma cells from growth inhibition by dmAMT with thymidine and hypoxanthine

Cells were cultured for 72 h; see “Materials and Methods.” Protection is expressed as a percentage of the growth of control cultures containing 20 μM dThd and/or 50 μM HX but no drug. The results shown are the means of two or more replicate experiments with a limit of variability of ±17%.

<table>
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<th>Drug + HX</th>
<th>Drug + dThd + HX</th>
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<td>31</td>
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<tr>
<td>2.2 μM</td>
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<td>95</td>
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<td>4.4 μM</td>
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<td>72</td>
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<tr>
<td>8.8 μM</td>
<td>9</td>
<td>45</td>
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<td>95</td>
</tr>
<tr>
<td>MTX (1.0 μM)</td>
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<td>20</td>
<td>100</td>
</tr>
<tr>
<td>PDDF (39 μM)</td>
<td>10</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ICI D1694 (0.2 μM)</td>
<td>6</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tbody>
</table>

Sublines H35R0.3 (decreased transport) and H35R10 (decreased transport and increased DHFR), and the FdUrd-resistant H35FF (increased TS) subsline were determined (Table 2). Additionally, to confirm that growth inhibition was due entirely to an antifolate effect, the H35 cells were grown in the presence of dThd (20 μM), HX (50 μM), or a combination of dThd (20 μM) and HX (50 μM) (Table 3). As shown in Table 2, dmAMT in standard growth medium had an IC50 of 0.11 μM against the parental H35 cells, but was 10-fold less potent than MTX (IC50 = 0.01 μM) and 5-fold less potent than ICI D1694 (IC50 = 0.002 μM). However, it was 35-fold more potent than PDDF (IC50 = 3.9 μM). Only partial protection was achieved with 20 μM dThd or 50 μM HX alone when the cells were treated with dmAMT or MTX at concentrations that resulted in >90% inhibition of growth in the absence of protection; on the other hand, protection was complete with a mixture of 20 μM dThd and 50 μM HX (Table 3), or when the medium contained 50 μM folic acid (data not shown). In contrast, dThd alone completely prevented growth inhibition by concentrations of PDDF or ICI D1694 that blocked the growth of unprotected controls by >90%. However, when dmAMT was used at concentrations that produced only 65 to 75% growth inhibition, dThd afforded relatively greater protection (e.g., 72% at 4.4 μM versus 45% at 8.8 μM dmAMT).

The IC50 of dmAMT against the MTX-resistant sublines H35R3 and H35R10 was found to be 100 and >5,000 μM, respectively, as compared with an IC50 of 0.11 μM against the parental cells (Table 2). Thus, H35R3 cells were 100-fold resistant to MTX and 910-fold resistant to dmAMT, whereas H35R0 cells were 10,000-fold resistant to MTX and >45,000-fold resistant to dmAMT. The H35R3 cells were only slightly cross-resistant to PDDF (IC50 = 8 μM versus 3.9 μM for the parental line), but were 170-fold cross-resistant to ICI D1694 (IC50 = 3.4 μM versus 0.02 μM for the parental line). Similarly

Table 4 Protection of cultured L1210 mouse leukemia cells from growth inhibition by dmAMT with thymidine, hypoxanthine, and 5-aminomimidazole-4-carboxamide

Cells were cultured for 72 h; see “Materials and Methods.” Protection is expressed as a percentage of the growth of control cultures containing 5 μM dThd, 100 μM HX, 100 μM AICA, 5 μM dThd plus 100 μM AICA, or 5 μM dThd plus 100 μM HX, but no dmAMT. The results shown are the means of two or more replicate experiments with a limit of variability of ±15%.

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</table>

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Although the difference is only 18-fold. Polyglutamylation had recently by this group for derivatives of ICI D1694 with up to five additional glutamates, with IC_{50} values in the 200 to 300 nM range. Thus, there were observed for compounds containing a total of four or five glutamates would bind better than dmAMT to certain enzymes of the folate pathway, our data with dmAMT suggest that the distal region of the active site of the enzyme may include a domain, perhaps containing basic lysine or arginine residues, with which the poly/anionic side chain of polyglutamate conjugates can interact. Our results also support the view that polyglutamates of 2,4-diamino antifolates do not inhibit DHFR very differently from the parent compounds because the latter bind almost stoichiometrically. In other words, replacement of the 2-amino group by a methyl substituent makes it possible to use ordinary kinetic methods to assess the effect of polyglutamylation on binding, whereas this is difficult to do with very tightly bound 2,4-diamino antifolates like AMT or MTX.

Our finding that dmAMT and its polyglutamates were weak inhibitors of GARFT (Table 1) was in qualitative agreement with the literature data for MTX and its polyglutamates (23), as well as with the more recent finding of Ferone et al. (49) that ICI D1694 and its polyglutamates likewise bind poorly to this enzyme (IC_{50} > 50 μM).

Polyglutamylation has been reported to bring about substantial (>100-fold) increases in the ability of MTX to bind to TS (22) and AICARFT (24). Although caution must be exercised in comparing data from different laboratories using enzymes from different sources, and in comparing K_{i} ratios to IC_{50} ratios, the magnitude of these effects appears to be somewhat different for dmAMT and MTX. Thus, in studies of the interaction of MTX and its polyglutamates with enzymes from MCF-7 human breast carcinoma cells, Allegra et al. (22) found the K_{i} of MTX+Glu_{4} to be 277-fold lower than that of MTX against TS (22), whereas against AICARFT the K_{i} of MTX+Glu_{4} was 2500-fold lower than that of MTX (24). By comparison, we find that dmAMT+Glu_{4} binds only 55-fold more tightly to TS and only 284-fold more tightly to AICARFT than does dmAMT, suggesting that polyglutamylation enhances both TS and AICARFT binding to a greater degree in MTX than in dmAMT. In the absence of data on polyglutamates of the 2-desamino-2-methyl analogue of MTX, we do not know whether the enhanced sensitivity of MTX to polyglutamylation in comparison with dmAMT is due more to the 2-amino group or to the N^{60}-methyl substituent. The possibility clearly exists, and is worth exploring, that dmAMT analogues can be synthesized that enhance the polyglutamates of which are more potent than those of the parent drug. However, given the fact that the IC_{50} of dmAMT+Glu_{4} against human DHFR is 5-fold higher than that of IC_{50} of dmAMT against LI210 leukemia in mice.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg/day)</th>
<th>Schedule</th>
<th>Range</th>
<th>Median</th>
<th>T/C (%)</th>
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<tr>
<td>dmAMT</td>
<td>6</td>
<td>Treatment A</td>
<td>8–10</td>
<td>9</td>
<td>113</td>
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<tr>
<td></td>
<td>12</td>
<td>Treatment A</td>
<td>8–10</td>
<td>9</td>
<td>113</td>
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<tr>
<td></td>
<td>24</td>
<td>Treatment A</td>
<td>7–10</td>
<td>9</td>
<td>100</td>
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<tr>
<td></td>
<td>12</td>
<td>Treatment B</td>
<td>13–18</td>
<td>17</td>
<td>242</td>
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<tr>
<td></td>
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<td>17–18</td>
<td>18</td>
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<tr>
<td></td>
<td>48</td>
<td>Treatment B</td>
<td>16–17</td>
<td>16</td>
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<tr>
<td></td>
<td>6</td>
<td>Treatment B</td>
<td>13–15</td>
<td>14</td>
<td>171</td>
</tr>
</tbody>
</table>

* Five animals were used at each dose; a group of 18 untreated animals served as controls.

** The median survival of control animals was 8 days, with a range of 7 to 9 days.

** Treatment A, once daily on Days 1 to 9; Treatment B, twice daily for 10 days.

** The doses per injection in the twice daily 10-day experiments were 6, 12, and 24 mg/kg for dmAMT and 1, 2, and 3 mg/kg for MTX.

** One of five mice treated with MTX on the twice daily 10-day schedule died on Day 11 at the 4-mg/kg dose, and two of five mice died on Days 9 and 10 at the 8-mg/kg dose. There were no toxic deaths at any of the listed doses of dmAMT on the once daily 9-day or twice daily 10-day schedule (higher doses were not tested).

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DISCUSSION

dmAMT was originally synthesized to test the concept that, while the absence of a 2-amino group would greatly diminish binding of the parent compound to DHFR, polyglutamylation in tumor cells might enhance binding not only to DHFR but also perhaps to other key enzymes of folate metabolism (29, 30). This would make dmAMT more truly a prodru...
of nonpolyglutamylated MTX (28), a major objective of such a program should be to discover compounds whose polyglutamate not only bind better than those of dmAMT, but also bind better than MTX.

Although our results show that the IC_{50} values of dmAMT polyglutamates in the enzyme assays are several times higher than the IC_{50} of the parent compound for growth inhibition, sustained intracellular concentrations of polyglutamates in the enzyme-inhibitory range should be achievable if the dmAMT undergoes concentrative uptake followed by rapid polyglutamylation. However, because polyglutamylation is critically important where the ability of dmAMT to inhibit DHFR and other enzymes is concerned, the effect of this compound on growth would be expected to be highly dependent on the level of FPGS activity in a particular cell. This may account for the fact that (a) the IC_{50} of dmAMT against WI-L2, L1210, H35, and CCRF-CEM cells can vary as much as 100-fold, whereas IC_{50} values for MTX span a much narrower range; and (b) the IC_{50} of dmAMT against CCRF-CEM/R15 cells, which have normal transport properties but are antifolate resistant because of a markedly impaired ability to form polyglutamates (50), is at least 100-fold higher than the IC_{50} against the parent cells (data not shown). Except for the evidence from our cross-resistance studies that dmAMT probably enters cells via the same carrier-mediated active transport pathway as MTX (Table 3), the mechanism of dmAMT uptake has not been studied in detail.

The cross-resistance data obtained with H35 rat hepatoma cells and the resistant H35R_{o,3}, H35R_{o,9}, and H35FF sublines (Table 2) provide indirect information about the mode of action of dmAMT. The fact that the MTX-resistant H35R_{o,9} cells, which owe their resistance to a severe defect in MTX and reduced folate active transport (10), were cross-resistant to dmAMT and ICI D1694, but not to PDDF, suggests that these 2-desamino compounds are transported via the same carrier as MTX and reduced folates, rather than via the one used by nonreduced folic acid and PDDF. Jackman et al. (47) have reported that murine leukemic cells resistant to MTX because of a transport defect are not cross-resistant to PDDF but are cross-resistant to 2-desamino-PDDF. It thus appears that removal of the 2-amino group does not abrogate the ability of an antifolate to use the MTX/reduced folate transport pathway, regardless of whether the antifolate is a 4-amino or 4-oxo derivative. When the effects of dmAMT, PDDF, and ICI D1694 on the growth of a rat hepatoma subline (H35R_{o,10}) with increased DHFR activity as well as decreased transport (40) were compared, the cells were found to be highly cross-resistant to dmAMT, minimally cross-resistant to PDDF, but not more cross-resistant than the H35R_{o,3} cells to ICI D1694. The greater degree of cross-resistance to dmAMT than to ICI D1694 in both H35R_{o,3} and H35R_{o,9} cells suggests that TS inhibition by dmAMT polyglutamates has a lesser influence on growth than does TS inhibition by ICI D1694 polyglutamates. The finding that H35FF cells, which are 360-fold resistant to PDDF by virtue of increased TS activity (41), were only 36-fold cross-resistant to dmAMT likewise supports the view that TS inhibition by polyglutamates of dmAMT is not the principal mechanism by which this drug inhibits cell growth. It is of interest to note, however, that the PDDF-resistant H35FF cells showed complete lack of cross-resistance to MTX, as previously reported (51). This would suggest that dmAMT is not simply acting like MTX once it becomes polyglutamyalted.

The question of whether the primary effect of an antifolate is on pyrimidine or purine biosynthesis was addressed by means of standard protection experiments (Tables 3 and 4). As expected, dThd and HX alone afforded only 20% protection of H35 rat hepatoma cells when MTX was used at a concentration sufficient to decrease cell growth by 95% relative to control cultures during 72 h of treatment. As with MTX, protection from a 90% cytotoxic dmAMT concentration could not be achieved with dThd or HX alone, but was possible with combined dThd and HX. Moreover, protection from dmAMT with dThd appeared to be somewhat less effective than protection from MTX, whereas with HX the opposite was observed. In contrast to the results with MTX and dmAMT, dThd alone afforded complete protection from >90% cytotoxic PDDF and ICI D1694 concentrations, as expected from the fact that these drugs act predominantly at the level of TS (52, 53). Qualitatively similar results were obtained with L1210 cells, full protection being afforded only with dThd and HX (Table 4). Interestingly, it was also noted that substitution of AICA for HX, at a concentration known to satisfy the purine requirements of L1210 cells (44), gave no protection. Since conversion of AICA to AICARFT is proximal to the AICARFT step (44), these findings indicate that the block in purine biosynthesis in cells treated with dmAMT is probably at the level of AICARFT. While dmAMT polyglutamates may be the species directly responsible for AICARFT inhibition, it is also conceivable that purine synthesis inhibition by dmAMT is a secondary effect involving AICARFT inhibition by dihydrofolate polyglutamates (54–56). Overall, we conclude from the reversal studies that, while dmAMT qualitatively resembles MTX in that DHFR is the primary target, other enzymes of folate metabolism, such as TS and AICARFT, may also be affected indirectly (57).

The finding that comparable survival increases are possible with dmAMT as with MTX in mice with L1210 leukemia (Table 5) demonstrated that the 2-amino group in antifolates targeted against DHFR is not an absolute requirement for in vivo antitumor activity, although more drug has to be used. The dose of dmAMT found to give the best therapeutic effect (T/C = 257%) on the twice daily 10-day treatment schedule was 24 mg/kg, whereas for MTX a comparable increase in survival was obtained with approximately one tenth of this dose. This was consistent with previous cell culture data showing the IC_{50} of dmAMT against L1210 cells during 72 h of constant exposure to be 5-fold higher than that of MTX (29, 30). However, the fact that the potency difference between the two drugs was greater in vivo than in vitro suggests that additional factors must be considered. Less efficient intracellular conversion of dmAMT than MTX to long-chain polyglutamates is one possibility. Even though dmAMT is used 10 times more efficiently than MTX in an assay of diglutamate synthesis by partially purified FPGS (29, 30), it is not known whether polyglutamates with more than two glutamate residues are formed from dmAMT in intact tumor cells in a physiological milieu. Recent evidence suggests that the K_m for the conversion of a monoglutamate to a diglutamate is not necessarily predictive for each step of the polyglutamylation sequence (58). Moreover, a potentially important role has been identified for intracellular polyglutamate hydrolase activity in determining the chain length of antifolate polyglutamates in intact cells (for a review, see Ref. 59). Finally, it is possible that the 10-fold difference in optimal therapeutic dose observed in vivo between dmAMT and MTX is due to a pharmacokinetic effect, with dmAMT being cleared more rapidly than MTX, e.g., via 7-hydroxylation.
Because dmAMT contains one less NH₂ group than MTX, it is more hydrophobic and thus may be a better substrate for hepatic aldehyde oxidase, as has been shown with other lipophilic MTX analogues (60).

In summary, replacement of the 2-amino group in AMT by a methyl substituent yields a novel antifolate (dmAMT) the biological activity of which appears to be polyglutamylation dependent and may therefore vary widely from cell to cell. Unlike MTX and other classical 2,4-diamino antifolates whose nonpolyglutamylated forms are already potent DHFR inhibitors, dmAMT can be considered a true prodrug. Protection of rat hepatoma cells from >90% cytotoxic concentrations of dmAMT is afforded by dThd and HX in combination, or by folic acid, but not by dThd or HX alone. A similar protection pattern is observed in mouse L1210 leukemia cells. Furthermore, protection of L1210 cells does not occur when AICA is substituted for HX, supporting the view that inhibition of de novo purine synthesis by dmAMT is probably at the level of AICARFT. Cross-resistance studies suggest that transport across the cell membrane is probably via the MTX/reduced folate pathway, and that the primary enzyme target of dmAMT after polyglutamylation is DHFR rather than TS or GARFT. However, the possibility is not excluded that secondary effects on cell growth may occur via expansion of the dihydrofolate pool and concomitant depletion of N⁵,N¹⁰-methylene tetrahydrofolate and N⁵-formyltetrahydrofolate. In assays of in vivo antitumor activity in mice with L1210 leukemia, dmAMT is less toxic than MTX and produces a similar increase in lifespan, although larger doses and more frequent treatment are required, as one might expect for any antifolate the activity of which depends on polyglutamylation. This requirement also appears to exist in the case of the potent desamino compound ICI 2154, which is of the 4-oxo rather than 4-amino type and has TS instead of DHFR as its primary target. Thus, provided that efficient uptake and metabolic enhancement of enzyme binding via polyglutamylation occur, deletion of the 2-amino group is as acceptable in antifolates targeted against DHFR as it is in antifolates targeted against TS.

ACKNOWLEDGMENTS

We are grateful to Drs. Carmen Allegra (National Cancer Institute, Bethesda, MD), Ann Jackman (Institute of Cancer Research, Sutton, Surrey, England), Terry Jones (The Agouron Institute, La Jolla, CA), Roy Kisliuk (Tufts University, Boston, MA), and Richard Moran (USC Comprehensive Cancer Center, Los Angeles, CA) for stimulating discussions during this work.

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