Polyglutamylation of the Dihydrofolate Reductase Inhibitor γ-Methylene-10-deazaaminopterin Is Not Essential for Antitumor Activity

S. Cao, A. Abraham, M. G. Nair, R. Pati, J. H. Galivan, F. H. Hau sher, and Y. M. Rustum


ABSTRACT

As part of a continuing program aimed at developing nonpolyglutamylatable inhibitors of dihydrofolate reductase that are less toxic and more specific in their action, we herein report the therapeutic efficacy and toxicity of γ-methylene-10-deazaaminopterin (MDAM) in athymic nude mice bearing advanced human HCT-8 ileocecal xenografts and its antitumor activity in C57BL/6 × DBA/2 F1 (hereafter called B6D2F1) mice bearing P388 murine leukemia. For the xenograft study, MDAM was administered at the maximum tolerated dose by the following dose schedules: (a) 5-day continuous i.v. infusion at 1.0 mg/kg/day (schedule I); and (b) i.v. push, daily for 5 days at 50 mg/kg/day (schedule II). The maximum tolerated dose values for methotrexate (MTX) under these conditions were 0.2 and 1.0 mg/kg/day for schedule I and schedule II, respectively. MTX did not exhibit any significant antitumor activity in this model system by both schedules; however, MDAM induced complete responses of 13 and 25% and partial responses of 28 and 50% by schedules I and II, respectively. MDAM also exhibited antitumor activity significantly superior to that of MTX in the P388 tumor model. One of the enantiomers of MDAM, which possesses the natural configuration at the γ-methylene glutamate moiety (L-MDAM), has been shown to be a better inhibitor of human recombinant dihydrofolate reductase and H35 hepatoma cell growth than d-L-MDAM. L-MDAM inhibited the uptake of radiolabeled folic acid to H35 hepatoma cells eight times more efficiently than MTX. The results indicate that the superior activity of MDAM relative to MTX may be partially due to a combination of enhanced transport to tumor cells and slower deactivation by aldehyde oxidase.

INTRODUCTION

Antifolates are compounds that interfere at various stages of folate metabolism (1, 2). The central role of tetrahydrofolate in cell growth and tissue proliferation has been the topic of extensive studies and reviews (1–4). DHFR, TS, glycaminamide ribonucleotide formyltransferase, and folypolyglutamate synthetase are folate-dependent enzymes that have been recognized as therapeutic targets for anticancer drug development. Among the various types of antifolates currently in clinical use or under development, MTX, which is a powerful, near-stoichiometric, competitive inhibitor of DHFR, continues to be the most widely used anticancer agent. As with any classical antifolate, MTX undergoes polyglutamylation in a dose- and time-dependent manner (5, 6), and these metabolites are believed to play significant roles in the therapeutic efficacy and toxicity of the drug (7–9).

Recently, the synthesis and biological evaluation of a number of interesting folate analogue inhibitors of DHFR that are inefficient in undergoing polyglutamylation have been reported (10–15). Among them, a tetrazole analogue of MTX (10, 11), an aminopterin analogue bearing a phthaloyl-protected ornithine moiety (PT 523; Refs. 12 and 13), and MDAM are not substrates of folypolyglutamate synthetase. Despite their inability to be metabolized to polyglutamates, all of the above compounds exhibited markedly superior growth inhibition of tumor cells in culture relative to the polyglutamylatable MTX under identical conditions of continuous exposure. F-MTX (14, 15) is an analogue of MTX in which the L-glutamate moiety is replaced with a 4-fluoroglutamate residue. The diastereomeric mixture of F-MTX exhibited comparable activity to MTX in inhibiting the growth of a number of tumor cells in culture on continuous exposure, although it is not polyglutamylated efficiently. F-MTX differs from MDAM with respect to its ability to be metabolized to the diglutamate level and the pronounced efficacy of MDAM to compete with folic acid transport to H35 hepatoma cells. The more active erythrodiastereomer of F-MTX and MTX have similar IC50 values to compete with folic acid transport to these cells (18.0 μM) compared with 2.3 μM for L-MDAM. L-MDAM has an influx advantage of 8-fold.

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over MTX by the RFT. Recently, it was reported that PT 523 is retained and bound to plasma proteins more efficiently than MTX, and it exhibited tumor growth delays superior to MTX in the murine and human xenograft models (13). Both F-MTX and the tetrazole analogue of MTX have not yet been evaluated for antitumor activity in an appropriate human tumor model in vivo. Both trimetrexate and piritrexim differ from MTX and MDAM with respect to their inability to be transported to tumor cells by the RFT. Because PT 523 has been reported to be moderately active against tumor cells that are resistant to MTX by virtue of defective transport via the RFT, it is reasonable to conclude that it is taken up by the cells partially by other mechanisms, such as facilitated diffusion. This is corroborated by the fact that PT 523 was able to overcome MTX resistance in the transport-defective 15/R1 squamous cell carcinoma cell line and its activity against the growth of the M 5076 ovarian reticulum cell sarcoma in mice, which is a tumor naturally resistant to MTX due to a transport defect (13). To our knowledge, MDAM is the first example of a "pure" folate analogue DHFR inhibitor capable of cellular influx solely by the RFT and unable to undergo polyglutamylation.

The biochemical and pharmacological consequences of antifolate polyglutamylation are diverse and intriguing. Once formed within the cells, MTX polyglutamates do not efflux efficiently, and the excess fraction that is not bound to DHFR directly inhibits TS (16, 17) and aminomimidazole carboxamid ribonucleotide formyltransferase (18). Because these two enzymes are involved in de novo pyrimidine and purine biosynthesis, respectively, the antipurine and antipyrimidine effects of MTX are potentiated by polyglutamylation. Although the cytotoxicity of MTX is enhanced by polyglutamylation, the losses of pharmacological control and target specificity of the drug are undesirable consequences that seriously limit its clinical utility. MDAM is an analogue of MTX that is incapable of polyglutamylation. It inhibits human DHFR to a degree similar to that of MTX and interacts with the RFT in H35 hepatoma cells more efficiently than MTX.

The objective of this study was to determine whether polyglutamylation of a classical folate analogue inhibitor of DHFR is an essential requirement for in vivo antitumor activity and whether it is capable of efficient transport to tumor cells via the RFT. In this article, we report a comparative evaluation of the therapeutic efficacy and toxicity of the polyglutamylatable MTX versus the nonpolyglutamylatable MDAM using the advanced human ileocecal HCT-8 xenograft in athymic nude mice and its antitumor activity in mice bearing P388 leukemia.

MATERIALS AND METHODS

Drugs. MDAM was synthesized at the University of South Alabama (Fig. 1). MTX was obtained from the National Cancer Institute through the courtesy of Dr. J. A. R. Mead. Stock solutions of MDAM and MTX were made by dissolving 100 mg of the drug in 1.0 ml 5% sodium bicarbonate solution by gentle warming (50°C) and shaking. The resultant clear solution was filtered through a 4.5-micropore filter and diluted as desired with 0.9% NaCl solution. Time-released pellets of MTX and MDAM were custom formulated by Innovative Research, Inc. (OH) such that uniform release at the predetermined dosage was accomplished during a 15-day period.

Mice. Eight- to 12-week-old female DBA/2N mice and athymic nude mice (body weight, 20–25 g), were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). They were kept five/cage with water and food ad libitum. Micro-Isolator cages were used for nude mice. B6D2F1 female mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were kept six/cage with water and food ad libitum.

Tumor. Human HCT-8 ileocecal carcinoma was obtained from the American Type Culture Collection (Rockville, MD) and was maintained in RPMI 1640 medium supplemented with 1 mm sodium pyruvate and 10% dialyzed horse serum. The cells have a doubling time of ~18 h. Initially, the growth of human HCT-8 ileocecal carcinoma was established in athymic nude mice by implanting 10^6 cells s.c. Subsequently, ~50-mg tumor pieces were transplanted s.c. into mice when tumor sizes reached ~1–1.5 g. Treatments were initiated when tumor sizes reached 150–200 mg (~8–10 days after transplantation) after passage through several generations. P388 lymphocytic leukemia cells obtained from the American Type Culture Collection were maintained as above, and 1 x 10^6 cells were i.p. implanted in B6D2F1 mice and propagated for two generations. Cells were harvested from the second generation, and the tumors were implanted in test and control animals by injecting 0.1 ml diluted ascitic fluid containing 1 x 10^6 cells for therapy experiments.

MTD. The MTD was defined as the maximum dose that could be administered to tumor-bearing mice without causing drug-related lethality and maximum weight loss ≤20%.

Tumor Measurements and Body Weight. Two axes (mm) of each tumor (L, longest axis; W, shortest axis) were measured with the aid of a Vernier caliper. Tumor weight (mg) was estimated as:

\[
\text{tumor weight} = \frac{1}{2} (L \times W^2)
\]

Tumor measurements were taken before treatment and once a day on days 1–8, every 2 days on days 8–20, and thereafter, twice a week after treatment. As a general policy, mice were
Inhibition of recombinant Human DHFR, folinic acid influx, and H35 hepatoma cell outgrowth by MTX, d-L-MDAM, and L-MDAM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_50 (M)</th>
<th>DHFR</th>
<th>Folinic acid influx</th>
<th>H35 cell growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>1.7 x 10^-8</td>
<td>18.0 x 10^-6</td>
<td>10 x 10^-9</td>
<td></td>
</tr>
<tr>
<td>d-L-MDAM</td>
<td>4.4 x 10^-8</td>
<td>5.5 x 10^-6</td>
<td>39.0 x 10^-9</td>
<td></td>
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<tr>
<td>L-MDAM</td>
<td>2.2 x 10^-8</td>
<td>2.3 x 10^-6</td>
<td>8.0 x 10^-9</td>
<td></td>
</tr>
</tbody>
</table>

*Data were provided by Roy L. Kislik.

Antitumor Activity Evaluation. Antitumor activity was assessed by the mean time for tumors to reach twice the initial size and the mean maximum inhibitory ratio of the relative tumor volumes of the treated over the control mice. PR represents a >50% reduction in tumor volume, and CR represents mice with no detectable tumors at the initial sites of tumor appearance. For the P388 murine tumor model, tumor-bearing animals were euthanized at the first sign of immobility. The %T/C was calculated on the basis of an increase in survival time of the test animals relative to untreated controls.

Treatment Schedules. MDAM and MTX were administered by: (a) continuous i.v. infusion (19) for 5 days (schedule I); and (b) i.v. push daily for 5 days (schedule II), according to 0.2 ml/20 g mouse weight on a mg/kg basis. Each group contained four or five mice, and experiments were repeated at least once; therefore, there were two to four separate experiments at each dose schedule. All studies were performed in accordance with Institutional Animal Care and Use Committee guidelines and under approved institution protocol.

DHFR Enzyme Assay. A DHFR enzyme assay was performed spectrophotometrically in a solution containing 50 μM dihydrofolate, 80 μM NADPH, 0.05 M Tris-HCl, 0.01 M 2-mercaptoethanol, and 0.001 M EDTA (pH 7.4) at 30°C. The reaction was initiated with an amount of enzyme yielding a change in A at 340 nm of 0.015/min. Pure human recombinant enzyme was used.

Statistical Evaluation. Student’s t tests were used for statistical evaluation, and differences were considered statistically significantly different when P < 0.05.

RESULTS

Inhibition of Recombinant Human DHFR, Folic Acid Influx, and H35 Hepatoma Cell Outgrowth by MTX, d-L-MDAM, and L-MDAM. The data in Table 1 constitute an outline of the inhibition of DHFR, folinic acid influx, and growth inhibition by MTX, d-L-MDAM, and L-MDAM. The data indicated that the effect on DHFR and cell growth by MIX and L-MDAM were similar; however, L-MDAM was more effective than MTX at inhibiting folinic acid influx.

MTD. To identify the MTD of MDAM and MTX, DBA/2N mice were treated via the i.v. route with different drug doses according to the two schedules of drug administration: (a) continuous i.v. infusion for 5 days (schedule I); and (b) i.v. push for 5 days (schedule II), and the results are summarized in Fig. 2. The data demonstrated a steep dose-response curve for both drugs when used on either schedule I or schedule II. The MTD of MDAM was found to be 1.0 mg/kg/day on schedule I and 50.0 mg/kg/day on schedule II. The corresponding values for MTX were 0.2 and 1.0 mg/kg/day, respectively (Table 1). Depending on the schedule, the MTD of MDAM was 5–50 times higher than that of MTX. The results in DBA/2N mice were confirmed in nude mice.

Antitumor Activity of MDAM and MTX at the MTD. The antitumor activity of MDAM and MTX in nude mice bearing HCT-8 ileocecal xenografts was determined for two different schedules. For 5-day, continuous i.v. infusion (Fig. 3 and Table 2), the data demonstrated superiority of MDAM over MTX in terms of the mean maximum inhibitory ratio, mean time for tumors to reach twice the initial size, PR, and CR. With this schedule, MTX at the MTD (0.2 mg/kg/day) did not exhibit any significant antitumor activity, with no PR or CR. Further escalation of the dose was not possible because of toxicity. In
Preclinical Evaluation of MDAM

complished by surgical insertion of time-release pellets was contrast, using the same schedule, MDAM at the MTD (1 mg/kg/day) produced a 13% CR and a 25% PR (38% objective response).

The antitumor activity of MDAM and MTX using an i.v. push daily for 5 days (schedule II) was also evaluated, and the results are shown in Fig. 4 and Table 2. MTX at the MTD (1 mg/kg/day) had no significant efficacy and showed greater but reversible toxicity than MDAM (body weight loss). MDAM at the MTD (50 mg/kg/day) produced a 25% CR and a 50% PR (75% overall response). The data presented in Fig. 3 and Table 2 indicate that, with both schedules, MDAM is more active and less toxic than MTX in this model system.

A continuous s.c. release of both MTX and MDAM accomplished by surgical insertion of time-release pellets was used for their antitumor evaluation in mice bearing P388 leukemia. At a dose of 30 mg/kg/day, MDAM and MTX exhibited %T/C values of 131 and 111% respectively. However, a schedule of ip injections of MDAM on days 1–3, 7–9, and 13–15 at 75 mg/kg/injection resulted in a dramatic increase of the %T/C value to 152%, indicating the schedule dependency of the antitumor activity of MDAM.

DISCUSSION

MDAM is a classic nonpolyglutamylatable DHFR inhibitor, which is transported to cells by the RFT (20). Previous pulse exposure studies from our laboratory using H35 hepatoma cells in culture demonstrated that MDAM is capable of enhanced clearance relative to MTX (20), which is consistent with the phenomenon associated with nonpolyglutamylatable antifolates. Polyglutamylation of antifolates with greater tissue retention and cytotoxicity has been well established (8, 9). With higher concentrations of polyglutamates derived from MTX, 10-DAM, and edatrexate that exceed the binding capacity of DHFR, these agents inhibit other folate-dependent enzymes, TS, and aminomimidazole carboxamide ribonucleotide formyltransferase (21–23). Although multiple inhibition of folate-dependent enzymes secondary to polyglutamylation potentiates cytotoxicity, the resultant loss of pharmacological control is a serious limitation of cancer treatment with classic antifolates. Classic nonpolyglutamylatable DHFR inhibitors capable of efficient influx to tumor cells, on the other hand, may be subjected to better pharmacological control in a clinical setting, due to their rapid tissue clearance and preservation of target enzyme specificity.

We have recently shown that, like MTX, MDAM is hydroxylated at position 7 with rabbit liver aldehyde oxidase (24). The rate of oxidation as determined by spectrophotometry and high-performance liquid chromatographic analysis was strikingly lower for MDAM relative to MTX. More significantly, 7-hydroxy-MDAM was considerably less cytotoxic than 7-hydroxy-MTX to CCRF-CEM human leukemia cells or H35 hepatoma cells in culture, despite the fact that 7-hydroxy-MDAM was equally as efficient as MDAM for influx to the latter cell line. One obvious explanation for the enhanced cytotoxicity of 7-hydroxy-MTX is its ability to be polyglutamylated and to interfere at multiple sites of folate metabolism in tissues such as liver and kidney, causing organ toxicity, as seen in patients receiving high-dose MTX therapy (25). It is reasonable to assume that a slower rate of 7-hydroxylation and the very low

| Treatment (mg/kg/day) | Schedule Schedule MIR (%) TD (days) CR (%) PR (%) |
|-----------------------|------------------------|-----------------|-----------------|
| Control               | 1 + II                 | 1.0 ± 0.5       | 0 0             |
| MTX<sub>2.0</sub>     | I                      | 50 ± 9          | 5.0 ± 0.5       | 0 0             |
| MDAM<sub>5.0</sub>    | II                     | 174 ± 1'        | 7.6 ± 1.2'      | 13<sup>d</sup> 25<sup>d</sup> |
| MTX<sub>10.0</sub>    | I                      | 30 ± 9          | 4.6 ± 0.6       | 0 0             |
| MDAM<sub>50</sub>     | II                     | 79 ± 6<sup>d</sup> 8.4 ± 0.8<sup>d</sup> | 25<sup>d</sup> 50<sup>d</sup> |

<sup>a</sup> See text for description of schedules.
<sup>b</sup> Values are mean ± SD.
<sup>c</sup> P < 0.01.
<sup>d</sup> P < 0.001.
cytotoxicity of 7-hydroxy-MDAM might be partially responsible for the observed more favorable toxicity profile of MDAM relative to MTX.

Using L-γ-methylene glutamate isolated from peanut seedlings, we have synthesized the L-enantiomer of MDAM (26). This enantiomer of MDAM exhibited strikingly superior activity relative to D,L-MDAM in inhibiting the growth of H35 hepatoma cells. The IC₅₀ of growth inhibition for D,L-MDAM and L-MDAM were 39 and 8 nM, respectively. Although D,L-MDAM was approximately three times more effective than MTX in inhibiting the uptake of radiolabeled folic acid in H35 hepatoma cells, L-MDAM was eight times more potent (Table 1) than MTX (26). Taken together, these results indicate that the observed anticancer activity and low toxicity of MDAM most probably are due to: (a) preservation of target enzyme (DHFR) specificity because of lack of polyglutamylation; (b) very efficient influx of MDAM to tumor cells by the RFT; and (c) diminished deactivation of MDAM by aldehyde oxidase.

Although a large body of evidence supports the view that polyglutamylation of classic antifolates generally potentiates cytotoxicity (8, 9), polyglutamylation per se does not seem to be a prerequisite for the therapeutic activity of folate analogues DHFR inhibitors (10, 13, 20, 27). The uncertainty surrounding the exact role of these important metabolites in host toxicity versus anticancer response is difficult to resolve unless and until a classic nonpolyglutamylatable folate antagonist capable of efficient influx to tumor cells via the RFT demonstrates an impressive anticancer response in vivo relative to a polyglutamylatable counterpart such as MTX.

In Figs. 3 and 4 and Table 2, we have summarized the antitumor activity and toxicity of MDAM and MTX at different doses, including their respective MTDs, under two different schedules in athymic nude mice bearing advanced human HCT-8 ileocecal carcinoma xenografts. MDAM exhibited greater antitumor activity and less toxicity relative to MTX in this model system. Using the human panel of the National Cancer Institute, both D,L-MDAM and L-MDAM exhibited a wide spectrum of cytotoxicity and were more active than MTX in at least 22 tumor cell lines, representing leukemia, non-small cell and small cell lung cancers, colon and central nervous system cancers, melanoma, and ovarian and renal cancers (26, 27). MDAM is also more active than MTX in increasing the mean survival of mice bearing P388 leukemia. Collectively, these preclinical data of the nonpolyglutamylatable MDAM versus the polyglutamylatable MTX indicate the potential superiority of the former as an anticancer agent. The preceding preclinical results have led to the introduction of MDAM in the clinics, which is presently undergoing a Phase I clinical trial in the United States. The results of this trial will undoubtedly contribute to a better understanding of the role of polyglutamylation in the therapeutic efficacy and toxicity of antitumor antifolates.

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