Antitumor Activity of Antifolate Inhibitors of Thymidylate and Purine Synthesis in Human Soft Tissue Sarcoma Cell Lines with Intrinsic Resistance to Methotrexate

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ABSTRACT

We examined the antitumor effects of two antifolate inhibitors of thymidylate synthesis, N-(5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thienyl-L-glutamic acid (D1694; Tomudex) and 1843U89 as well as a folate-based inhibitor of purine synthesis, 5,10-dideazatetrahydrofolic acid (DDATHF) on human soft tissue sarcoma cell lines having intrinsic resistance to methotrexate (MTX) due to impaired accumulation of polyglutamates of MTX (HS-16 and HS-42 cells) and to increased levels of dihydrofolate reductase and thymidylate synthase activity (HS-18 cells). Growth inhibition studies showed that ED₅₀ values for D1694 and 1843U89 after a 24-h exposure were 11-19-fold and 22-222-fold lower, respectively, than those for MTX in HT-1080, a MTX-sensitive cell line, and the three MTX-resistant cell lines. In contrast, DDATHF was less cytotoxic than MTX in both the MTX-sensitive and the three resistant sarcoma cell lines. Uptake of D1694, 1843U89, or DDATHF was 2.5-4.5-fold lower than MTX in these sarcoma cell lines. However, D1694 and 1843U89, unlike MTX, accumulate in HS-16 and HS-42 cells as polyglutamate forms, reaching 70% of the total intracellular drug level after 24 h. DDATHF polyglutamates (9.4-24%) were less in the same cell lines. Much lower Kₘ values for D1694 and 1843U89 as compared to MTX for folylpolyglutamate synthase were measured in the sarcoma cell lines, with Vₘₐₓ values equal to or slightly higher than those obtained with MTX.

D1694 and 1843U89 are significantly more cytotoxic than MTX in intrinsically MTX-resistant sarcoma cell lines as a result of extensive formation of polyglutamates. These two thymidylate synthase inhibitors should be evaluated in patients with soft tissue sarcomas.

INTRODUCTION

Soft tissue sarcomas remain one of the most difficult human cancers to treat by chemotherapy. For example, MTX has been shown to have only marginal activity against this disease, although this drug is effective in the treatment of other cancers which include acute lymphocytic leukemia, choriocarcinoma, head and neck cancer, and breast cancer (1, 2). Impaired MTX transport, decreased accumulation of long chain MTX polyglutamates, and increased DHFR levels, are causes of resistance to MTX in soft tissue sarcomas (3-5). Among these mechanisms, decreased accumulation of long chain polyglutamates of MTX was found to be the major mechanism of intrinsic resistance. New antifolates such as trimetrexate and 10-ethyl-10-deazaaminopterin are in clinical trial, based on improved antitumor properties compared to MTX in experimental tumors and some human cancers. 10-Ethyl-10-deazaaminopterin is better transported and polyglutamylation than MTX, and trimetrexate does not require polyglutamylation for retention (6, 7). However, these drugs also act on DHFR, and their effects on soft tissue sarcomas are limited by toxicity or may share similar resistant mechanisms as MTX (5, 8).

In recent years, newer antifolates including D1694, 1843U89, and DDATHF have been synthesized as inhibitors of folate-dependent enzymes other than DHFR. The quinazoline antifolate D1694 and benzoquinazoline antifolate 1843U89 are inhibitors of TS (9, 10), and DDATHF inhibits de novo purine biosynthesis by acting on GARFT (11). These drugs therefore may circumvent MTX resistance due to an increased or an altered DHFR enzyme. Also, these drugs are retained in cells due to polyglutamate formation, a property that may enhance cytotoxicity especially after short-term exposure to drugs (12-14). D1694 has been shown to have significant antitumor activity against colon and breast cancer in Phase II studies (15). D1843U89, scheduled for Phase I studies, has potent antitumor effects versus human tumor cell lines (10). DDATHF is also highly active against experimental tumors in vitro and in vivo and is currently undergoing clinical trials (16). However, little information is available concerning the cytotoxicity and mechanisms of action of these drugs in soft tissue sarcomas, especially in tumors with intrinsic resistance to MTX. In this article, we demonstrate that D1694 and 1843U89 are more potent, and DDATHF is less potent than MTX in sarcoma cell lines intrinsically resistant to MTX.

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MATERIALS AND METHODS

Chemicals. MTX was obtained from Lederle Laboratories. [1H]MTX (20 Ci/pmol) was purchased from Moravek Biochemical (Brea, CA). D1694 and [5-3H]D1694 (19.1 Ci/pmol) were supplied by Zeneca Pharmaceuticals. D1694, in the free acid form as a pale yellow powder, was dissolved in 50 mM NaHCO₃, and the pH was adjusted to 9–9.5 using NaOH. 1843U89 and [1H]1843U89 (31 Ci/pmol) were gifts from Dr. R. Ferone of Burroughs Wellcome. 1843U89 was dissolved in 10 mM NaHCO₃, and the pH was adjusted to 9-9.5 using NaOH. (6R)-Ferone of Burroughs Wellcome. 1843U89 was dissolved in 10 mM NaHCO₃, and the pH was adjusted to 9-9.5 using NaOH. (6R)-DDATHF and [14C]DDATHF (7.5 mCi/pmol) were supplied by Lilly Laboratories. DDATHF was dissolved in 0.1 N NaOH, and the pH was adjusted to 7.0. Polyglutamate standards were gifts from Dr. R. Ferone (1843U89 + Glu₄) and Dr. A. Jackman (D1694 + Glu₄). MTX polyglutamates (MTX + Glu₂₋₃) were purchased from B. Schirke Laboratories (Jona, Switzerland). 1-[2,3-3H]Glutamic acid (NET-395, 25 Ci/ml) was obtained from New England Nuclear (Boston, MA). All other chemicals were reagent grade and obtained from standard commercial sources.

Cell Lines. HT-1080, a human fibrosarcoma cell line, was obtained from American Type Culture Collection. HS-16 (mesenchymal chondrosarcoma), HS-18 (liposarcoma), and HS-42 (mixed mesodermal tumor ranging from liposarcoma to rhabdomyosarcoma) were established from untreated patients (5). All cell lines were grown as monolayer cultures at 37°C in a 5% CO₂ humidified atmosphere in RPMI 1640 media containing 10% fetal bovine serum. Growing cells were counted using a Model ZB Coulter Counter, and ED₅₀ values were determined from growth inhibition data (17).

Drug Uptake Studies. Exponentially growing cells were harvested by centrifugation after trypsinization. Cells were suspended in RPMI 1640 containing 10% fetal bovine serum at a density of 10⁵ cells/ml and incubated at 37°C in the presence of 1 μM [1H]D1694 (1020 cpm/pmol), [1H]1843U89 (1700 cpm/pmol), or [14C]DDATHF (35 cpm/pmol), respectively. At specified times, 100-μl aliquots of cells were removed, added to 700 μl ice-cold 0.9% NaCl solution and 200 μl 9% sucrose in microfuge vials, washed twice with cold 0.9% NaCl, boiled for 5 min, and centrifuged for 2 min at 15,000 × g. The radioactivity in the supernatant fraction was then measured using a liquid scintillation counter.

Analysis of Intracellular Levels of D1694, 1843U89, and DDATHF Polyglutamates. Radiolabeled drugs (final specific activity, 680, 395, and 13.8 cpm/pmol for D1694, 1843U89, and DDATHF, respectively) were added to flasks of exponentially growing cells to yield a 1.0 or 10 μM concentration. At indicated times, cells were harvested by scraping, centrifuged (4°C), and washed twice with ice-cold 0.9% NaCl solution. The cell pellets were suspended in 500 μl of boiling 50 mM sodium phosphate (pH 5.5), boiled for 5 min, and centrifuged at 20,000 × g for 10 min at 4°C. Supernatants were stored at −20°C until analysis using HPLC. HPLC analysis of cell extracts was performed using a modification of a previously described method (12, 13, 18). Separations were done on an absorbosphere C18 column (5-μm particle size, 4.6 × 250 mm) equipped with a 1-cm guard cartridge column of the same material from Alltech Associates using a Spectra Physics 8800 HPLC gradient pump. A 100-μl sample was injected. For D1694, the mobile phase consisted of 0.1 M sodium acetate buffer (pH 5.5) and a linearly increasing gradient of acetonitrile (4–14%) over 60 min at a flow rate of 1 ml/min. For 1843U89, the mobile phase consisted of 0.1 M sodium acetate buffer (pH 5.5) and a linearly increasing gradient of acetonitrile (3–30%) over 50 min at a flow rate of 1 ml/min. For DDATHF, the mobile phase consisted of 0.1 M sodium acetate buffer (pH 5.5) and a linearly increasing gradient of methanol/acetonitrile (10–60%). Unlabeled standards were added to the samples to provide internal controls. Radioactivity in each 1-ml fraction was measured by a Beckman LS-5801 liquid scintillation counter. The effluent was monitored at 312, 265, and 260 nm for D1694, 1843U89, and DDATHF, respectively, by a Waters 99 photodiode array detector.

FPGS Activity Assay. FPGS activity in cell extracts was determined as described by McGuire et al. (19). Each assay contained, in a final volume of 250 μl, 100 mM Tris-HCl (pH 8.85), 10 mM ATP, 20 mM MgCl₂, 20 mM KCl, 100 mM β-mercaptoethanol, 4 mM [1H]glutamate (4.1 cpm/pmol), enzyme, and substrate (either D1694, 1843U89, or DDATHF). After the reaction was stopped at different time points with the addition of 1 ml cold 5 mM glutamate (pH 7.5) containing 25 mM 2-mercaptoethanol, the unreacted [1H]glutamate was separated from the polyglutamates by DE-52 minicolumn chromatography. Enzyme activity is expressed as the incorporation of pmol glutamate/h/mg at 37°C.

RESULTS

Inhibition of Cell Growth by D1694, 1843U89, and DDATHF. Mid-log sarcoma cells were exposed to antifolates for 24 h or 5 days. ED₅₀ values for D1694 and 1843U89 after 24-h exposure were 11–19-fold and 22–222-fold lower than that for MTX, respectively, in HT-1080, a MTX-sensitive cell line, and three MTX-resistant cell lines (Table 1). In contrast, higher ED₅₀ values for DDATHF were observed compared to MTX in both the MTX-sensitive HT-1080 cell line and the three resistant cell lines. Compared with 24-h exposure, 5-day exposure to the antifolates resulted in more marked cell growth inhibition in all four cell lines. ED₅₀ values for D1694 and 1843U89 were lower than that for MTX. However, ED₅₀ values for DDATHF were still higher than that for MTX in all cell lines.

Uptake of ICI-D1694, 1843U89, and DDATHF. Uptake of radiolabeled D1694, 1843U89, and DDATHF in the four cell lines at an extracellular concentration of 1 μM is shown in Fig 1. Uptake of the three drugs increased in a time-dependent
Fig. 1 Uptake of MTX, ICI-D1694, 1843U89, and DDATHF in soft tissue sarcoma cell lines. Cells were exposed to 1 μM radiolabeled drugs. At the indicated times, the cells were assayed for radiolabeled activity as described in "Materials and Methods." O, HT-1080 cells; ●, HS-16 cells; △, HS-18 cells; ▲, HS-42 cells.

Table 1 Inhibitory effects of MTX, D1694, 1843U89, and DDATHF on growth of sarcoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>24 h</th>
<th>5 day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTX</td>
<td>D1694</td>
</tr>
<tr>
<td>HT-1080</td>
<td>170</td>
<td>9.0</td>
</tr>
<tr>
<td>HS-16</td>
<td>2700</td>
<td>69.2</td>
</tr>
<tr>
<td>HS-18</td>
<td>1627</td>
<td>65.4</td>
</tr>
<tr>
<td>HS-42</td>
<td>8000</td>
<td>97.3</td>
</tr>
</tbody>
</table>

Mean of two to three different experiments. SE is less than 20%. See "Materials and Methods" for details.

Accumulation of Intracellular Polyglutamates of D1694, 1843U89, and DDATHF. Intracellular levels of D1694, 1843U89, and DDATHF polyglutamates in the four cell lines exposed to 1 or 10 μM radiolabeled drugs for 24 h are shown in Table 2. Intracellular D1694 and its polyglutamates were 71.1 pmol/10^7 cells in HT-1080 cells and 32.8, 88.0, and 39.0 pmol/10^7 cells in HS-16, HS-18, and HS-42 cell lines, respectively, after a 24-h exposure to the drug (1 μM). Long chain polyglutamates (Glu6-) formed were about 70–80% of the total amount in all four cell lines, similar to the amount of
Table 2  Accumulation of D1694, DDATHF, and 1843U89 and their polyglutamates in four soft tissue sarcoma cell lines

<table>
<thead>
<tr>
<th>Glutamate (pmol/10^7 cells)</th>
<th>N = 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Total</th>
<th>3–6 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICI-D1694</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-1080</td>
<td>12.1</td>
<td>7.1</td>
<td>16.5</td>
<td>33.1</td>
<td>2.3</td>
<td>1.1</td>
<td>71.1</td>
<td>53.0 (74.5)</td>
</tr>
<tr>
<td>HS-16</td>
<td>8.0</td>
<td>0.5</td>
<td>11.4</td>
<td>11.8</td>
<td>1.1</td>
<td>1.1</td>
<td>32.8</td>
<td>24.3 (74.0)</td>
</tr>
<tr>
<td>HS-18</td>
<td>9.3</td>
<td>3.7</td>
<td>16.3</td>
<td>54.8</td>
<td>2.8</td>
<td>1.1</td>
<td>88.0</td>
<td>75.0 (85.2)</td>
</tr>
<tr>
<td>HS-42</td>
<td>8.2</td>
<td>2.9</td>
<td>6.7</td>
<td>18.7</td>
<td>2.0</td>
<td>0.5</td>
<td>39.0</td>
<td>27.9 (71.5)</td>
</tr>
<tr>
<td>DDATHF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-1080</td>
<td>160.6</td>
<td>5.9</td>
<td>16.6</td>
<td>22.2</td>
<td>13.4</td>
<td>25.3</td>
<td>227.4</td>
<td>77.5 (34.0)</td>
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<tr>
<td>HS-16</td>
<td>166.6</td>
<td>2.8</td>
<td>11.4</td>
<td>6.2</td>
<td>187.0</td>
<td>17.6</td>
<td>246.2</td>
<td>9.4 (9.4)</td>
</tr>
<tr>
<td>HS-18</td>
<td>117.6</td>
<td>8.0</td>
<td>17.3</td>
<td>12.7</td>
<td>7.3</td>
<td>3.7</td>
<td>166.6</td>
<td>41.0 (24.6)</td>
</tr>
<tr>
<td>HS-42</td>
<td>184.3</td>
<td>11.3</td>
<td>11.3</td>
<td>6.6</td>
<td>8.1</td>
<td>20.1</td>
<td>246.2</td>
<td>46.2 (18.7)</td>
</tr>
<tr>
<td>1843U89</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-1080</td>
<td>7.8</td>
<td>149.2</td>
<td>6.1</td>
<td>6.7</td>
<td>170.7</td>
<td>12.8</td>
<td>170.7</td>
<td>12.8 (7.5)</td>
</tr>
<tr>
<td>HS-16</td>
<td>12.2</td>
<td>31.3</td>
<td>43.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10.5</td>
<td>11.9 (11.9)</td>
</tr>
<tr>
<td>HS-18</td>
<td>5.8</td>
<td>71.9</td>
<td>5.9</td>
<td>5.7</td>
<td>89.3</td>
<td>11.6</td>
<td>89.3</td>
<td>13.0 (13.0)</td>
</tr>
<tr>
<td>HS-42</td>
<td>5.9</td>
<td>55.5</td>
<td>2.7</td>
<td>3.6</td>
<td>67.7</td>
<td>6.3</td>
<td>67.7</td>
<td>6.3 (9.4)</td>
</tr>
</tbody>
</table>

MTX polyglutamates formed in HT-1080 cells but 5.5- and 12-fold higher than the amount of MTX in HS-16 and HS-42 cell lines, respectively. Total amounts of DDATHF and its polyglutamates accumulated were comparable to that of MTX obtained in the MTX-resistant cell lines, but long chain polyglutamates were only 9.4–24.6% of the total amount of intracellular DDATHF, even in cells exposed to 10 μM DDATHF for 24 h. 1843U89 and its polyglutamates were 170.7, 43.5, 89.3, and 67.7 pmol/10^7 cells in HS-1080, HS-16, HS-18, and HS-42 cell lines. However, the major form of 1843U89 polyglutamates was the diglutamate (more than 70% of the total amount) in all cell lines, although some tri- and tetruglutamates of 1843U89 were observed in HS-1080, HS-18, and HS-42 cells. In order to determine whether the diglutamate of 1843U89 was retained intracellularly, cells were incubated in drug-free medium for an additional 4 h after exposure to 1 μM 1843U89 for 24 h. Approximately 60%, 60%, 36%, and 21% of the diglutamate of 1843U89 persisted in this form in HT-1080, HS-16, HS-18, and HS-42 cells, respectively (Table 2).

FPGS Activity. FPGS activities were measured using D1694, 1843U89, and DDATHF as substrates. As shown in Table 3, FPGS activities using D1694, 1843U89, and DDATHF as substrates were equal to or greater than with MTX as the substrate. In contrast, Km values for D1694 were 0.2, 0.2, 0.7, and 0.4 μM for HT-1080, HS-16, HS-18, and HS-42 cell lines, respectively; 15–185-fold lower than that for MTX. Similar Km

values for 1843U89 comparable to those for D1694 were obtained (0.1–0.4 μM). $K_m$ values for FPGS for DDATHF were also lower than MTX $K_m$ values in HT-1080 and HS-16, HS-18 and HS-42 cells (8-, 23-, 6-, and 30-fold lower, respectively).

**DISCUSSION**

Our previous studies indicated that HS-16 and HS-42 cells were intrinsically resistant to short-term (24-h) exposure to MTX due to the lack of retention of this drug as a consequence of decreased accumulation of long chain MTX polyglutamates (5). Further studies in HS-16 cells showed that this difference was likely due to an increased breakdown of MTX polyglutamates because of increased glutamyl hydrolase activity (20). The reason for low level accumulation of MTX polyglutamates in HS-42 cells is still unclear as FPGS activity and hydrolase activity are similar to the levels in HT-1080 cells (Table 3 and Ref. 20). MTX resistance in HS-18 cells is associated with an increase in DHFR enzyme activity and an increase in DHFR mRNA levels; MTX polyglutamylation is equivalent to 1080 cells (21). The present study shows that D1694 and 1843U89 were 25–83-fold and 22–222-fold more potent inhibitors of growth in these cell lines than MTX after a 24-h exposure to the drugs (Table 1). To explain the more potent effects of D1694 and 1843U89, uptake and polyglutamate accumulation of these drugs were determined. Uptake of D1694 and 1843U89 was 3-fold higher than MTX in HS-18 and HS-42 cells. Although the uptake of D1694 and 1843U89 was similar to that of MTX in HS-16 cells, a 6-fold higher level of long chain polyglutamates of D1694 than that of MTX was found in this cell line, similar to the intracellular D1694 polyglutamate levels measured in two other MTX-resistant cell lines. Thus, higher levels of D1694, compared to MTX, retained intracellularly as polyglutamate forms, likely explain the increased growth inhibition noted with this antifolate, since polyglutymates of this compound are retained longer and bind to TS enzyme more tightly than the monoglutamate form (22). In contrast to D1694, both 1843U89 and its diglutamate form are tight binding inhibitors of TS (15).

HPLC analysis showed that the MTX-resistant sarcoma cell lines polyglutamated 1843U89 to the diglutamate (70–80% of total amount) or above after a 24-h incubation with this drug (1 μM). Substantial amounts of the diglutamate were retained in the cells after a 4-h efflux, levels that were 180–290-fold higher than that of the MTX diglutamate retained in the cells under the same conditions (data not shown). Thus, unlike MTX diglutamate that effluxes rapidly from cells, the 1843U89 diglutamate is slowly effluxed. DDATHF, a folate analogue GARTF inhibitor, is more potent than MTX against some tumor cells resistant to MTX due to decreased transport (23). However, MTX-resistant tumor cells with low FPGS activity were also resistant to DDATHF (14, 24). In soft tissue sarcoma cell lines, higher concentrations of DDATHF, compared with that of MTX, were required for equivalent growth inhibition after short-term exposure to this drug (24 h), especially HS-16 and HS-42. This result was not due to a decreased uptake of DDATHF. However, accumulation of long chain polyglutamates of DDATHF was low (9–24% of total DDATHF). DDATHF polyglutamates are 100-fold more potent inhibitors of GARFT than DDATHF (25).

Synthesis of intracellular polyglutamates is a function of FPGS activity ($V_{max}$) and substrate affinity ($K_m$) for this enzyme. Enzyme activity with all four drugs as substrates was equivalent; however, $K_m$ values for D1694 and 1843U89 were much lower compared to the $K_m$ values for MTX. Thus, extensive and rapid accumulation of polyglutamates of D1694 and 1843U89 is likely due to the excellent substrate properties ($K_{cat}$) of these drugs for FPGS. $K_m$ values for DDATHF were higher than those for two TS inhibitors although lower than that for MTX, explaining in part the lack of accumulation of DDATHF polyglutamates in these cell lines.

Compared to the HT-1080 cell line, these MTX-resistant cell lines are still relatively resistant to D1694, 1843U89, and DDATHF (24-h exposure). Increased breakdown of antifolate polyglutamates and efflux in HS-16 cells may explain the relative resistance to these drugs in HS-16 cells, as this cell line contains high glutamyl hydrolase activity (20). Resistance to D1694 and 1843U89 in HS-18 cells may be related to the increased TS activity present in these cells, while decreased uptake of DDATHF may result in resistance in this cell line. Relative resistance to these drugs in the HS-42 cell line may result from relatively low levels of polyglutamate formation.

Growth inhibition by MTX, D1694, 1843U89, and DDATHF in all four cell lines, as expected, was time dependent. As these cell lines have doubling times ranging from 26 to 38 h, a longer exposure ensures that these cell cycle agents are present during S-phase. 1843U89 was more potent than D1694 after a 5-day exposure, probably due to more potent binding of 1843U89 to TS (26). The marked increase in cytotoxicity of DDATHF after a 5-day exposure may be mainly due to a progressive depletion of ribonucleoside triphosphate pools by prolonged inhibition of purine synthesis (27).

1843U89 and DDATHF are significantly more cytotoxic than MTX in intrinsically MTX-resistant sarcoma cell lines as a result of extensive formation of polyglutamates. These two TS inhibitors should be evaluated in Phase II trials in soft tissue sarcomas.

**REFERENCES**

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