In Vitro Uptake, Anabolism, and Cellular Retention of 1843U89 and Other Benzoquinazoline Inhibitors of Thymidylate Synthase

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ABSTRACT

1843U89 is a potent inhibitor (Ki = 0.09 nM) of thymidylate synthase (TS; EC 2.1.1.45) that is in clinical trial for the treatment of solid tumors. Although it is an excellent substrate for the folate anabolizing enzyme for poly(4hydroxypentamidine synthetase (FPS)), 1843U89 differs from other folate-based inhibitors of TS (e.g., CIB3711, D1694, and LY231514), in that the parent compound is as potent an enzyme inhibitor as its polyglutamated analogues. As reported (D. S. Duch et al., Cancer Res., 53: 810–818, 1993), 1843U89 is 10–80-fold more cytotoxic than the close structural analogue 1031U89, which is an equipotent inhibitor of TS but is a less efficient substrate for FPS. This correlation between substrate efficiency for FPS and cytotoxicity suggests that polyglutamyl activity of 1843U89 contributes to its cytotoxicity. In the current study, we measured intracellular levels of polyglutamyl anabolics of 1031U89, 1843U89, and three other benzoquinazoline inhibitors of TS as well as anabolics of D1694 in HCT-8 ileocecal carcinoma cells. Each TS inhibitor was anabolized to polyglutamated analogues with one to five added glutamyl residues after exposure for 24 h to IC50 concentrations (those that inhibit growth by 90% after 72 h of constant exposure). D1694, which requires polyglutamyl for potent enzyme inhibition as well as for cytotoxicity, was anabolized mostly to penta- and hexaglutamates, whereas approximately 90% of intracellular 1843U89 was the diglutamate analogue. The substrate efficiency of the benzoquinazolines for FPS was predictive of the extent of intracellular anabolism. The diglutamate analogue of 1843U89 was only 1/10 as efficient a substrate for further glutamation as was 1843U89 itself. The efficient anabolism to the diglutamate analogue and the lack of dependence on further polyglutamation for enzyme inhibition or cytotoxicity provide a rationale for the reported 1843U89 sensitivity of cells with impaired FPS activity.

As part of an investigation of the effects of polyglutamation, we measured the retention of intracellular 1843U89 and D1694 anabolics after 24 h of exposure to 20 nM of each compound. After 48 h in drug-free medium, 7% of intracellular 1843U89 (mostly diglutamate analogue) and 36% of D1694 (mostly penta- and hexaglutamates) remained in the cells. Because prolonged retention (associated with tissue storage of polyglutamates) can contribute to clinical toxicities, 1843U89 may present fewer long-term toxicities than D1694.

INTRODUCTION

Duch et al. (1) reported biological properties of a series of benzoquinazoline inhibitors of TS2 (EC 2.1.1.45). Compounds 1396U88, 1944U88, 428U89, 1031U89, and 1843U89 (Fig. 1) are potent TS inhibitors that are increasingly cytotoxic to a number of cell lines (Table 1). Although the two most potent, 1031U89 and 1843U89, inhibit the isolated enzyme equally (Ki = 0.9 pm), 1843U89 is a somewhat better substrate for the reduced folate transporter, is a much better substrate for hog liver FPGS, and is 10–80-fold more toxic in cell lines. These data suggest, but do not directly demonstrate, that 1843U89 is anabolized intracellularly to polyglutamated forms and that polyglutamation makes it more cytotoxic than 1031U89. In the present study, we measured anabolism of the above benzoquinazolines by the ileocecal tumor line HCT-8. In addition, we measured the intracellular persistence of 1843U89 and its anabolics after removal of extracellular drug. Comparisons of cellular uptake, anabolism, and retention were made with the quinazoline TS inhibitor D1694 (Tomudex), for which anabolism to polyglutamates is a prerequisite for cytotoxicity (2).

MATERIALS AND METHODS

TS Inhibitors and Polyglutamates. The benzoquinazoline inhibitors 1396U88, 1944U88, 428U89, 1031U89, and 1843U89 (Fig. 1), as well as D1694, were synthesized as described previously (3). Tritium-labeled drugs (21–40 Ci/mmol) were prepared from nonradioactive compounds at Moravek Biochemicals by catalytic exchange with tritium. Minor radiolabeled contaminants were removed from all drug solutions by reverse-phase HPLC as described previously (1) or by extraction on 100-mg bond elute DEA cartridges (Varian Bond Elut) as follows. Cartridges were prepared by sequential 800-μl washes with acetonitrile, water, and 10 mM NH4Ac. Up to 1 μmol of radiolabeled compound in up to 2 ml of 10 mM NH4Ac was applied (1 ml/min), followed by 1.9 ml each of methanol and 200 mM NH4Ac. Compounds were desorbed with 1.9 ml of 400 mM NH4Ac or 500 mM NH4Ac in 50% acetonitrile. Solvent was removed under reduced pressure (Savant Speed Vac). The residues were dissolved in 500 μl of 50% ethanol, chilled (4°C), and centrifuged (8000 × g) for 10 min to remove particulates. Polyclonal antibodies were prepared by enzymatic synthesis with Escherichia coli FPGS and isolated on a Waters 19-mm × 15-cm μBondapak C18 column as described previously (4).

Hog Liver FPGS. The relative efficiency of compounds as substrates for hog liver FPGS was determined as described previously (1). The assay measures the addition of one glutamyl residue to a folate-like substrate. The addition of multiple glutamyl residues to a substrate was determined in a similar assay with increased FPGS (8 U/ml) and increased incubation time (18 h). Reactions were terminated by immersion in a boiling water bath for 5 min. After cooling, reaction tubes were centrifuged at room temperature for 10 min at 8000 × g. The supernatants were filtered and examined on analytical HPLC as described below.

Cell Culture. HCT-8 ileocecal carcinoma cells were obtained from ATCC and adapted to and maintained in folate-free RPMI 1640 (Life Technologies, Inc.) containing 10 nM (6R,S)-5-formyl-tetrahydrofolic acid (leucovorin) and 10% fetal bovine serum (Hyclone) that had been dialyzed against charcoal. Seventy-two-h IC50 concentrations were determined as described (1). Six-h IC50 concentrations were determined similarly, except that after 6 h of drug exposure, the medium was removed, cells were washed twice with PBS, and incubation was continued for 66 h in drug-free medium. For studies of intracellular accumulation and drug anabolism, cells in logarithmic growth were harvested with trypsin/EDTA and seeded onto T25 flasks at 1–2 million cells/flask. Titrated compounds were added after 24 h. Six or 24 h later, cells were washed and extracted either in pH 7 phosphate buffer as described previously (5) or with ZnSO4-acetonitrile as described below. For the longer drug-efflux studies, cells were seeded at 0.3 million cells/flask. The medium was replaced after 24 h with 3 ml fresh medium containing either [3H]D1694 or [3H]1843U89 and other additions as indicated. After 24 h, media was removed, cells were washed four times with 5 ml ice-cold PBS, and 5 ml efflux

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POLYGLUTAMATION OF BENZOQUINAZOLINE TS INHIBITORS

RESULTS

Extraction of Cells. In initial studies of inhibitor uptake, cells were harvested with trypsin/EDTA, washed with cold PBS, suspended in hypotonic buffer [10 mM potassium phosphate (pH 7), 0.1 mM 2-mercaptoethanol with internal standards], and lysed and extracted in a boiling water bath as described previously (5). This procedure was assumed to release polyglutamated anabolics from cytosolic proteins and to inactivate lysosomal enzymes that might degrade them. The recovery of radioactivity was high (80–93%) for two of the compounds (1396U88 and 1944U88), as was the recovery of polyglutamated internal standards added to the cells before lysis. However, the recovery of radioactivity was sometimes quite low (36–74%) for the other benzoquinazoline inhibitors, and in some cases, internal standards of polyglutamates were degraded rapidly to less polyglutamated forms during the cell extractions. For example, when polyglutamates of 1031U89 were added to a 0.5-ml cell suspension immediately before immersion into a boiling water bath, they were degraded in the short time interval between cell lysis and protein denaturation. Because the isolated polyglutamates of this compound were stable to boiling in the lysis buffer, and the identical extraction procedure had preserved polyglutamated HCT-8 anabolics of other folate analogues (5), it was likely that the benzoquinazoline polyglutamates were especially sensitive to degradation by lysosomal FPGH. To avoid anabolite degradation, the ZnSO4/acetoin trile extraction procedure described in “Materials and Methods” was developed. This procedure resulted in excellent recovery of both radioactivity and polyglutamated internal standards (86–99%) in part because of inhibition of FPGH by ZnSO4 (7).

Accumulation and Anabolism of Benzoquinazoline TS Inhibitors. In initial studies of inhibitor uptake/accumulation (Table 2), HCT-8 cells were exposed for 24 h to drug concentrations that inhibited growth by 90% during constant exposure for 72 h (72-h IC50) or 20 times that concentration. After 24 h of exposure to the IC50 concentrations of 1396U88, 1944U88, 428U89, 1031U89, or 1843U89, intracellular concentrations of the drugs (plus anabolics) were 15.8, 6.0, 6.6, 1.2, and 0.7 pmol/million cells, respectively. Each compound (Table 2) was polyglutamated by HCT-8; anabolics with up to four added glutamates were observed for each inhibitor. However, even after 24 h of exposure, only 30% of intracellular 428U89 or 1031U89 was metabolized. In contrast, 80–95% of 1396U88, 1944U88, and 1843U89 was metabolized, and the predominant intracellular forms were those with one additional glutamate. (These are referred to as diglutamates of 1396U88 and 1944U88. Because 1843U89 is a glutamate analogue (Fig. 1), the major anabole is referred to as the diglutamate analogue.) These anabolic profiles are unusual for most polyglutamateable folate analogues; a polyglutamate distribution similar to that of naturally occurring folates (mostly pentaglutamate) was expected. Surprising as it was, anabolism of 1843U89 to a form with one additional glutamate was associated with intracellular accumulation to several hundredfold above the extracellular concentration.

Intracellular Accumulation and Polyglutamation in the Presence of Leucovorin. To explore the question of whether glutamate was necessary for intracellular accumulation of 1843U89, we exposed HCT-8 cells simultaneously to 1843U89 and a folate that could compete for polyglutamation. As a control, we compared the intracellular accumulation and polyglutamataion of 1843U89 with that of the quinazoline TS inhibitor D1694, which is polyglutamated extensively by L1210, Wi12, and HT-29 cells in culture (8). In the experiment represented in Fig. 2, cells were seeded at 2 million/flask. After 24 h, the media was made 20 mM of either [1H]1843U89 or [1H]D1694 ± 5 μM leucovorin. Intracellular accumulation of drug (and anabolics) is shown in Fig. 2. Without leucovorin, D1694
accumulated predominantly as the pentaglutamate; both accumulation and polyglutamation were decreased in the presence of leucovorin. In contrast, the 24-h accumulation of 1843U89 was similar with and without leucovorin. However, in the presence of leucovorin, the majority (57%) of the drug was not anabolized, and the remainder had only one glutamyl residue added; no higher polyglutamates were seen. The cell could accumulate 1843U89 against a concentration gradient even when anabolism to the diglutamate was reduced. Thus, the benzoquinazoline 1843U89 and the quinazoline D1694 TS inhibitors responded differently to an agent that reduced their intracellular anabolism. D1694 (which requires polyglutamation for activity) was poorly accumulated by HCT-8 when polyglutamation was inhibited; 1843U89 was accumulated, although more slowly, under the same conditions.

Assay of 1843U89 and Anabolites in Media. In the following experiment, we tested whether 1843U89 and anabolites were stable in spent media from HCT-8 cells. HCT-8 cells were exposed to 8 nm 1843U89 for 24 h, after which the drug-containing medium was replaced. The accumulation of tritiated materials in the media was determined in the ensuing 48 h. In this time period, the cells became swollen because of exposure to the cytotoxic agent. Although nonmetabolized 1843U89 was the predominant form of tritium in the efflux media, the di-, tri-, and pentaglutamate analogues were also observed. Although these 1843U89 polyglutamates in the medium came from damaged cells, this experiment was significant in that it demonstrated that polyglutamates of 1843U89 were assayable and stable in the spent efflux medium from HCT-8 cells. In the efflux experiments described below, in which cells were protected from the cytotoxic effects of 1843U89, greater than 97% of the drug equivalents in the efflux media were nonmetabolized 1843U89. Small amounts of the diglutamate analogue were seen; polyglutamated species of 1843U89 did not appear in the media.

Efflux of TS Inhibitors (and Anabolites) after Removal of Extracellular Drug. There are reports in the folate/antifolate literature (8–11) of mono- and diglutamates that diffuse rather rapidly from cells, whereas the conjugation of three or more glutamyl residues is associated with prolonged cellular retention. Size, charge, and increased binding to intracellular proteins serve to limit diffusion of polyglutamates across cell membranes. In the following experiment, we investigated whether the anabolites of 1843U89 would be retained by HCT-8 after removal of external drug from the media. We compared the intracellular retention of 1843U89 anabolites to those of the quinazoline TS inhibitor D1694. Cells were seeded at 0.3 million/flask. Thymidine (10 μM) and hypoxanthine (50 μM) were included in the medium to prevent growth inhibition. Our previous data in this cell line had revealed that 10 μM thymidine alone reversed the growth-inhibitory effects of 20 nm 1843U89, whereas the addition of 50 μM hypoxanthine was necessary to achieve reversal of growth inhibition when the cells were exposed to 20 nm D1694 (12).

After 24 h of exposure to either 20 nm [3H]1843U89 or 20 nm [3H]D1694, HCT-8 cells accumulated the compounds and their polyglutamated anabolites to a similar extent (9.45 and 10.6 pmol/million cells, respectively). Intracellular retention of [3H]1843U89 and [3H]D1694 equivalents in the 48 h after removal of extracellular drug is shown in Fig. 3. In the first 24 h after removal of extracelluldr drug, 93% of intracellular 1843U89 (and anabolites) was lost from the cells. There was no further loss of [3H] from the cells in the second 24 h of efflux. In contrast, D1694 and its anabolites were more avidly retained. Forty-five % of the intracellular drug was lost from the cells in the first 24 h of efflux, and an additional 19% left the cells in the next

### Table 2: HCT-8 24-h accumulation and anabolism of TS inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Extracellular concentration (nM)</th>
<th>Intracellular concentration (pmol/10⁶ cells)</th>
<th>Extraction method</th>
<th>Polyglutamates % of intracellular tritium identified as:</th>
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</thead>
<tbody>
<tr>
<td>1396U88</td>
<td>30,000⁰⁺</td>
<td>15.8</td>
<td>pH 7⁺</td>
<td>G₁, G₂, G₃, G₄, G₅</td>
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<tr>
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<td>10,000⁰⁺</td>
<td>6.0</td>
<td>pH 7⁻</td>
<td>G₁, G₂, G₃, G₄, G₅</td>
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<tr>
<td>428U89</td>
<td>25,000⁰⁺</td>
<td>6.6</td>
<td>Zn/ACN</td>
<td>G₁, G₂, G₃, G₄, G₅</td>
</tr>
<tr>
<td>1031U89</td>
<td>160⁰⁺</td>
<td>1.2</td>
<td>Zn/ACN</td>
<td>G₁, G₂, G₃, G₄, G₅</td>
</tr>
<tr>
<td>1031U89</td>
<td>3200⁰⁺</td>
<td>1.5</td>
<td>Zn/ACN</td>
<td>G₁, G₂, G₃, G₄, G₅</td>
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<tr>
<td>1843U89</td>
<td>1.5⁻</td>
<td>0.7</td>
<td>pH 7⁻</td>
<td>G₁, G₂, G₃, G₄, G₅</td>
</tr>
<tr>
<td>1843U89</td>
<td>30⁻</td>
<td>3.2</td>
<td>Zn/ACN</td>
<td>G₁, G₂, G₃, G₄, G₅</td>
</tr>
</tbody>
</table>

* The concentration is equal to the 72-h IC₅₀.
* The cells were lysed and extracted in hot hypotonic pH 7 buffer as described in text.
* The cells were lysed in 2 mm ZnSO₄ and extracted in 60% acetonitrile (ACN) at 4°C.
* The concentration is equal to 20 times the 72-h IC₅₀.

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![Fig. 2. HCT-8 intracellular accumulation of parent and polyglutamated anabolites of 1843U89 (A) and D1694 (B). Insets, accumulation after exposure to 20 nm of the inhibitor alone (●) or to inhibitor plus 5 μM leucovorin (●). Bars, anabolite accumulation after 24 h exposure to inhibitor alone (●) or to inhibitor plus leucovorin (●). G₁, parent compound; G₂-G₅, polyglutamated anabolites. Data are the averages of two or three determinations.](cancerres.aacrjournals.org)
carboplatin was 0—12 μg/ml and for cisplatin was 0—10 μg/ml. DX, cell lines established from patients at the time of diagnosis; PD-md, cell lines derived from patients with progressive disease during induction chemotherapy (PD-Ind) and cell lines established at relapse after myeloablative therapy (PD-BMT) in patients who relapsed after myeloablative chemoradiotherapy followed by BMT. These values were calculated from the dose-response curves shown in Figs. 2—4 and 6.

Accumulation and Efflux of 1843U89 Anabolites in the Presence of Folic Acid. Because folic acid modulates the toxicity of 1843U89 (13), we examined the effects of folic acid on accumulation, anabolism, and efflux of the drug. HCT-8 cells were exposed to 20 μM folic acid and 20 nM [3H]1843U89 in the presence of 10 μM thymidine and 50 μM hypoxanthine. After 24 h (Table 3, bottom section; t = 0), the intracellular accumulation of [3H]1843U89 and its anabolites was less than half that observed in a similar experiment without folic acid. While the percentage of intracellular compound that was anabolized to the diglutamate analogue (87%) was similar to that observed without folic acid (82%), the longer-chain polyglutamates (tetra- and pentaglutamate analogues) were not present. Within 24 h of the removal of extracellular [3H]1843U89, 84% of the tritium had left the cells, and the polyglutamate profile had shifted such that the majority (52%) of the intracellular 1843U89 was parent compound and 47% was the diglutamate analogue. Forty-eight h after removal of extracellular 1843U89, only 7% of the accumulated 1843U89 remained in the cells. This result is almost identical to that observed without folic acid. Thus, folic acid appeared to reduce 1843U89 accumulation and anabolism in HCT-8 cells and to have little effect on efflux of accumulated drug.

Efficiency of Polyglutamates as Substrates for FPGS. 1843U89 and its di- and triglutamate analogues were tested as substrates of hog liver FPGS. As shown in Table 4, the relative velocities with the glutamated analogues as substrates were less than 1/100 of the velocities of the parent compound. Similar results were obtained with polyglutamates of the benzoxquinazolines 1031U89, 1936U88, and 1944U88 (data not shown). These results contrast with that of a natural substrate for this enzyme: the diglutamate of [6S]-tetrahydrofolic acid was about 1/3 as efficient a substrate as its parent. Substrate efficiency decreased with further polyglutamation, but even the tetraglutamate was a relatively efficient substrate. When the synthesis reaction was extended by increasing the FPGS concentration and by incubation for 18 h with 1843U89 as substrate (Table 5), the predominant product was the diglutamate analogue. A similar assay with D1694 as substrate resulted in production of mostly tetra- and pentaglutamates. Thus, in a cell-free system devoid of the folate catalyzing enzyme FPGRH, 1843U89 was polyglutamated to a limited extent, whereas D1694 was highly polyglutamated, qualitatively reproducing the anabolite profiles observed in whole cells. Therefore, the intracellular anabolite profiles of the benzoxquinazolines were due to the inherent substrate efficiencies of the parent and polyglutamated compounds themselves.

**DISCUSSION**

When we began studies of *in vitro* metabolism of the benzoxquinazoline TS inhibitors, we expected to find that they would be polyglutamated intracellularly and that long-chain polyglutamates would predominate, as we and others had observed for other folate analogues and as reported in L1210, Wi12, and HT-29 cells exposed to D1694 (8). Surprisingly, the diglutamates (and the 1843U89 analogue of a diglutamate) were the predominant intracellular forms, and although long-chain polyglutamates were synthesized and accumulated, they accounted for a small percentage of the intracellular drugs. To prove that the polyglutamate distributions were not artifacts of the cell extraction procedure, we monitored the recovery of internal standards in each experiment. The pH 7 extraction procedure resulted in good (about 85%) recoveries of polyglutamates of 1396U89 and 1944U88 as well as good recovery of tritium present in the cell lysates. However, with 428U89, 1031U89, and 1843U89, we noticed a parallel loss.

**Table 3** HCT-8 retention of intracellular 1843U89 and D1694 and polyglutamated anabolites after removal of extracellular drug

<table>
<thead>
<tr>
<th>Compound</th>
<th>Additions</th>
<th>Efflux (h)</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
<th>G6</th>
<th>G7b</th>
<th>G8b</th>
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<tr>
<td>D1694</td>
<td></td>
<td>0'</td>
<td>0.09</td>
<td>0.14</td>
<td>0.29</td>
<td>0.35</td>
<td>4.89</td>
<td>1.62</td>
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<td></td>
<td></td>
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<td>0</td>
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<td></td>
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<td>0</td>
<td>0.12</td>
<td>0.12</td>
<td>1.23</td>
<td>1.04</td>
<td>0.19</td>
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<td>0.37</td>
<td>4.77</td>
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<td>0.04</td>
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<td>1843U89</td>
<td>Folic acid</td>
<td>0'</td>
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* Average; n = 3.

b The identity of the hepta- and octaglutamates of D1694 was suggested by retention times on the chromatograms; standards were unavailable.

* Cells were exposed 24 h to 20 nM compound (t = 0).

* Cells were exposed 24 h to 20 nM 1843U89 and 20 μM folic acid.

* Media contained 20 μM folic acid during efflux.
of tritium and of internal standards. Much of the tritium in the cell lysates remained associated with the cell pellets, and significant amounts of extracted radioactivity could not be identified as parent drug or as polyglutamates. With 1031U89 polyglutamate standards in particular, there was a selective loss of the higher polyglutamates. Such losses could have resulted from decreased solubility of the longer-chain polyglutamates, selective binding of polyglutamates to the cell pellet and/or FPFG activity in the lysate. Because loss of polyglutamates (in the pH 7 extraction procedure) was sometimes accompanied by an increase in monoglutamate, FPFG did seem to be involved in the loss of polyglutamated standards.

Extraction with acetonitrile in the presence of the FPFG inhibitor ZnSO₄ resulted in excellent recovery of both radioactivity and polyglutamated standards. No significant nonidentifiable tritiated material was seen on chromatograms of cells extracted by this method. The method was also used to identify 1031U89 anabolites in MOLT-4 and L1210 cells and 1843U89 anabolites in HT-29 cells (data not shown). These polyglutamate profiles were very similar to those found in HCT-8; 1031U89 was anabolized very little, whereas the predominant intracellular form of 1843U89 was the diglutamate analogue. Experiments with hog liver FPFG revealed that compared to the parent drug, the di- and triglutamate analogues of 1843U89 were very inefficient substrates for glutaminylation. These data suggest that the restricted conformations of the γ-carboxyl of 1843U89 is excellent for the addition of one glutamyl residue by FPFG, but is a poor conformation for further glutamyl.

Because polyglutamation is not required for target enzyme inhibition (14), and because the difference in cytotoxicity between 1031U89 and 1843U89 is correlated with the extent of anabolism to the diglutamate and diglutamate analogue, respectively, we asked whether the 1843U89 diglutamate analogue was retained longer in the cell than the parent compound, and whether the diglutamate analogue left the cell directly or was first degraded back to the parent compound. To address these questions, we analyzed media samples from all the 1843U89 experiments described here, looking for the presence of 1843U89 anabolites and in particular the diglutamate analogue. In preliminary experiments, polyglutamates of 1843U89 were shown to be stable in fresh media at 37°C for up to 48 h and in spent media under conditions of the efflux assay. Our data showed that almost all of the tritium in the efflux media of cells that had been preloaded with 1843U89 polyglutamates (mostly the diglutamate analogue) was in the form of parent drug. Because the parent compound did not accumulate intracellularly in this experiment, the rate-limiting step in the efflux of the 1843U89 diglutamate analogue was the degradation to parent compound; once formed, it left the cell rapidly. These data suggest that the intracellular addition of one (or more) glutamyl residue(s) to 1843U89 served to aid intracellular retention of the drug. The fact that intracellular 1843U89 equivalents remained constant between 24 and 48 h of efflux suggested that the remaining intracellular drug was bound, possibly to TS. Perhaps rapid anabolism to the diglutamate analogue, associated with a modest increase in intracellular half-life, aided the titration of the intracellular target and thus made 1843U89 such a potent cytotoxic agent when compared to its close structural analogue 1031U89, which was inefficiently anabolized. A further observation supports the importance of anabolism, with increased intracellular retention, to the cytotoxicity of 1843U89. The 6- and 72-h IC₅₀ growth-inhibitory concentrations differed by 20-fold (Table 1). As reported (8), a low ratio of these values is associated with rapid formation of a retained (active) drug form. 1031U89 was polyglutamated poorly and had a high 6-h:72-h IC₅₀ ratio, supporting further the importance of diglutamate formation for cytotoxicity.

Others (2, 8) have reported the reduction of polyglutamation of D1694 by cells in the presence of leucovorin, accompanied by reduced intracellular accumulation of the drug, and reduced cytotoxicity. The results presented here for HCT-8 are in agreement. In the presence of leucovorin, intracellular accumulation of D1694 was reduced by 90%, and the major anabolite was the tri- instead of the pentaglutamate. We likewise observed a reduction in glutamation of 1843U89 and intracellular accumulation in the presence of leucovorin. Although the experiment measured net cellular accumulation (which is effected by influx and efflux rates as well as anabolism), clearly anabolism of each drug was associated with increased intracellular accumulation.

1843U89 is currently in Phase I clinical trials in the US; the clinical protocol for use of 1843U89 includes the administration of oral folic acid prior to i.v. 1843U89. This treatment reduces the gut toxicity of 1843U89 in dogs and in mice (13). The mechanism of protection is proposed to be competition for uptake and is thought to be selective for cells (such as gut cells) that can transport folic acid (13, 15, 16). HCT-8 is more sensitive to folic acid reversal of toxicity than are seven other cell lines tested in a clonogenic growth assay (13). The data reported here suggest a reason for the moderate HCT-8 sensitivity to folate reversal. After continuous exposure to 1843U89 and folic acid, intracellular accumulation of 1843U89 was reduced 59%. This suggests that in HCT-8 cells, as well as in the human intestine, 1843U89 and folic acid share a common uptake route. Indirect evidence of folic acid uptake by HCT-8 was provided by the polyglutamate profiles after 24 h of continuous exposure to folic acid and 1843U89 and after 48 h efflux in the presence of folic acid. The higher polyglutamates of 1843U89 did not accumulate intracellularly, presumably because elevated levels of intracellular folates (from folic acid) competed with the diglutamate analogue of 1843U89 for polyglutamation. Thus, folic acid can modulate the anabolism of 1843U89 in vitro.

Anticancer compounds BW1843U89 and D1694 (Tomudex) inhibit the same target enzyme, TS (17), and are substrates for the folate anabolizing enzyme FPFG (8, 14). The drugs differ markedly in that BW1843U89 is a very potent inhibitor of its target enzyme, the Kᵅₙ of which changes very little when it is polyglutamated (14), whereas D1694 is a weaker TS inhibitor, the activity of which is enhanced up to two orders of magnitude by polyglutamation (8). Because 1843U89 is an excellent substrate for FPFG and because anabolism to only the diglutamate analogue is sufficient to enhance intracellular retention, its spectrum of activity includes cells with impaired ability to make and retain polyglutamates (18, 19). Soft-tissue sarcoma-derived cell lines that are resistant to methotrexate because of impaired polyglu-
Drug resistance patterns expressed by neuroblastoma cell lines appears to increase with the intensity of chemotherapy and BMT. Thus, expression of a drug resistant phenotype in patients treated with myeloablative chemotherapy and BMT. Higher drug resistance was seen in cell lines derived from patients treated with myeloablative chemotherapy containing cyclophosphamide, carboplatin, etoposide, vincristine, and dacarbazine due to increased drug resistance in cell lines exposed to these drugs in patients. Consideration may be given to substituting novel non-cross-resistant agents for etoposide and doxorubicin, since etoposide and doxorubicin may mostly contribute to drug-related toxicities without effectively contributing to tumor cell-kill in many patients when used in vitro. Doxorubicin may mostly contribute to drug-related toxicities without effectively contributing to tumor cell-kill in many patients when used in vitro.
In Vitro Uptake, Anabolism, and Cellular Retention of 1843U89 and Other Benzoquinazoline Inhibitors of Thymidylate Synthase

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