ABSTRACT

5-Deazaacyclotetrahydrofolate is a cytotoxic tetrahydrofolate analogue which inhibits glycaminide ribonucleotide transformylase (Kelley et al., J. Med. Chem., 33: 561-567, 1990). Cultured mouse L-cells and human MCF-7 and MOLT-4 cells concentrated the drug several hundred-fold after 24 h of continuous exposure to a cytotoxic level (100-200 nM) of radiolabeled drug. High performance liquid chromatography analysis revealed that each cell type metabolized ≥80% of the internalized drug to polyglutamated forms, which are more potent glycaminide ribonucleotide transformylase inhibitors. In L-cells, 45% of the polyglutamated metabolites were also N-formylated. The pharmacokinetics and distribution of [14C]-deazaacyclotetrahydrofolate were studied in C57BL/6 male mice. Its plasma half-life was 2.15 h. Radiolabel was concentrated to well above plasma level in the kidney, pancreas, and liver. Metabolism was examined in tumor-bearing and in normal mice. Twenty-four h after a single i.p. injection (50 mg/kg), drug equivalents were 0.6 nmol/g (83% polyglutamated) in colon-38 adenocarcinoma carried s.c., 2.4 nmol/g (100% polyglutamated) in ascitic P388 cells, and 3.7 nmol/g (76% polyglutamated and ~20% formylated) in mouse liver. Elimination was mostly in the urine as unmethylated drug. Feces contained 5-deazaacyclotetrahydrofolate (parent compound less glutamate). In conclusion, 5-deazaacyclotetrahydrofolate was shown to be concentrated to well above the extracellular level and metabolized to more active polyglutamated forms by transformed cells grown in culture and in mice.

INTRODUCTION

Various reports in the literature (1-3) have demonstrated higher levels of de novo purine biosynthetic enzymes and greater incorporation of simple precursors into purines in cancer cells, compared to normal cells. Thus, the enzymes of purine de novo synthesis may be selective targets for cancer chemotherapy. Additional selectivity might be obtained by inhibition of a target enzyme with a prodrug which could be selectively activated in tumor cells. A cyclic analogue which inhibits purine de novo synthesis and which could be activated by polyglutamation might fit these criteria. Several such inhibitors, homofolate (4), reduced homofolate analogues (5, 6), 5,10-dideazatetrahydrofolate(7), and 5-DACTHF (8), have been described.

5-DACTHF is a cytosytic tetrahydrofolate acid analogue (Fig. 1) which inhibits the two folate-requiring enzymes of purine de novo synthesis, GAR and 5-amino-4-imidazole carboxamide ribonucleotide transformylases (8). Previous work (8) demonstrated that 5-DACTHF is a good substrate for mammalian folylpolyglutamate synthetase, that polyglutamation makes it a more potent inhibitor of both transformylases, and that its cytotoxicity can be reversed by hypoxanthine alone and by leucovorin alone, which suggests that it interrupts purine bio-

MATERIALS AND METHODS

Preparation of 5-DACTHF and Analogues

5-DACTHF and N-CHO-5-DACTHF were made as described (8). [14C]-5-DACTHF was synthesized by John Hill of these laboratories by condensation of dimethyl [benzoyl-carbonyl-14C]-N-(4-aminobenzoyl)-l-glutamate (which had been dialyzed against 0.3% charcoal in 0.9% NaCl). With 3-(2-acetylamino-4-diacylamino-6-hydroxy-5-pyrimidinyl)propanaldehyde, as described (9). Before each experiment, radioactive contaminants were removed from the drug solution by extraction on a 500-mg C8 cartridge, as described below. The eluent (in 5% ammonium acetate) was lyophilized and redissolved in PBS, pH 7, or cell culture medium. Both radiochemical and UV HPLC analysis showed that all 5-DACTHF solutions used were ≥98% pure. Escherichia coli folypolyglutamate synthetase (10) was used to synthesize the analogues of 5-DACTHF with one and two additional glutamates. These products were extracted on a 100-mg C18 cartridge, as described below, and separated by C18 HPLC. Fractions containing the reaction products were collected, brought to 100 mM 2-mercaptoethanol, lyophilized to dryness, and redissolved in water. These and all solutions containing analogues and metabolites of [14C]-5-DACTHF were protected from air and light by saturation with oxygen-free argon and storage in amber gas-tight vials. 5-Deazaacyclotetrahydrofolate (5-DACTHF less the glutamate) and the hexaglutamated analogue were chemically synthesized. Partial enzymatic hydrolysis of the latter compound with hog kidney γ-glutamyl hydrolase resulted in a mixture of analogues of 5-DACTHF with three to six glutamates. One g of dried hog kidney (Dico) was extracted (11), precipitated with 55% ammonium sulfate, and centrifuged. The pellet was dissolved in 4 ml of 0.04 M EDTA, 1 mM l-cysteine, and dialyzed against the same solution. Dialyzed hog kidney extract (10 µl) was added to 120 µl of 100 µM hexaglutamated substrate in 40 mM sodium acetate, pH 4.7, 2 mM l-cysteine. After 2 h at 37°C, the hydrolysis was terminated by acidification and extraction on a 100-mg C18 cartridge, as described below.

Cell Cultures

MOLT-4 cells, human MCF-7 breast adenocarcinoma cells, and mouse L-cells (a connective tissue carcinoma) were obtained from the American Type Culture Collection. All cells were maintained at 37°C in 95% air/5% CO2, in folate-free RPMI 1640 medium (GIBCO) supplemented with 10 nm (6-R,S)-Ca leucovorin and 10% fetal calf serum (which had been dialyzed against 0.3% charcoal in 0.9% NaCl). For metabolism studies, MOLT-4 cells were cultured in 25-cm2 flasks

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: 5-DACTHF, N-4-[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propylamino]-benzoyl-l-glutamic acid; GAR, glycaminide ribonucleotide; N-CHO-5-DACTHF, N-4-[3-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)-N-formylpropylamino]-benzoyl-l-glutamic acid; FGPS, folylpolyglutamate synthetase; TFA, trifluoroacetic acid; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; SAX, strong anion exchange.

administered either i.v. (through the tail vein) or i.p. The total 14C content in the plasma was determined by combustion, as described below. That fraction of the total plasma 14C which was parent drug was determined by HPLC analysis. Pharmacokinetic parameters were estimated with a nonlinear least-squares regression computer program, NONLIN (12). Mean plasma drug concentrations were fit to a two-compartment open model with first-order elimination. The bioavailability of 5-DACTHF after i.p. administration was determined from the ratio of the areas under the concentration-time curves.

Detection of Radioactivity

Total radioactivity was determined in all tissues described in this study. Whole tissues, aliquots of plasma and crude tissue homogenates, and all cell pellets and precipitates were combusted in a model 306 Packard oxidizer. The generated 14C was trapped in a column filled with Packard Carbo-sorb, flushed into a vial using the scintillant Permafluor V (Packard), and counted in a Beckman model LS230 liquid scintillation counter. Aliquots of all cell lysates and tissue homogenates were counted in the Beckman liquid scintillation counter in 10 ml of Beckman Ready-Safe. Recovery of radioactivity through all stages of sample preparation for HPLC was ≥85%.

Extraction and Preparation of Samples for HPLC

Urine. Urine was diluted with an equal volume of aqueous 2% (w/v) TFA. Up to 100 μl of the acidified urine were applied to a 100-μg C18 Bond Elut cartridge (Analytichem International) which had been prewashed sequentially with 1 ml acetonitrile, 1 ml water, and 1 ml 0.1% TFA. After sample application, the column was washed with 8 ml 0.1% TFA. The 14C-metabolites were eluted in 1 ml of either 0.1% TFA in 50% acetonitrile (for SAX HPLC) or 5% ammonium acetate in ethanol (for C18 HPLC). The TFA-acetonitrile eluents were filtered (Millipore HV) and examined directly on SAX HPLC. The ammonium acetate-ethanol eluents were dried under an argon stream at 45°C, dissolved in 5% ammonium acetate in water, and filtered. Up to 3 ml of this filtrate were loaded onto the C18 HPLC column.

Plasma. An aliquot of plasma (5–300 μl) was diluted with water to 900 μl, made pH ≤2 by the addition of 100 μl 10% TFA, and extracted on a C18 cartridge, as described above.

Cell Pellets. MCF-7, MOLT-4, and P388 cells were lysed (10⁷ cells/ml) in boiling extraction buffer [50 mM Na,K phosphate, pH 7; 100 mM Na citrate (to inhibit cellular γ-glutamyl hydrolases); 100 mM β-mercaptoethanol (to protect the drug and its metabolites from oxidation)] and boiled for 10 min. Lyases were clarified by centrifugation (30 min, 4°C, 17,000 g). The supernatants were adjusted to pH ≤2 with 0.15 volumes of 40% TFA, centrifuged, and extracted on 100-μg C18 cartridges, as described above.

Colon-38, Livers, and Feces. Samples were removed from the −70°C freezer, held at room temperature for 2 min, shattered with a hammer, added to a tube containing 6 ml/g sample of ice-cold extraction buffer, and homogenized with a Brinkman Polytron (20 sec at setting 11). The homogenate was held in boiling water for 10 min, cooled on ice, and centrifuged (30 min, 4°C, 17,000 × g). The pellet was re-extracted as above. The two supernatants were pooled, adjusted to pH ≤2 with 0.15 volumes of 40% TFA, and centrifuged (15 min, 4°C, 3,000 × g). Tumor supernatants were extracted on 100-μg C18 cartridges, as described above. Up to 10 ml of liver supernatant were extracted on a 500-μg C18 cartridge, which was developed as above except that the volume of all washes and elutions was increased 5-fold. A 1-ml aliquot of fecal supernatant was extracted on a 500-μg C18 cartridge, which was developed like the 500-μg C18 cartridge. This cartridge was used for fecal samples because much of the radioactivity in these samples could not be removed from a C18 cartridge.

High Pressure Liquid Chromatography and Mass Spectral Analyses

Two HPLC columns were used to separate metabolites of [14C]-5-DACTHF. A Whatman SAX column (25 cm), developed with a 1222-s linear gradient from water to 650 mM KH2PO4, pH 6, at 1.5 ml/min, gave superior baseline separation of the drug and its polyglutamated analogues. UV absorbance was monitored with Waters 480, Perkin
Elmer LC95, and LKB 2140 detectors. The latter provided spectra of metabolites. The column eluent was mixed with Fisher Scinintverse LC at 4.5 ml/min and \(^{14}C\) was detected by a radioactivity flow detector (Flo-one; Radiomatic, Inc.). A Waters C\(_{18}\) Novapak Radial Compression Column, developed with a 1222-s linear gradient from 0 to 13% acetonitrile in 140 mM ammonium acetate, pH 5.5, at 1.5 ml/min, gave optimum separation of several N-substituted drug analogues and monoglutamated metabolites. This eluent was mixed with Flo-scint III (Radiomatic) at 4.5 ml/min for detection of radioactivity. The output from each of the detectors was captured by a Digital Specialties minicomputer and analyzed as described previously (13).

Liquid chromatography–mass spectral analysis was done with a Waters C\(_{18}\) \(\mu\)-Bondapak column, developed with a 600-s linear gradient from 6 to 24% acetonitrile in 140 mM ammonium acetate, pH 5.5, coupled to a Finnigan MAT 4500 mass spectrometer with a Vestec Corporation thermospray interface.

**Rat Plasma \(\gamma\)-Glutamyl Hydrolase and GAR Transformylase Reactions**

Metabolism of \([^{14}C]\)-5-DACTHF other than polyglutamation was examined following enzymatic hydrolysis of added glutamates. Sample aliquots (200 \(\mu\)l in 5% ammonium acetate, 30 mM 2-mercaptoethanol) were incubated for 2 h at 37°C with 50 \(\mu\)l dialyzed rat plasma, which contains \(\gamma\)-glutamyl hydrolase activity (14). Following this hydrolysis, the samples were HV filtered and fractionated by C\(_{18}\) HPLC. Those fractions which contained \(^{14}C\) were collected and further characterized. Those which co-eluted with synthetic N-CHO-5-DACTHF were lyophilized, subjected to an enzymatic treatment (see below) which had previously been shown to deformylate N-CHO-5-DACTHF, and re-examined on HPLC.

N-CHO-5-DACTHF and samples suspected of containing N-CHO-5-DACTHF were dissolved in 400 \(\mu\)l 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 8.5. Twenty-five \(\mu\)l 5 mM GAR and 6 units hog liver GAR transformylase were added, and the mixture was incubated at 37°C. The enzymatic deformylation was followed by examination by HPLC of aliquots taken at intervals up to 20 h. Identifications of substrate and product peaks were based on retention times and UV spectra.

**RESULTS**

**In Vitro Metabolism.** Each cell line was exposed to a cytotoxic level (100 nm for MCF-7 and 200 nm for MOLT-4 and L-cells) of \([^{14}C]\)-5-DACTHF for up to one cell doubling time. At the times indicated in Table 1, the cells were extracted and the \(^{14}C\)-metabolites were identified. After one cell doubling, intracellular concentrations of radioactivity were 21–26 pmol/10\(^6\) cells in each cell line. This represents a concentration 108–228-fold greater than extracellular levels (if the intracellular volume is estimated to be 1 \(\mu\)l/10\(^6\) cells). This intracellular accumulation of drug was accompanied by rapid metabolism. As early as 1 h after drug exposure, polyglutamated metabolites were seen. By 19–24 h, almost all of the intracellular drug was highly polyglutamated (>2 glutamates added). For convenience, polyglutamated metabolites are tabulated as containing one and two additional glutamates or as containing greater than two additional glutamates. The individual metabolites were determined by HPLC analysis. Polyglutamated drug analogues were the only metabolites of 5-DACTHF observed in MCF-7 and MOLT-4 extracts, but additional metabolites were seen in the material extracted from L-cells. Fig. 2 shows a chromatogram of a 19-h L-cell extract before and after treatment with hog liver \(\gamma\)-glutamyl hydrolase. Before this treatment (Fig. 2A), much of the radioactivity eluted from the C\(_{18}\) column earlier than the polyglutamated analogues of 5-DACTHF. The \(\gamma\)-glutamyl hydrolase treatment resulted in two radioactive materials (Fig. 2B), which co-chromatographed with N-CHO-5-DACTHF and 5-DACTHF, respectively. The UV spectra of these materials were also similar to those of N-CHO-5-DACTHF and 5-DACTHF, respectively. This suggested that, after a 19-h exposure to 5-DACTHF, all of the drug extracted from L-cells was polyglutamated and much of it was also formylated (which would decrease retention times on HPLC). In similar experiments, exposure of the metabolites extracted from MCF-7 and MOLT-4 cells to \(\gamma\)-glutamyl hydrolase resulted in retention of all of the radioactive metabolites to a single material, which eluted at the position of 5-DACTHF. We had previously observed that a structural analogue of 5-DACTHF, N-CHO-5-DACTHF (Fig. 1), could serve as a formyl donor for GAR transformylase from L-cells, MOLT-4 cells, and chicken and hog livers. In this reaction, N-CHO-5-DACTHF is deformedylated to 5-DACTHF. To determine if the earlier eluting metabolite in Fig. 2B was N-CHO-5-DACTHF, we collected this peak from HPLC, lyophilized it, redisolved it, exposed it to hog liver GAR transformylase as described in previous studies.

**Table 1 In vitro polyglutamation of 5-DACTHF**

<table>
<thead>
<tr>
<th>Source</th>
<th>Exposure (h)</th>
<th>5-DACTHF + 1 or 2 glutamates</th>
<th>5-DACTHF + 3-7 glutamates</th>
<th>Total (^{14}C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cells</td>
<td>200</td>
<td>3</td>
<td>0</td>
<td>6.4(^a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>6.4(^a)</td>
<td>21.1(b)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>100</td>
<td>1</td>
<td>2.8</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>2.0</td>
<td>3.6</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>200</td>
<td>24</td>
<td>0.4</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>21.6</td>
<td>25.6</td>
</tr>
</tbody>
</table>

\(^a\) 81% were formylated as well as polyglutamated.

\(^b\) 45% were formylated as well as polyglutamated.

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\(^{14}C\) for cancer research.

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“Materials and Methods,” and rechromatographed it. Fig. 2C shows that after this treatment all of the radioactivity cochromatographed with 5-DACTHF. Thus, we concluded that the earlier eluting metabolite observed in Fig. 2B was N-CHO-5-DACTHF.

Pharmacokinetics and Distribution. Plasma concentrations of [14C]-5-DACTHF exhibited biexponential decay after both i.v. and i.p. administration. The mean concentration-time data were very similar for both routes of administration and are displayed in Fig. 3, with the model-fit profiles. After i.v. administration, the estimated distribution and terminal elimination half-lives of 5-DACTHF were approximately 0.2 and 1.8 h, respectively. The model-predicted clearance was approximately 1300 ml/min/kg, indicating appreciable distribution of the drug into tissues. Model-predicted areas under the concentration-time curves were 65 and 72 h-nmol/ml after i.p. and i.v. administration, respectively, resulting in an estimated i.p. bioavailability of 90%. In a preliminary experiment (data not shown), bioavailability was estimated to be between 13 and 32% following p.o. drug administration.

Table 2 shows the distribution of 14C in mouse organs at 1.5 and 24 h following i.p. drug administration. Radioactivity accumulated over the plasma level in most of the tissues examined, particularly in the kidney, pancreas, and liver. In the first 24 h after dosing, 74% of the administered 14C was excreted in the urine and 2% was recovered in the feces; recoveries were 1 and 6%, respectively, in the second 24 h.

In Vivo Metabolism. HPLC analysis of mouse urine revealed two radioactive components. Eighty-eight % of the radioactivity was parent drug through the first 24 h and 34% was parent drug in the second 24 h. The remainder was a metabolite which eluted from the SAX column prior to 5-DACTHF and which was not further characterized. Two radioactive species were extracted from the feces. Less than half of the 14C in each fecal sample was parent drug. The remainder of the radioactivity was a metabolite which was identified as 5-deazaacyclotetrahydropteroyl. Its chromatography on C18 HPLC and its mass, determined by liquid chromatographic-mass spectral analysis (data not shown), were identical to those of the pteroate analogue.

Table 3 shows the concentration of 14C-metabolites in mouse liver and in tumors at the indicated times after drug administration. The liver data are from mice implanted with colon-38; livers from non-tumor-bearing mice gave similar results. Polyglutamated analogues of [14C]-5-DACTHF accumulated in the liver over the 24 h studied. They were first observed between 2 and 5 h in the livers of non-tumor-bearing mice. At 24 h after drug administration, 62% of the intracellular drug was highly polyglutamated and 10–20% of it was also formylated. Total drug equivalents (parent drug plus polyglutamated metabolites) in the liver were 3.7 nmol/g at this time. The concentration of drug equivalents at 24 h was 6-fold lower in colon-38 taken from the same animals and only 33% was highly polyglutamated. In contrast, P-388 cells grown in mice rapidly concentrated and polyglutamated the drug, such that at 24 h intracellular drug equivalents were 2.4 nmol/g and 100% was highly polyglutamated.

DISCUSSION

We have observed the metabolism of 5-DACTHF in tissue culture, in mice, and in tumors implanted into mice. Each of the in vitro cell lines studied here is highly sensitive to the drug. Growth IC50 values for 72-h continuous exposure are 37, 58,
These cells rapidly concentrated and polyglutamated the drug. Since polyglutamates of this drug are better inhibitors of GAR and 5-amino-4-imidazole carboxamide ribonucleotide transformylases than is the monoglutamated parent drug (8), 5-DACTHF was activated in situ. We have also observed that mouse L-cells and mouse liver formylate 5-DACTHF; however, the contribution of the formylated metabolites to the drug's cytotoxicity is unclear, since 5-DACTHF is toxic to other cells which do not formylate it.

The in vivo studies showed that, after a single 50-mg/kg dose of 5-DACTHF, plasma levels of the drug which were toxic in cell culture (>100 nM) could be maintained in mice for 24 h. The drug was concentrated to well above the plasma level in kidney, pancreas, and liver, as well as in P388 tumor cells. Elimination was principally as unmetabolized drug, in the urine. The feces contained deglutamated drug. The source of this metabolism was not examined, but it was likely a product of bacterial metabolism, as has been seen with other folate analogues (15, 16).

Polyglutamates of 5-DACTHF were observed in each of the tissues examined for metabolites. Since intracellular retention of antifolates against a concentration gradient is thought to be a function of polyglutamation (17), the active polyglutamated metabolites are expected to be retained in cells longer than the parent drug. This phenomenon was clearly demonstrated in the in vivo data shown in Table 3. In each of the tissues examined, the amount of total drug equivalents (parent plus all polyglutamated metabolites) present at 24 h after drug administration was almost identical to the amount of polyglutamated drug present at 7 h. Thus, drug polyglutamation was clearly predictive of intracellular drug retention; ongoing studies are directed at seeing if there is a correlation between in vivo polyglutamation of 5-DACTHF and tumor sensitivity.

The in vivo studies described here did not address the possibility of selective activation (polyglutamation) of 5-DACTHF by tumor cells, but other researchers have shown that malignant cells can be more efficient than host cells at transporting and polyglutamating an antifolate drug. Ehrlich tumor cells accumulate polyglutamates of methotrexate to well over the level in mouse gut (18), and L1210 cells synthesize more methotrexate polyglutamates than small intestine, liver, and marrow (19). Galivan et al. (20) showed that the antifolate methotrexate is polyglutamated to a greater extent in hepatoma cells than in hepatocytes and that the relative enzyme activities of FPGS and folypolyglutamate hydrolase favor greater polyglutamation in the hepatoma cells. Similarly, other researchers assayed these enzyme activities in a number of malignant and nonmalignant cells and showed higher levels of FPGS and higher ratios of FPGS/folypolyglutamate hydrolase activities in malignant cells (21, 22). In addition, rapidly dividing cells show a greater ability to polyglutamate than do confluant cells (20). Thus, the spectrum of activity of 5-DACTHF, as well as its toxicity to host cells, will most likely be a function of the ability of a cell or tissue to transport and polyglutamate 5-DACTHF as well as of its dependence on purine de novo biosynthesis.

ACKNOWLEDGMENTS

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In Vivo and in Vitro Metabolism of 5-Deazaacyclotetrahydrofolate, an Acyclic Tetrahydrofolate Analogue

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