0026-895X/91/010085-05$03.00/0

MOLECULAR PHARMACOLOGY, 39:85-89

Intracellular Metabolism of 5,10-Dideazatetrahydrofolic Acid in Human Leukemia Cell Lines

GIUSEPPE PIZZORNO, JOHN A. SOKOLOSKI, ARLENE R. CASHMORE, BARBARA A. MOROSON, ANN D. CROSS, and G. PETER BEARDSLEY

Departments of Pediatrics (A.R.C., P.A.M., A.D.C., G.P.B.) and Pharmacology (G.P., J.A.S., G.P.B.), Yale University School of Medicine, New Haven, Connecticut 06510

Received August 15, 1990; Accepted October 11, 1990

SUMMARY

5,10-Dideazatetrahydrofolic acid (DDATHF) is a new potent antitumor agent that specifically inhibits purine biosynthesis, primarily through inhibition of glycinamide ribonucleotide transformylase, the first of the tetrahydrofolate-requiring enzymes in the *de novo* synthesis pathway. DDATHF has been shown to be an excellent substrate for mouse liver folylpolyglutamate synthetase *in vitro*, suggesting that intracellular conversion to polyglutamates could play an important role in the action of this antifolate. In this report, metabolic studies of the 6*R*-diastereomer of DDATHF in the cultured human leukemia cell lines CCRF-CEM and HL-60 are presented. At both 1 and 10 μM (6*R*)-DDATHF was rapidly converted to polyglutamates in both cell lines. DDATHF$(Glu)_5$ and DDATHF$(Glu)_6$ were the main intracellular metabolites. After incubation in drug-free medium, (6*R*)-DDATHF polyglutamates were better retained intracellularly with increasing glutamate chain length. (6*R*)-DDATHF showed reduced cytotoxicity toward a folylpolyglutamate synthetase-deficient cell line, CCRF-$CEM_{30/6}$ related to a dramatically diminished accumulation of polyglutamates. The activity of (6*R*)-DDATHF in CCRF-$CEM_{30/6}$ cells was decreased after both short and prolonged exposures. These results suggest that polyglutamylation of (6*R*)-DDATHF not only represents a mechanism for trapping the drug inside the cells but also produces a more potent inhibitor of the target enzyme.

DDATHF (Fig. 1) is representative of a new series of folate antimetabolites. DDATHF is a potent inhibitor of cell growth in culture and a highly active antitumor agent *in vivo* (1, 2). DDATHF is a close analog of tetrahydrofolic acid, differing only by replacement of the 5- and 10-position nitrogen atoms by carbon, which renders DDATHF incapable of participating in any of the one-carbon transfers or cofactor interconversions characteristic of folate metabolism. The locus of DDATHF action lies along the pathway of *de novo* purine biosynthesis. The primary target is glycinamide ribonuclide transformylase, the first of two tetrahydrofolate cofactor-requiring enzymes of that pathway (1, 2). DDATHF can exist in two diastereomeric forms, differing in configuration at a carbon 6. (6*R*)-DDATHF (Fig. 1) has the configuration analogous to that of natural tetrahydrofolate (3). Both diastereomers of DDATHF are potent inhibitors of cell growth in culture (4). The 6*R*-diastereomer is currently undergoing clinical trials.

Both diastereomers are excellent substrates for mouse liver FPGS *in vitro* (2, 4), suggesting that conversion to polyglutamates would be a major feature of the intracellular metabolism of DDATHF. In this paper we report studies using (6*R*)-[^{3}H]DDATHF that demonstrate the formation of polyglutamates in cultured cells and we present evidence suggesting that conversion to polyglutamates produces metabolites of (6*R*)-DDATHF that are better retained intracellularly and that are more effective inhibitors of the intracellular target enzyme.

Materials and Methods

(6*R*)-DDATHF was kindly supplied by Dr. Chuan Shih, Lilly Research Laboratories (Indianapolis, IN). Solutions of (6*R*)-DDATHF were made in 0.1 N NaOH and the pH was adjusted to 7.0. The concentration was checked using a value of $\epsilon_{273\ nm}$ of $9.150 \times 10^3\ M^{-1}\ cm^{-1}$ in 0.1 N NaOH.

[^{3}H]DDATHF was obtained by reduction of diethyl-2-acetyl-5,10-dideaza-9,10-didehydrofolate (5). The specific activity of the individual diastereomers was 11.2 Ci/mmol. The locations of the incorporated tritium atoms were completely determined by ^{1}H-decoupled 320 MHz ^{3}H NMR, by comparison with the 500 MHz ^{1}H NMR spectra of (6*S*)-[^{1}H]DDATHF, (6*R*)-[^{1}H]DDATHF, and [5-^{2}H] (50% ^{2}H), 6-^{2}H (100% ^{2}H), 7-^{2}H (50% ^{2}H), 9-^{2}H (50% ^{2}H), and 10-^{2}H (50% ^{2}H) (6*RS*)-

This work was supported by National Institutes of Health Research Grants CA 42367 and CA57320. G.P. is a Special Fellow of the Leukemia Society of America. G.P.B. is the recipient of an American Cancer Society Faculty Research Award.

ABBREVIATIONS: DDATHF, 5,10-dideaza-5,6,7,8-tetrahydrofolic acid; DDATHF$(Glu)_n$, *n* indicates the total number of glutamyl residues, i.e., DDATHF = DDATHF$(Glu)_1$; HPLC, high performance liquid chromatography; MTX, methotrexate, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamate; ED_{50}, the concentration of drug required to decrease the cell count to 50% of control; FPGS, folylpolyglutamate synthetase.

DDATHF. The details of the preparation, isolation, and ^3H NMR characterization will be reported separately.[1]

The tri-, penta-, and hepta-γ-L-glutamate conjugates of 5,10-dideaza-5,6,7,8-tetrahydropteroic acid were made by preparing the fully protected oligo-γ-glutamates, as described by Drey and Priestly (6), and condensing them with 5,10-dideaza-5,6,7,8-tetrahydropteroic acid, using the rationale developed by Konig and Geiger as discussed by Kemp (7). The reaction mixture was treated as described by D'Ari and Rabinowitz (8), and the product was further purified by passage through a silica gel column developed with ethanol/ethyl acetate (1:7, v/v). Protecting groups were removed according to the method of Godwin *et al.* (9), modified to include the scavenger 1,2-ethanedithiol, as described by Lundt *et al.* (10), to react with the *t*-butyl trifluoroacetate formed. Analytically pure product was obtained after preparative paper chromatography on Whatman DEAE-cellulose in the ascending mode, using 0.6 M ammonium bicarbonate. HPLC retention times are given below.

MTX was obtained from the Drug Development Branch, National Cancer Institute (Bethesda, MD). L-[3,4-^3H]Glutamic acid was purchased from New England Nuclear (Boston, MA). Horse and fetal bovine serum, medium, and antibiotics were acquired from GIBCO (Grand Island, NY). All other chemicals were obtained, in the highest purity available from Sigma Chemical Co. (St. Louis, MO).

Cell lines. The parent CCRF-CEM human lymphoblastic leukemia cell line (11) and the MTX-resistant subpopulation CCRF-CEM$_{30/6}$ (12) were routinely cultured in RPMI 1640 medium supplemented with 10% horse serum. HL-60 human promyelocytic leukemia cells (13) were grown in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to all media and cells were grown at 37° in a 5% CO_2 atmosphere. All cell lines were tested every 3 months for *Mycoplasma* (Gen-Probe, San Diego, CA).

Cell volume determination. The internal cellular volume was calculated as the difference between the total pellet volume (^3H_2O) and the extracellular surface-bound radioactivity ([^{14}C]inulin) (14). The cell volume was 6.43 ± 0.37 μl/10^7 cells for CCRF-CEM and CCRF-CEM$_{30/6}$ and 4.32 ± 0.12 μl/10^7 cells for HL-60 cells.

Cell growth-inhibition studies. Exponentially growing cells at a density of 1–2 × 10^5 cells/ml were treated with the indicated compounds in 25-cm^2 flasks. After 72 hr, cell densities were determined with a model B Coulter counter (Coulter Electronics, Hialeah, FL). For shorter exposures, cells were incubated for 4 hr in the presence of the drug, washed, resuspended in drug-free medium, and counted after a total of 72 hr. ED_{50} values were determined by plotting of cell growth versus inhibitor concentration and interpolation to 50% inhibition.

FPGS activity. FPGS was assayed using the method of McGuire *et al.* (15), with minor changes. L-[3,4-^3H]Glutamic acid was used instead of the L-[2,3-^3H]glutamic acid. Potassium glutamate (50 mM) in addition to 25 mM 2-mercaptoethanol was used to stop the reaction at the end of the incubation period. These changes minimized background counts to 75 cpm/ml of column eluant. Activity was verified to be linear with respect to both time and enzyme concentration.

Analysis of (6*R*)-DDATHF polyglutamates. CCRF-CEM cells at a density of 1–2 × 10^5 cells/ml were incubated for the indicated times with 1 or 10 μM (6*R*)-DDATHF, in the presence of 2 μCi/ml (6*R*)-[^3H]DDATHF. In the experiment in which polyglutamates were determined after efflux, cells were washed twice in prewarmed complete medium and resuspended in drug-free medium. After 24 hr, the cells were washed twice with ice-cold 0.9% saline and collected by centrifugation at 900 × *g* for 10 min. The cell pellet was suspended in 1 ml of 50 mM sodium phosphate, pH 6.0, containing 200 mM 2-mercaptoethanol, and was boiled 5 min. The cellular debris was removed by centrifugation at 2000 × *g* for 10 min. In a separate experiment, the radiolabeled material was exposed to the extraction conditions and recovered unchanged.

DDATHF polyglutamate analysis utilized HPLC reverse phase ion pair chromatography. The 5-μm C_{18} Ultrasphere column (4.6 mm × 25 cm; Rainin, Woburn, MA) was eluted with a linear gradient from 20% methanol/acetonitrile (2:1) to 40% methanol/acetonitrile (2:1) in 100 mM ammonium phosphate, 1.5 mM tetrabutylammonium bromide, pH 6.5, at 1 ml/min. A second system utilized the same C_{18} column, but the elution was carried out with a linear gradient from 20 to 50% methanol in water. Unlabeled standards were added to the sample to provide internal controls. The column effluent was collected in 1-ml fractions. Radioactivity was determined by scintillation counting after the addition of scintillation cocktail (Optifluor; Packard Instrument Co., Downers Grove, IL).

The peaks in the radiochromatograph were identified as polyglutamates by treatment of the cell extract with partially purified hog kidney γ-glutamyl hydrolase (16) in 50 mM sodium acetate, pH 4.5, 10 mM glutamic acid, 2% 2-mercaptoethanol, for 2 hr at 37° (17). The identity of the hydrolase product as (6*R*)-DDATHF was confirmed by its identical retention characteristics in both the ion pair and reverse phase HPLC systems.

Protein determination. Protein was determined by the method of Bradford (18), using bovine serum albumin as the standard.

Results

Identification of (6*R*)-DDATHF metabolites. (6*R*)-DDATHF was converted intracellularly to polyglutamates in

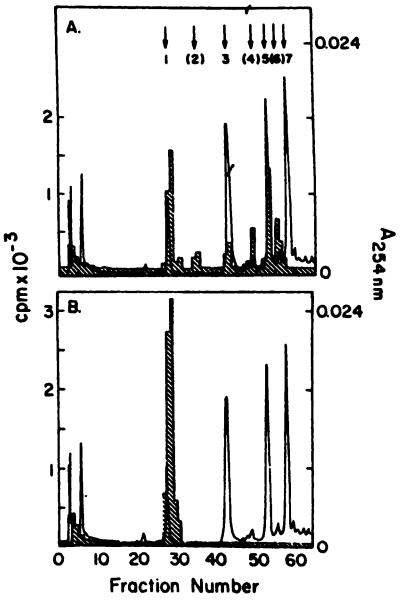

Fig. 2. Radiochromatogram of CCRF-CEM cell extract following a 24-hr exposure to 10 μM (6*R*)-[^3H]DDATHF was directly analyzed by HPLC (A) and 1-ml fractions were collected and counted for radioactivity. An aliquot of the same extract was then incubated for 2 hr with partially purified hog kidney hydrolase and analyzed by HPLC (B). *Numbers without parentheses*, retention times of the chemically synthesized DDATHF polyglutamate standards. *Numbers with parentheses*, interpolated retention times for the other polyglutamates. *Hatched area*, radioactivity; *solid line*, absorbance at 254 nm.

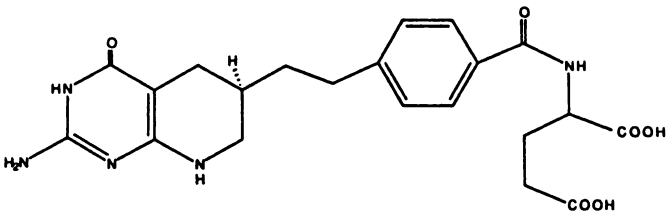

Fig. 1. Structure of (6*R*)-DDATHF.

[1] A. D. Cross, H. Morimoto, P. G. Williams, and G. P. Beardsley. Chemical reduction in the synthesis of triatiated 5,10-dideaza-5,6,7,8-tetrahydrofolate. Submitted to J. of Labelled Compounds and Radiopharmaceuticals. Submitted for publication.

all of the cell lines tested. For example, CCRF-CEM cells were exposed to 10 μM (6*R*)-[^{3}H]DDATHF for 24 hr and extracted. Fig. 2A shows the radiochromatogram obtained upon HPLC analysis of the extract. The earliest eluting peak (28 min) was identified as the parent compound, (6*R*)-DDATHF, by comparison of its retention time with that of the authentic unlabeled compound in both chromatographic systems. The retention times of the authentic chemically prepared polyglutamates were DDATHF(Glu)$_3$, 43 min; DDATHF(Glu)$_5$, 53 min; and DDATHF(Glu)$_7$, 58 min. By interpolation, the additional peaks in the radiochromatogram could be assigned as follows (6*R*)-DDATHF(Glu)$_2$, 35 min; (6*R*)-DDATHF(Glu)$_4$, 49 min; and (6*R*)-DDATHF(Glu)$_6$, 56 min.

As shown in Fig. 2B, treatment of the extract with hog kidney γ-glutamyl hydrolase before HPLC analysis eliminated the additional chromatographic peaks and augmented the parent peak, thus further identifying the additional peaks as polyglutamates of (6*R*)-DDATHF.

Intracellular formation and retention of (6*R*)-DDATHF polyglutamates. The time course of (6*R*)-DDATHF polyglutamate accumulation in CCRF-CEM cells exposed to 10 μM drug is shown in Fig. 3. Significant accumulation of polyglutamates occurred within 4 hr and continued to increase for up to 24 hr. Fig. 3 also shows the result of an experiment in which cells were exposed to 10 μM (6*R*)-[^{3}H]DDATHF for 24 hr, incubated in drug-free medium for an additional 24 hr, and then analyzed. Polyglutamates of (6*R*)-DDATHF were better retained intracellularly with increasing chain length. After efflux, approximately 55% of the 24-hr level of (6*R*)-DDATHF(Glu)$_5$ was present, and the amount of (6*R*)-DDATHF(Glu)$_6$ was actually slightly more than that present at the start of the efflux period. In contrast, intracellular levels of the parent monoglutamate had fallen to less than 10% of the pre-efflux values.

Conversion of (6*R*)-DDATHF to polyglutamates was similarly investigated using cultured HL-60 human promyelocytic leukemia cells. In this cell line, (6*R*)-DDATHF inhibited growth with an ED_{50} of 0.05 μM at 72 hr (19), similar to its potency against CCRF-CEM cells, 0.009 μM (Table 1).

Table 2 shows comparative accumulation and distribution of various (6*R*)-DDATHF polyglutamates in CCRF-CEM and HL-60 cells after 24-hr exposure to 1 and 10 μM drug. These concentrations used in the culture media are well within the range of clinically relevant plasma (6*R*)-DDATHF levels in patients.[2] At each concentration, the accumulation of total intracellular drug and (6*R*)-DDATHF polyglutamates was very similar in both cell lines. At the lowest external concentration, a 6–7-fold concentration of total intracellular drug and polyglutamate metabolites was achieved in both cell lines. The CCRF-CEM and HL-60 cell lines also showed strikingly similar profiles for distribution of (6*R*)-DDATHF polyglutamates, at both 1 and 10 μM, with the predominant polyglutamates being (6*R*)-DDATHF(Glu)$_5$ and -(Glu)$_6$. Table 2 also shows the accumulation of (6*R*)-DDATHF in CCRF-CEM$_{30/6}$ cells. These latter cells are deficient in the ability to accumulate polyglutamates of MTX as a result of decreased activity of FPGS (12, 20). In Table 3, the FPGS activity of CCRF-CEM$_{30/6}$ is compared with that of the parental line, using various substrates. The resistant line contained about 10% of the FPGS enzymatic activity of the parental line, irrespective of whether an antifolate or a natural folate was used as the substrate. Compared with HL-60 or CCRF-CEM cells, these FPGS-deficient CCRF-CEM$_{30/6}$ cells accumulated lesser amounts of (6*R*)-DDATHF polyglutamates, and the distribution was strongly shifted toward lower polyglutamates.

The growth-inhibitory effects of long (72-hr) and short (4-hr) exposure of sensitive CCRF-CEM and resistant CCRF-CEM$_{30/6}$ cell lines to MTX and (6*R*)-DDATHF are compared

[2] R. Bowsher (Eli Lilly & Co.), personal communication.

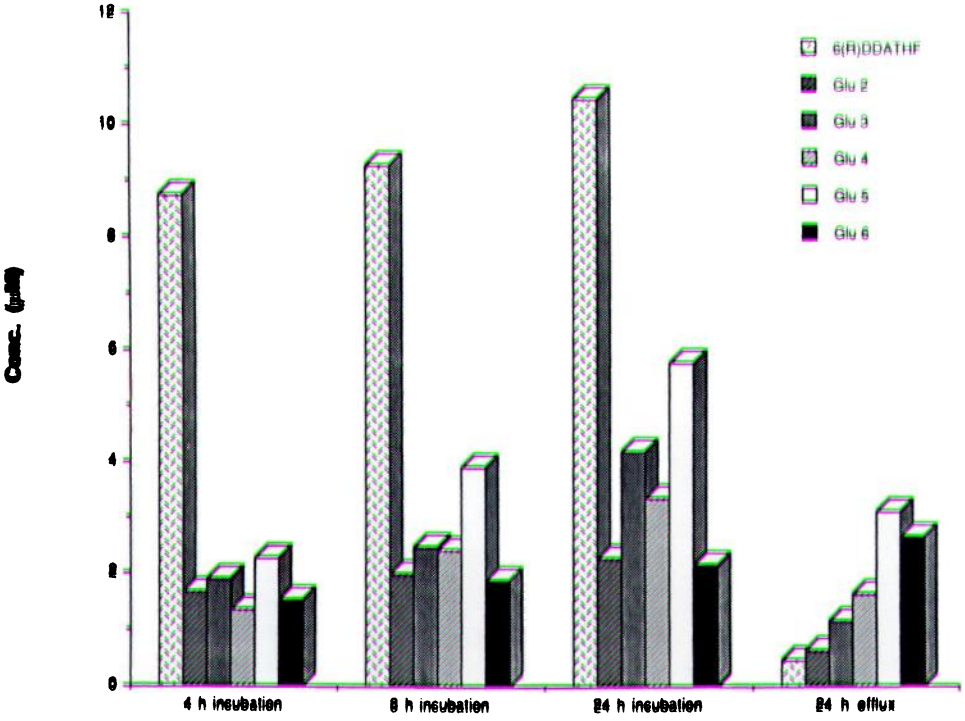

Fig. 3. Time course of the accumulation and distribution of (6*R*)-DDATHF polyglutamates in the CCRF-CEM cell line. CCRF-CEM cells were incubated for the indicated time periods (4, 8, and 24 hr) with 10 μM (6*R*)-[^{3}H]DDATHF, and the cell extract was analyzed by HPLC, as described in Materials and Methods. In addition, cells were incubated with (6*R*)-[^{3}H]DDATHF for 24 hr, collected, further incubated in drug-free medium for an additional 24 hr, and then analyzed as above (*24 h efflux*).

TABLE 1

Comparative sensitivities toward varying exposure times to (6R)-DDATHF and MTX of parental CCRF-CEM cells and an FPGS-deficient subline, CCRF-CEM$_{30/6}$

Cells were incubated for 4 or 72 hr with the indicated drug and counted as described in Materials and Methods.

Cell line	EC_{50}			
	(6R)-DDATHF		MTX	
	4 hr	72 hr	72 hr	4 hr
	μM			
CCRF-CEM	0.16	0.009	0.085	0.017
CCRF-CEM$_{30/6}$	16.0	0.085	26.0	0.029

in Table 1. The CCRF-CEM$_{30/6}$ cells were relatively resistant to both long (10-fold at 72 hr) and short exposures (100-fold at 4 hr) to (6R)-DDATHF. In contrast, whereas there was more than a 300-fold decrease in the growth inhibition sensitivity of these cells, compared with the parent line, for a 4-hr MTX exposure, the relative decrease was only 1.7-fold for a 72-hr exposure. Thus, the CCRF-CEM$_{30/6}$ cells retain most of their sensitivity toward MTX at long exposure times but are relatively more resistant to short MTX exposures.

Discussion

The results demonstrate clearly that conversion to polyglutamates is a major metabolic fate of (6R)-DDATHF in cells that are not deficient in FPGS activity. In both CCRF-CEM and HL-60 cells, similar degrees of overall conversion to higher polyglutamates were found at concentrations of 1 and 10 μM, within the clinically achievable plasma level in patients, and the distribution of the various species was strikingly similar. This result is consistent with the close structural relationship between (6R)-DDATHF and tetrahydrofolate and the previously demonstrated efficiency with which (6R)-DDATHF is utilized as a substrate by FPGS *in vitro* (4).

No metabolites of (6R)-DDATHF other than its polyglutamates were detected in these human cell lines. The intracellular metabolites, after treatment with γ-glutamyl hydrolase, were homogeneous in two distinctly different chromatographic systems and had retention characteristics identical to those of (6R)-DDATHF. Although an unknown metabolite of DDATHF could conceivably be undetected because of identical chromatographic properties, such an event is unlikely. The structural design of DDATHF (4) precludes its serving as a substrate for any of the tetrahydrofolate cofactor interconversions. The structure also makes it unlikely to undergo oxidation at position 7, as occurs with folates and analogs such as MTX. Cleavage between positions 9 and 10 is also rendered unlikely by the structure of DDATHF. The distribution of tritium in the (6R)-[^{3}H]DDATHF (position 5, 6, 7, 9, or 10) used in these experiments was such that products arising from such reactions would remain radiolabeled and probably would have been detected. The pteroic acid analog that would result from cleavage of (6R)-DDATHF has distinctly different chromatographic properties from the parent compound. None of this material was detected.

The data shown in Fig. 3 demonstrate efficient uptake and conversion of (6R)-DDATHF to polyglutamates. After 4 hr of exposure to 10 μM drug, CCRF-CEM cells had accumulated a total intracellular (6R)-DDATHF concentration approximately 1.8-fold higher than the drug concentration in the medium, with about 45% of the total intracellular drug being present as polyglutamyl metabolites. The major metabolite was the pentaglutamate, with a significant fraction present as hexaglutamate. This distribution contrasted with that found for MTX, where under the same conditions the triglutamate predominated, with little conversion beyond the tetraglutamate, although the total intracellular uptake was similar (12). The uptake and conversion of (6R)-DDATHF may also be compared with those reported for N^{10}-propargyl-5,8-dideazafolic acid (CB3717) in L1210 cells (21). The distribution of CB3717 polyglutamates was strikingly similar to that reported here for (6R)-DDATHF; however, CB3717 achieved a maximum total intracellular concentration that was only 10% of the concentration of the drug in the medium, 50 μM.

Fig. 3 also demonstrates the intracellular retention of higher polyglutamates of (6R)-DDATHF, even after prolonged incubation in drug-free medium. In clinical treatment with (6R)-DDATHF, potentially active drug metabolites could, thus, remain present intracellularly long after plasma drug levels have fallen.

FPGS activity is a major determinant of the intracellular accumulation of folate analogs such as MTX and aminopterin (22). Our studies using the FPGS-deficient line CCRF-CEM$_{30/6}$ showed markedly decreased accumulation of (6R)-DDATHF polyglutamates. Comparison of the sensitivities of the parental and FPGS-deficient cell lines toward varying exposure times to (6R)-DDATHF and MTX (Table 1) suggest differing roles for polyglutamylation in the mechanism of (6R)-DDATHF cytotoxicity, compared with that of MTX. Although conversion to polyglutamates serves to anchor MTX intracel-

TABLE 2

Accumulation and distribution of polyglutamyl metabolites after a 24-hr exposure to 1 and 10 μM (6R)-DDATHF)

Cells were incubated with 10 μM (6R)-DDATHF for 24 hr, washed with saline, and boiled in 50 mM sodium phosphate, pH 6.0, containing 200 mM 2-mercaptoethanol. The cell extracts were analyzed by HPLC, as described in Materials and Methods. Data represent the average of three experiments, each conducted in duplicate, with a standard deviation not exceeding 15%.

Cell type	(6R)-DDATHF	Fraction present						Internal concentration	
		1[a]	2[a]	3[a]	4[a]	5[a]	6[a]	Total	Polyglutamates
	μM	%						μM	
CCRF-CEM	10	34.7	8.3	15.3	12.7	21.3	7.7	28.5	18.6
CCRF-CEM	1	17.5	13.1	14.8	15.5	22.8	16.3	6.3	5.2
CCRF-CEM$_{30/6}$	10	79.4	6.4	5.5	3.5	3.7	1.6	14.7	3.1
HL-60	10	39.8	4.7	7.5	10.3	21.8	15.8	27.4	16.5
HL-60	1	18.4	15.1	13.0	15.2	21.2	17.2	7.0	5.7

[a] Total number of glutamyl residues.

TABLE 3

FPGs activity in CCRF-CEM and CCRF-CEM$_{30/6}$ cell extracts

FPGS activity was assayed with the indicated substrates at a concentraion of 50 μM, as described in Materials and Methods. The incubation time for CCRF-CEM$_{30/6}$ was 2 hr. The values have been normalized to 1 hr. The values are the mean ± standard deviation of at least two experiments done in triplicate.

Cell line	Aminopterin	(6R)-DDATHF	Tetrahydrofolate
		pmol/hr/mg of protein	
CCRF-CEM	1297 ± 72	895 ± 66	1021 ± 122
CCRF-CEM$_{30/6}$	90 ± 27	101 ± 11	79 ± 27

lularly, MTX is itself a potent inhibitor of its primary target enzyme. In polyglutamylation-proficient cells, there is a relatively small difference between the cytotoxic effect of a 4-hr and a 72-hr exposure to MTX, presumably because during the shorter exposure, MTX polyglutamates are formed that maintain effective intracellular drug levels. In the FPGS-deficient cells, lesser amounts of MTX polyglutamates are formed during short exposure, resulting in a marked decrease in toxicity. Longer exposure to MTX maintains intracellular levels of drug without the need for polyglutamylation. Thus, MTX cytotoxicity is almost fully expressed. This is evidenced by the less than 2-fold difference in ED_{50} between the FPGS-proficient and -deficient lines for 72-hr MTX exposure. In contrast, the polyglutamylation-deficient line is 10-fold resistant to (6*R*)-DDATHF, even after prolonged drug exposure. This suggests that conversion of (6*R*)-DDATHF to higher polyglutamates produces more effective inhibitors of its target enzyme, in addition to simply retaining the drug intracellularly. In preliminary experiments using the pentaglutamate form of DDATHF, we have found a 100-fold increase in the capacity to inhibit glycinamide ribonucleotide transformylase purified from CCRF-CEM cells, compared with the monoglutamate (23). This kind of activation by FPGS has been described for other antifolates (24). MTX polyglutamates, in fact, can inhibit thymidylate synthase, AICAR transformylase, and other folate-requiring enzymes, and such inhibition may play some role in the cytotoxicity of MTX (25), although dihydrofolate reductase remains the primary target. The nearly equivalent cytotoxicity seen (Table 1) for 72-hr MTX exposure with both FPGS-deficient and parental cell lines makes it appear that the conversion to polyglutamates is less necessary for the expression of MTX toxicity than it is for (6*R*)-DDATHF toxicity.

In summary, our results demonstrate that the conversion to polyglutamates is a significant pathway of (6*R*)-DDATHF metabolism. Higher polyglutamates of (6*R*)-DDATHF are retained intracellularly even after prolonged incubation in drug-free medium. These higher polyglutamates probably represent the true intracellular active metabolites of (6*R*)-DDATHF.

Acknowledgments

A large measure of gratitude is due Prof. E. C. Taylor, Department of Chemistry, Princeton University, who carried out the original synthesis of DDATHF and has been a continuing collaborator and source of inspiration. We thank also Drs. John J. McGuire and John Galivan for critical review of the manuscript.

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Send reprint requests to: G. Peter Beardsley, Department of Pediatrics, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510.