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DETERMINANTS OF THE DISPARATE ANTITUMOR ACTIVITIES OF (6*R*)-5,10-DIDEAZA-5,6,7,8-TETRAHYDROFOLATE AND METHOTREXATE TOWARD HUMAN LYMPHOBLASTIC LEUKEMIA CELLS, CHARACTERIZED BY SEVERELY IMPAIRED ANTIFOLATE MEMBRANE TRANSPORT

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Abstract—We previously reported (Matherly *et al.*, *J Biol Chem* **267**: 23253–23260, 1992) that impaired methotrexate transport in a drug-resistant CCRF-CEM variant (CEM/MTX) involved the synthesis of a structurally altered isoform of the "classical" carrier for methotrexate and related derivatives. Although CEM/MTX cells were highly resistant (162- to 300-fold) to assorted antifolate substrates for the classical transporter, including methotrexate, aminopterin, 10-ethyl-10-deazaaminopterin, ICI D1694, and 1843U89, they were only 3.6-fold resistant to (6*R*)-5,10-dideaza-5,6,7,8-tetrahydrofolate (DDATHF). These divergent antifolate sensitivities were not associated with appreciable differences in the levels of dihydrofolate reductase, thymidylate synthase, and 5′-phosphoribosylglycinamide (GAR) transformylase, or the expression of a high affinity membrane folate binding protein receptor in either line. The initial rate of [^{14}C]DDATHF influx was increased 2.9-fold over that for [^{3}H]methotrexate in parental cells (at 2 μM). Whereas [^{14}C]DDATHF initial uptake was, likewise, increased over [^{3}H]methotrexate in CEM/MTX cells (5.3-fold), influx of both compounds was impaired substantially (95–97%). For the parent, influx of [^{14}C]DDATHF was inhibited by substrates for the classical transporter including unlabeled DDATHF, methotrexate, (6*R*,*S*)-5-formyl tetrahydrofolate, 10-ethyl-10-deazaaminopterin, ICI D1694, 1843U89, and folic acid. The synthesis of a modified transporter in CEM/MTX cells was accompanied by significant changes in the binding of all these transport substrates. In spite of its impaired transport, [^{14}C]DDATHF (at 2 μM), unlike methotrexate, continued to accumulate in CEM/MTX cells, eventually reaching 62% of the parental drug levels after 4 hr. At this time, 53% (parent) and 71% (CEM/MTX) of the intracellular radioactivity from [^{14}C]DDATHF was identified as polyglutamates. DDATHF polyglutamates in CEM/MTX cells after 4 hr reached 90% of the levels measured in parental cells. While significant levels of methotrexate polyglutamates were detected in the parental line, methotrexate polyglutamylation was negligible in intact CEM/MTX cells. The specific activity of folylpolyglutamate synthetase was measured in cell-free extracts from parental and CEM/MTX cells using aminopterin, methotrexate, and DDATHF as substrates; in each case, CEM/MTX cells showed 2-fold higher enzyme activity than parental cells. These data show that even for tumor cells with severely impaired antifolate transport, the extensive conversion of DDATHF to polyglutamyl forms required for GAR transformylase inhibition preserves high levels of antitumor activity.

Key words: antifolate, 5,10-dideazatetrahydrofolate, drug resistance, methotrexate, polyglutamate

In recent years there has been a growing interest in the development of new antifolate chemotherapeutic drugs that exhibit membrane transport and/or FPGS§ substrate properties superior to those of MTX. This rationale for drug design is typified by DDATHF, an analog of tetrahydrofolic acid with carbon substitutions for nitrogens at positions 5 and 10 [1–4]. These structural modifications render DDATHF incapable of participating in the one-carbon transfer and interconverting/isomerization reactions characteristic of tetrahydrofolate cofactors.

While sharing many features with other antifolates, DDATHF binds poorly to the enzymes commonly targeted by this class of drugs, including dihydrofolate reductase and thymidylate synthase. Rather, the antitumor effects of DDATHF derive from its potent inhibition of GAR transformylase (EC 2.1.2.1; [3–5]), the first folate-dependent enzyme in the *de novo* biosynthetic pathway leading to the purine nucleotides. In this fashion, intracellular purine nucleotide pools are depleted [4, 6]. DDATHF can exist in two stereospecific configurations centered at the asymmetric 6-carbon. Both stereoisomers are amongst the best known substrates for mammalian

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§ Abbreviations: DDATHF, (6*R*)-5,10-dideaza-5,6,7,8-tetrahydrofolate (Lometrexol); DPBS, Dulbecco's phosphate-buffered saline; FPGS, folylpolyglutamate synthetase; GAR, 5′-phosphoribosylglycinamide; mFBP, membrane folate binding protein; MTX, methotrexate; SDS, sodium dodecyl sulfate; and K_t, Michaelis–Menten constant for transport.

FPGSs [3, 7]. Moreover, both the 6*R*- and 6*S*-isomers are toxic to cultured cells [3]. This likely results from the accumulation of DDATHF polyglutamates, which are highly retained within cells [8] and are up to 100-fold more inhibitory than the underivatized antifolate toward GAR transformylase [5].

A potential therapeutic advantage of antifolate drugs that inhibit intracellular targets other than dihydrofolate reductase involves the circumvention of MTX resistance resulting from elevated [9] or structurally altered [10, 11] enzyme. Conversely, widespread cross-resistances to other antifolates may occur in MTX-resistant tumors expressing impaired drug transport [12, 13]. Although DDATHF binds to and is apparently transported [2, 14, 15] by the "classical" membrane carrier which mediates the uptake of both MTX and tetrahydrofolate cofactors in mammalian cells, with severely transport-impaired tumor cell variants, cross-resistance to DDATHF is often not observed [15, 16]. In at least some cases, this response correlates with the synthesis of a separate, high affinity mFBP, which facilitates DDATHF uptake [14, 16, 17].

The present study explores the respective roles of membrane transport and polyglutamylation as determinants of the disparate pharmacologic activities of MTX and DDATHF toward a highly MTX-resistant CCRF-CEM human lymphoblastic leukemia line (designated CEM/MTX; [18]). These cells express levels of dihydrofolate reductase and GAR transformylase similar to those of the wild type, but are severely deficient in MTX membrane transport. Impaired MTX transport in this cell variant is associated with the synthesis of a structurally modified isoform of the classical membrane carrier for MTX and related derivatives [19].

MATERIALS AND METHODS

Chemicals. [3′,5′,7-^{3}H]MTX (20 Ci/mmol) and [5-^{3}H]2′-deoxyuridine monophosphate (22 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Both unlabeled and [*benzoyl carboxyl*-^{14}C]-(6*R*)DDATHF (13 μCi/mg) were synthesized at Lilly Research Laboratories (Indianapolis, IN) and provided by Drs. Chuan Shih and Gerald Grindey. Benzamidine–HCl was from Calbiochem (La Jolla, CA) and Prefabloc from Boehringer Mannheim (Indianapolis, IN). [^{125}I]-Protein A was purchased from Dupont/New England Nuclear (Boston, MA). Unlabeled MTX, aminopterin, and (6*R*,*S*)-5-formyl tetrahydrofolate were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD. Folic acid was purchased from the Sigma Chemical Co. (St Louis, MO). Several antifolate drugs were provided as follows: 10-ethyl-10-deazaaminopterin (CIBA GEIGY Corp., Summit, NJ); ICI D1694 (*N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid; ICI Pharmaceuticals, Cheshire, UK); and 1843U89 ((*S*)-2-(5-(((1,2-dihydro-3-methyl-1-oxo-benzo(F)quinazolin-9-yl)methyl)amino)1-oxo-2-isoindolinyl)glutaric acid; Burroughs Wellcome Co., Research Triangle Park, NC). Both labeled and unlabeled MTX, and [^{14}C]DDATHF were repurified prior to use by reversed-phase HPLC [20]. The other antifolates were used as provided. Bio-Rad (Richmond, CA) was the source of the majority of the electrophoresis reagents. Tissue culture reagents and supplies were purchased from assorted vendors with the exception of fetal bovine serum and iron-supplemented calf serum, which were from the Grand Island Biological Co. (Grand Island, NY) and Hyclone Laboratories, Inc. (Logan, UT), respectively. Assorted chemicals or biochemicals were purchased from the Sigma Chemical Co. Rabbit antiserum to the soluble folate binding protein from KB cells was a gift of Dr. Sheldon Rothenberg (Brooklyn, NY).

Cell culture. Wild-type CCRF-CEM cells and an MTX-resistant subline (CEM/MTX; [18]) were gifts from Dr. Andre Rosowsky (Boston, MA). KB human epidermoid carcinoma cells were obtained from the American Type Culture Collection. All cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified atmosphere at 37° in the presence of 5% CO_2/95% air. CEM/MTX cells were maintained continuously in the presence of 1 μM MTX. Cells were subcultured routinely every 72–96 hr. KB cells were subcultured weekly with trypsin (0.05%):EDTA (0.53 mM). Cell numbers were determined by direct microscopic counting with a hemacytometer. For large scale culture of parental CCRF-CEM and CEM/MTX lines, iron-supplemented calf serum was substituted for fetal bovine serum in the above medium. For the large scale culture of wild-type CCRF-CEM cells as a source of purified FPGS, cells were grown in medium containing 10% horse serum.

For cytotoxicity determinations, cells were cultured in 24-well culture dishes at 70,000/mL in 2 mL of RPMI 1640 medium containing 10% dialyzed fetal bovine serum [12]. Cells were counted after 72–96 hr. The IC_{50} values were calculated as the concentrations of drug necessary to inhibit growth by 50% compared to control cells grown under identical conditions, except that the growth inhibitor was omitted. For experiments assessing folic acid growth requirements, cells were cultured in 24-well dishes using folate-free RPMI 1640 with dialyzed fetal bovine serum and antibiotics, and known concentrations of folic acid.

Assays of dihydrofolate reductase, thymidylate synthase, GAR transformylase, and FPGS levels. Dihydrofolate reductase enzyme levels were quantitated by measuring the extent of [^{3}H]MTX binding in the presence of high concentrations of NADPH [20, 21]. Cells (approx. 10^7) were sonicated in 2 mL of a buffer composed of 50 mM sodium citrate (pH 6), 150 mM KCl, 1 mM EDTA, 50 mM 2-mercaptoethanol, and 100 μM NADPH. Following a 10-min incubation at 23° with [^{3}H]MTX (1 nmol), unbound and dihydrofolate reductase-bound [^{3}H]-MTX were fractionated by chromatography of cell-free extracts on columns of Biogel P6 (200–400 mesh) by rapid centrifugation [20, 21]. Thymidylate synthase levels were determined by measurements

of catalytic activity involving the release of ^3H_2O during the conversion of [5-^{3}H]deoxyuridine monophosphate to thymidine monophosphate [22]. GAR transformylase activity in cell-free homogenates was assayed spectrophotometrically as described by Daubner *et al.* [23] with 10-formyl-5,8-dideazafolate as the formyl donor.

For FPGS assays, cell-free extracts were prepared by suspending frozen cell pellets (1.5×10^8 cells) in 0.8 to 1 mL of iced extraction buffer (100 mM Tris–HCl, pH 8.85, containing 0.1 mM Na_2-EDTA, 1 mM benzamidine–HCl, 0.5 mM Pefabloc, and 50 mM 2-mercaptoethanol). Cells were lysed by two cycles of freezing and thawing using a dry ice–methanol bath, and the crude supernatant was obtained by centrifugation at 35,000 *g*. FPGS activity was assayed as described previously [24]. For all antifolate substrates, radiolabeled polyglutamates were quantitatively retained and recovered from the DEAE-cellulose mini-columns used in the standard FPGS assay. Maintenance of time and linearity was verified for aminopterin in each experiment. For use in mixing experiments, FPGS was partially purified from wild-type CCRF-CEM cells by ammonium sulfate fractionation and gel sieving chromatography [25]; the preparation was purified further by phosphocellulose chromatography (McGuire JJ, unpublished method).

Membrane transport methodology. For membrane transport measurements, logarithmically growing cells were washed with DPBS [26] and suspended into phosphate-buffered Hanks' balanced salts [27]. CEM/MTX cells were cultured for 3–4 generations in the absence of MTX prior to assay of drug transport. Transport experiments with [^{3}H]MTX were performed exactly as described previously [28]. For parental cells, initial rates were generally recorded from 0–180 sec; for CEM/MTX cells, [^{3}H]-MTX influx was measured from 0–1200 sec. For [^{14}C]-DDATHF, an analogous experimental design was used; however, because of the low radiospecific activity (13.01 μCi/mg) for this compound, total cell numbers were increased 3- to 5-fold for each data point. Cell densities during transport assays for both drugs were maintained at $2–5 \times 10^7$ cells/mL. For both radioactive DDATHF and MTX, the levels of intracellular radioactivity were expressed in picomoles per milligram of protein, calculated from direct measurements of radioactivity and protein contents of the cell homogenates. Kinetic constants (i.e. K_t and V_{max}, and K_i values) for assorted folate and antifolate substrates were calculated from Lineweaver–Burk and Dixon plots, respectively.

HPLC analysis of polyglutamyl derivatives of MTX and DDATHF. For analysis of intracellular polyglutamyl forms of DDATHF and MTX, cells were incubated with the radiolabeled drugs as for the transport assays. Following three washes with ice-cold DPBS, cell pellets were suspended in 50 mM sodium phosphate, pH 6.0, containing 100 mM 2-mercaptoethanol and boiled (5 min) to extract the radiolabeled drug metabolites. The precipitated proteins were removed by centrifugation; the supernatants were analyzed using a reversed-phase HPLC method and an ISCO gradient liquid chromatograph equipped with a 5-μm octadecylsilyl column (4.5 × 250 mm; ISCO Inc.). The HPLC analyses consisted of a gradient from 0–15% acetonitrile in 0.1 M sodium acetate buffer (pH 5.5) over 20 min, followed by a 5-min isocratic elution at 15% acetonitrile. The flow rate was 2 mL/min; 0.3-min fractions were collected and measured for radioactivity. For MTX, the radioactive polyglutamates were identified by comparison of elution times to those of unlabeled standards [20]. Since no polyglutamyl standards were commercially available for DDATHF, confirmation of the polyglutamates involved their hydrolysis to the parent compound by an overnight treatment with a preparation of partially purified hog kidney conjugase [20, 29].

For both MTX and DDATHF, the intracellular drug forms were calculated from the percentage of each compound, determined by the chromatographic analysis, and the total intracellular radiolabel, calculated in picomoles per milligram of protein, as described above.

Immunoblot assay of mFBPs. Plasma membranes were isolated from CCRF-CEM, CEM/MTX, and KB cells as described previously [19]. The membrane pellets following the 200,000 *g* centrifugation step were solubilized in 10 mM Tris–HCl (pH 7.0) containing 1–2% SDS and proteolytic inhibitors (0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, 10 μg/mL antipain, 10 μg/mL bestatin, 3 μg/mL aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride).

Samples were diluted with Laemmli sample buffer [30], freeze-thawed 10–15 times in a dry ice–ethanol bath to facilitate disaggregation [28], and electrophoresed on 4–10% linear gradient gels in the presence of SDS [30]. Proteins were electrophoretically transferred to Immobilon P (Millipore Corp.) for 2 hr using a Bio-Rad Transblot cell and a buffer system composed of 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, pH 11, and 10% methanol [31]. Immunoblot analysis was performed, using rabbit antiserum against the purified folate binding protein from KB cells [32], or non-immune serum. Blots were developed using [^{125}I]Protein A. The immunoblot methodology was detailed in our earlier report [19]. The blots were autoradiographed (Kodak X-Omat AR film) at −70° with DuPont Kronex Intensifying screens. Approximate molecular weights of immunoblotted proteins were calculated from the relative migrations of unstained molecular weight standard proteins, which were coelectrophoresed and transferred to Immobilon P. The molecular weight standards were detected by staining the Immobilon P with Coomassie Brilliant Blue R250 [31].

Other measurements. Radioactivity was measured with a Tracor Analytic liquid scintillation counter. Counting efficiencies were corrected by internal standardization with [^{14}C]- or [^{3}H]toluene. Proteins were measured by the methods of Lowry *et al.* [33] or Bradford [34] using bovine serum albumin as standard.

RESULTS

Antifolate sensitivities of parental CCRF-CEM and CEM/MTX cells. Parental CCRF-CEM and CEM/MTX cells showed only minor differences in their

Table 1. Antifolate sensitivities of parental CCRF-CEM and CEM/MTX cells

Antifolate	IC_{50}* (nM)		
	Parent	CEM/MTX	Ratio†
MTX	10.5	2550	243
AMT‡	3	900	300
(6*R*)-DDATHF	16.2	59	3.6
10-EDAM‡	2.6	480	185
ICI D1694	4.7	760	162
1843U89	2.4	540	225

* Growth inhibition was measured after 72–96 hr in the continuous presence of the antifolates.

† Ratio of IC_{50} values for CEM/MTX and parent cells.

‡ Undefined abbreviations: AMT, aminopterin; and 10-EDAM, 10-ethyl-10-deazaaminopterin.

levels of dihydrofolate reductase (3.12 and 4.29 pmol/mg protein, respectively), thymidylate synthase (128 and 214 pmol/min/mg, respectively), and GAR transformylase (2.17 and 1.57 nmol/min/mg protein, respectively). High levels of MTX resistance in the CEM/MTX line result from a severe impairment of drug uptake [18]. Table 1 compares the growth inhibitory sensitivities of the parental CCRF-CEM and CEM/MTX cells to assorted antifolates that are reported substrates for the classical MTX membrane transporter.

Similar levels of resistance (162- to 300-fold) were observed for a number of antifolates including both inhibitors of dihydrofolate reductase (i.e. MTX, 10-ethyl-10-deazaaminopterin [35] and aminopterin [36]) and thymidylate synthase (i.e. ICI D1694 [37] and 1843U89 [38]). By contrast, the *de novo* purine nucleotide biosynthetic inhibitor, (6*R*)-DDATHF, was only 3.6-fold less active against CEM/MTX than parental cells.

Immunoblot analyses of mFBPs. Earlier reports

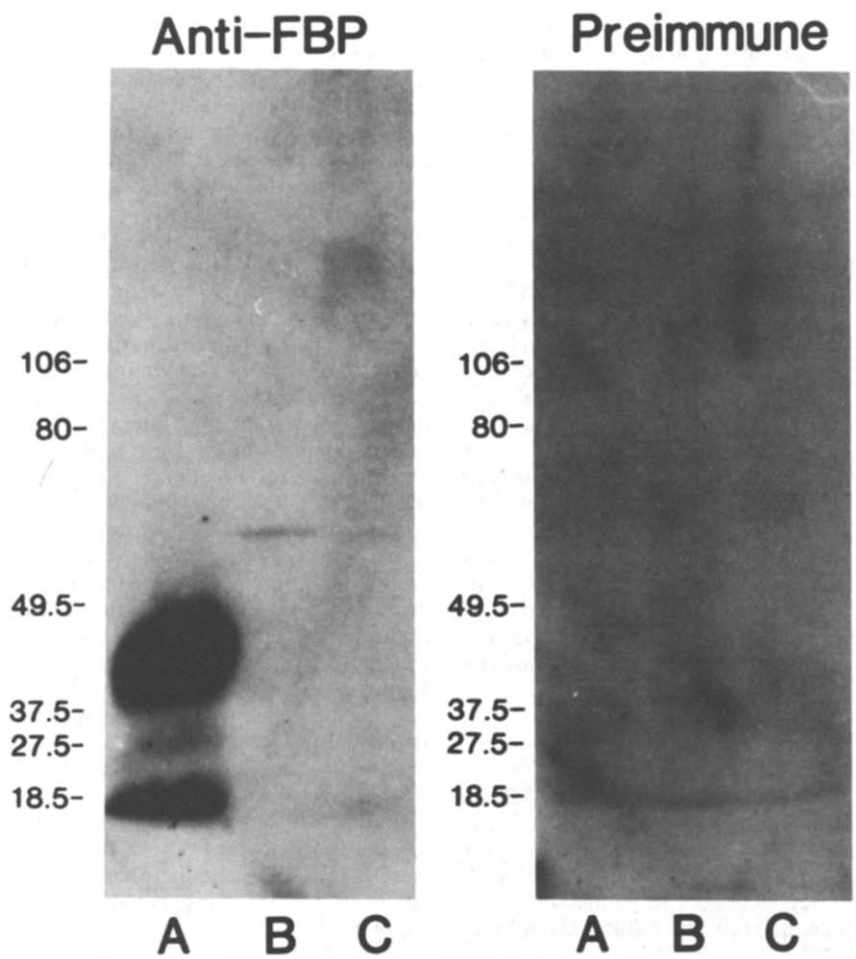

Fig. 1. Immunoblot analyses of mFBPs. Plasma membranes were prepared from KB (lane A), CEM/MTX (lane B), or parental CCRF-CEM (lane C) cells. Aliquots (40 μg) were electrophoresed on 4–10% polyacrylamide gels in the presence of SDS. Proteins were analyzed on western blots using antiserum to the folate binder from KB cells ("anti-FBP"), or preimmune serum. The immune complexes were detected with [^{125}I]Protein A and autoradiography.

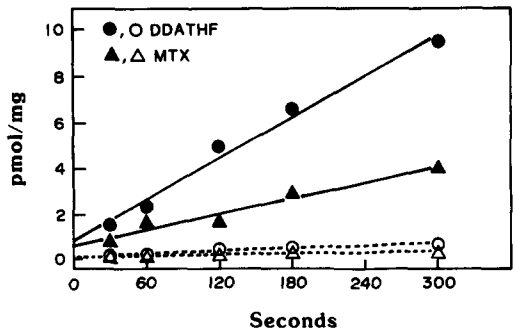

Fig. 2. Initial influx of [^{14}C]DDATHF and [^{3}H]MTX in parental CCRF-CEM (solid lines) and CEM/MTX (broken lines) cells. Cells were incubated at 37° in Hanks' balanced salt solution with the radioactive compounds. Uptake was measured as described in Materials and Methods.

described the avid binding of DDATHF to mFBPs in cultured murine and human leukemic cells, and the circumvention of MTX resistance in this fashion [16, 17]. Likewise, it seemed possible that the presence of mFBPs in CEM/MTX cells could contribute to their remarkable sensitivities to DDATHF. Although their high folic acid growth requirements (50% maximal growth at 280 and 690 nM for parent and CEM/MTX, respectively; data not shown) seemed incompatible with mFBP expression in either line, this possibility was directly evaluated by western blotting, using antiserum to the folate binder from KB human epidermoid carcinoma cells [32]. Plasma membranes from KB cells were included as a positive control. As illustrated in Fig. 1, a large amount of the 40-kDa mFBP was detected in KB plasma membranes (lane A); however, no analogous immunoreactive mFBPs were detected in plasma membranes prepared from either wild-type CCRF-CEM or CEM/MTX cells (lanes C and B, respectively).

Kinetic analysis of [^{14}C](6R)-*DDATHF membrane transport.* Experiments were performed to correlate the growth inhibitions by MTX and DDATHF in Table 1 with drug influx capacities. In CEM/MTX cells, initial [^{3}H]MTX uptake was 3% of that for parental cells (measured at 2 μM; Fig. 2). This was reflected in a reduced influx V_{max} (5.9-fold) and an increased K_t (3.3-fold; Table 2).

The initial influx rates for [^{14}C]DDATHF were increased over those for [^{3}H]MTX in both parental and CEM/MTX cells (2.9- and 5.3-fold, respectively, at 2 μM; Fig. 2). This was primarily due to decreased K_t values for DDATHF binding over MTX (8- and 77-fold, respectively; Table 2). Whereas a large disparity in relative [^{14}C]DDATHF influx was preserved between the lines (approx. 20-fold in Fig. 2), decreased uptake of DDATHF into CEM/MTX cells was due entirely to a lower V_{max} (Table 2). Indeed, in contrast to the results with MTX, the K_t calculated for DDATHF in the CEM/MTX line was 2.9-fold *lower* than that for the wild-type transporter ($P < 0.001$).

The inhibitory effects of assorted (anti) folates on [^{14}C]DDATHF influx were evaluated. Drug uptake was assayed at 2 μM [^{14}C]DDATHF over a range of inhibitor concentrations; K_i values were calculated from Dixon plots (not shown) and the K_t and V_{max} values in Table 2. K_i values for these inhibitions are summarized in Table 3. Uptake of [^{14}C]DDATHF in parental cells was potently inhibited by MTX, (6*R*,*S*)-5-formyl tetrahydrofolate, 10-ethyl-10-deazaaminopterin, ICI D1694, 1843U89, and unlabeled DDATHF. Folic acid was much less inhibitory. Notably, for the CEM/MTX transporter, differences from the parent were found not only in the binding of DDATHF and MTX, as noted above (i.e. Table 2), but also for the other transport substrates, as well. The K_i values for unlabeled DDATHF and MTX in Table 3 closely approximated the calculated K_t values for these radioactive substrates in Table 2. Moreover, when certain inhibitors (folic acid, 5-formyl tetrahydrofolate, and DDATHF) were tested against [^{3}H]MTX as the transport substrate, nearly identical K_i values were calculated (data not shown).

These results strongly argue that mediated uptake of DDATHF in CCRF-CEM cells involves the well-documented classical membrane carrier for MTX and reduced folates, as previously suggested [2, 14, 15]. For the CEM/MTX variant, the synthesis of a structurally modified carrier isoform [19] was accompanied by pronounced changes in the binding of a number of transport substrates.

Polyglutamylation of DDATHF and MTX in parental and CEM/MTX cells. Although the relative rates of [^{14}C]DDATHF and [^{3}H]MTX *initial* influx were comparably reduced in the CEM/MTX subline

Table 2. Kinetic constants for MTX and DDATHF*

Cell line	MTX influx parameters				DDATHF influx parameters			
	K_t (μM)	V_{max} (pmol/mg/min)	V_{max}/K_t	N	K_t (μM)	V_{max} (pmol/mg/min)	V_{max}/K_t	N
Parent	3.77 ± 0.98	4.56 ± 1.03	1.24 ± 0.13	3	0.47 ± 0.04	3.22 ± 0.53	7.10 ± 1.80	3
CEM/MTX	12.57 ± 3.86	0.78 ± 0.28	0.08 ± 0.03	5	0.16 ± 0.02	0.17 ± 0.03	1.03 ± 0.20	3

* Values are means ± SEM, determined from Lineweaver–Burk analyses of N experiments. The concentration ranges were 0.4 to 4 μM for MTX and 0.2 to 4 μM for DDATHF.

Table 3. K_i values of assorted (anti)folate transport substrates

(Anti)folate	K_i (μM) WT	K_i (μM) CEM/MTX	Ratio†
(6*R*)-DDATHF‡	0.68 ± 0.11	0.17 ± 0.07	4.00
Folic acid	167.27 ± 33.04	8.95 ± 2.30	18.69
(6*R*,*S*)5-CHO-H_4PteGlu§	2.37 ± 0.78	0.74 ± 0.09	3.21
MTX	4.48 ± 0.84	9.74 ± 1.52	0.46
10-EDAM§	0.64 ± 0.10	2.03 ± 0.35	0.32
ICI D1694	1.05 ± 0.29	1.59 ± 0.29	0.66
1843U89	0.21 ± 0.06	1.40 ± 0.39	0.15

* K_i values are expressed as means ± SEM for the inhibition of [^{14}C]DDATHF (2 μM) influx into wild-type (WT) and CEM/MTX cells. K_i values were calculated from Dixon plots for 2–7 separate experiments.

† K_i ratio for wild-type to CEM/MTX.

‡ Unlabeled (6*R*)-DDATHF was used as the inhibitor.

§ Undefined abbreviations: 10-EDAM, 10-ethyl-10-deazaaminopterin; 5-CHO-H_4PteGlu, 5-formyl tetrahydrofolate.

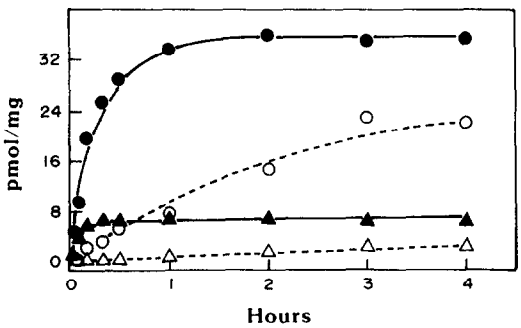

Fig. 3. Accumulation of [^{14}C]DDATHF and [^{3}H]MTX in parental CCRF-CEM (solid lines) and CEM/MTX (broken lines) cells during extended incubation. Cells were incubated at 37° in Hanks' balanced salt solution with a 2 μM concentration of the radioactive compounds. Antifolate accumulation was measured as described in Materials and Methods. Key: (▲, △) MTX; and (●, ○) DDATHF.

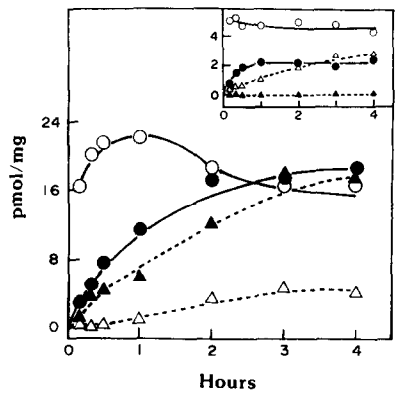

Fig. 4. Time course of accumulation of antifolate mono- and polyglutamates in parental CCRF-CEM (solid lines) and CEM/MTX (broken lines) cells. Cells were incubated with [^{3}H]MTX (inset) or [^{14}C]DDATHF (main graph). At the indicated times, the intracellular monoglutamate (○, △) and polyglutamate (●, ▲) forms were quantitated as described in Materials and Methods.

(Fig. 2), a far different picture emerged when drug accumulations were followed during an extended interval. Indeed, whereas intracellular MTX was maximal in CEM/MTX cells at a level approximating dihydrofolate reductase, [^{14}C]DDATHF continued to accumulate over 4 hr (Fig. 3), eventually reaching 62% of the parent level. Total DDATHF accumulation in parent cells at this time exceeded that for MTX (including both unbound and dihydrofolate reductase-bound drug) by 5.4-fold; for the CEM/MTX line, this difference was even greater (7.9-fold).

The disparate [^{3}H]MTX and [^{14}C]DDATHF accumulations illustrated in Fig. 3 reflect, in part, the differences in their membrane transport (i.e. Table 2). However, HPLC analyses of the intracellular drug forms established that there were also marked differences in the extent of metabolism of these antifolates to their polyglutamate forms. Three MTX polyglutamates (corresponding to di-, tri-, and tetraglutamates*) were synthesized in parent cells incubated with 2 μM MTX for 4 hr; 30% of the total intracellular MTX was polyglutamylated (Fig. 4, inset).

By contrast, only a small amount of MTX diglutamate was detected in CEM/MTX cells in this experiment (2.5% of total tritium after 4 hr; Fig. 4, inset). Even when intracellular monoglutamyl MTX was elevated (exceeding dihydrofolate reductase by 3.9-fold), by incubation with 20 μM medium MTX,

* The designations of di-, tri-, and tetraglutamates within this context refer to MTX (or DDATHF) containing one, two, and three additional glutamates, respectively. Likewise, monoglutamyl MTX (or DDATHF) refers to the unmetabolized antifolate.

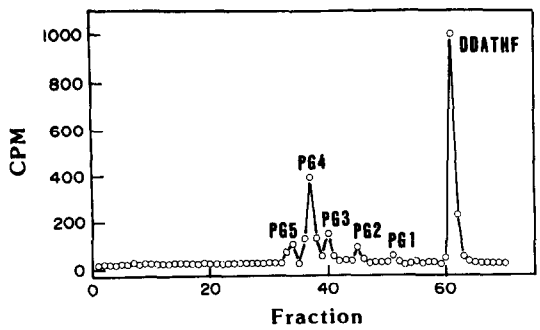

Fig. 5. HPLC analysis of DDATHF metabolites. Wild-type CCRF-CEM cells were incubated for 2 hr with 2 μM [^{14}C]DDATHF. The intracellular radiolabel was extracted and analyzed by reversed-phase HPLC as described in Materials and Methods.

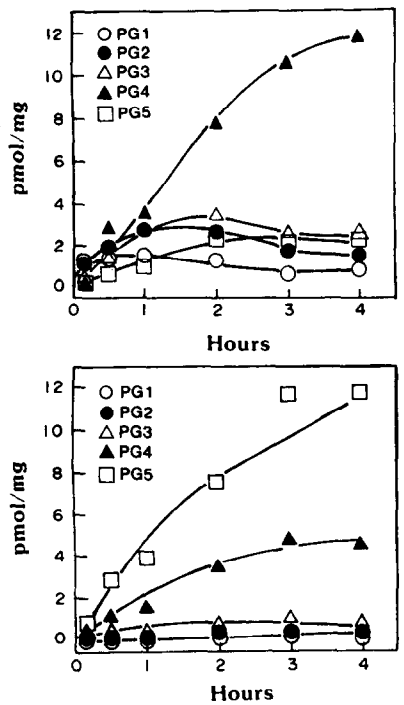

Fig. 6. Kinetics of formation of DDATHF polyglutamates. Parental CCRF-CEM (upper panel) and CEM/MTX (lower panel) cells were incubated with 2 μM [^{14}C]DDATHF. At the indicated times, the intracellular radioactive drug derivatives were quantitated as described in Materials and Methods. The polyglutamate derivatives are designated as in Fig. 5.

less than 3% (corresponding to 0.5 pmol/mg protein) was converted to MTX diglutamate (data not shown). In none of these experiments were other MTX polyglutamates detected. Similar findings of dramatically reduced MTX polyglutamylation in CEM/MTX cells have been described by Wright *et al.* [39].

Five ^{14}C-labeled metabolites (designated PG1–5 in Fig. 5) were resolved by HPLC of extracts from parental CCRF-CEM cells incubated with [^{14}C]-DDATHF. When an extract containing these derivatives was incubated with a partially purified preparation of hog kidney conjugase, PG1–5 *quantitatively* reverted to monoglutamyl DDATHF (not shown).

The conversions of [^{14}C]DDATHF to polyglutamates were extensive and nearly identical between the lines, in spite of the very large differences in initial rates of drug influx and intracellular levels of monoglutamyl substrate (Fig. 4, main graph). For parental cells, the majority of the intracellular ratiolabel at early times was unmetabolized DDATHF. This monoglutamyl DDATHF pool subsequently declined, accompanying its conversion to polyglutamates. By contrast, for CEM/MTX cells, *over* 90% of the intracellular drug was polyglutamylated within 10 min (the earliest time at which radioactive drug was detectable). After 4 hr, 53% (parent) and 71% (CEM/MTX) of the intracellular radioactivity was polyglutamyl DDATHF (Fig. 4). The net accumulations of these DDATHF metabolites at this time exceeded those for MTX by 8.4- and 237-fold, respectively.

The time dependence for the syntheses of the individual DDATHF polyglutamates is illustrated in Fig. 6. The early appearance of polyglutamyl DDATHF in parental cells (upper panel) was associated with shorter chain-length forms (PG1–3 in Fig. 5). These derivatives were detected within 10 min, peaked by 1–2 hr, and declined as PG4 and PG5 accumulated. After 4 hr, PG4 was, by far, the most abundant polyglutamate derivative, comprising 29.5% of the total intracellular radiolabel form [^{14}C]-DDATHF. Although the metabolism of DDATHF in CEM/MTX cells was considerable, as described above, the only polyglutamyl forms that accumulated to any appreciable extent were PG4 and PG5 (Fig. 6, lower panel). After 4 hr, 51% of the intracellular drug was identified as PG5.

The distributions of the individual DDATHF forms after 4 hr are compared in Table 4. These data further illustrate that, in spite of the slightly different polyglutamate chain length distributions for the two lines, the total levels of these metabolites are nearly identical. Data are also included to show the extent to which the DDATHF polyglutamates were retained in parent cells following resuspension in drug-free medium for 45 min. In this experiment, a major portion (94.52%) of the unmodified DDATHF exited the cells. Some decline in the levels of PG1 and PG2 was evident (53 and 19%, respectively); however, this seemed to be largely due to their conversions to longer chain derivatives (PG3–5) during the efflux interval. Similar results were obtained during drug efflux with CEM/MTX cells (not shown).

Measurements of FPGS catalytic activities in parental CCRF-CEM and CEM/MTX cells. The disparate polyglutamylation of MTX and DDATHF between parental CCRF-CEM and CEM/MTX cells could be caused by differences in the levels and/or substrate specificities for FPGS. Accordingly, the specific activity of FPGS was measured in cell-free extracts from each line, using aminopterin, MTX, and

Table 4. Distribution of DDATHF polyglutamates in parental and CEM/MTX cells*

Cell line	Total (pmol/mg)	DDATHF forms (pmol/mg)						Total PG1–5 (pmol/mg)
		DDATHF	PG1	PG2	PG3	PG4	PG5	
Parent	32.68	16.40	0.51	1.29	2.19	9.63	2.69	16.31
CEM/MTX	20.17	5.44	0.11	0.21	0.69	3.47	10.22	14.70
Parent†	19.41	0.90	0.24	1.04	2.86	10.88	3.47	18.49

* Cells were incubated for 4 hr with 2 μM [^{14}C]DDATHF accumulations and HPLC analysis of [^{14}C]-DDATHF drug forms were performed as described in Materials and Methods.

† Cells were incubated in drug-free medium for 45 min prior to assay of total cellular DDATHF and the individual drug derivatives.

† Cells were incubated in drug-free medium for 45 min prior to assay of total cellular DDATHF and the individual drug derivatives.

Table 5. Folylpolyglutamate synthetase specific activity in extracts from parental CCRF-CEM and CEM/MTX cell lines measured with different antifolate substrates

Substrate	Concn (μM)	FPGS specific activity (pmol/hr/mg)		Ratio*
		Parent	CEM/MTX	
Aminopterin†	100	877 ± 48	2058 ± 101	0.43
MTX‡	200	473 ± 6	1079 ± 20	0.44
DDATHF‡	2	143 ± 9	310 ± 6	0.46
DDATHF‡	10	333 ± 3	769 ± 50	0.43

* Ratio of FPGS specific activities of parent to CEM/MTX.

† Mean ± SD (N = 6).

‡ Average ± range (N = 2).

DDATHF as substrates (Table 5). Concentrations of aminopterin and MTX previously found to be saturating for parental FPGS were used [40]; for DDATHF, both a saturating concentration (10 μM) and a concentration near its reported K_m (2 μM) were tested [3]. With each substrate, extracts prepared from CEM/MTX cells showed approximately 2-fold higher FPGS activity than parental cells (Table 5). The different activities of the crude homogenates were unrelated to the presence of soluble modulators, since when extracts were mixed with purified CCRF-CEM FPGS, 85–91% of the expected additive activities were measured (data not shown). A similar increased FPGS activity in CEM/MTX cells was reported previously using (6*R*,*S*)tetrahydrofolate as the substrate [41].

DISCUSSION

Our earlier study [19] showed that impaired drug transport in MTX-resistant CCRF-CEM cells involves the synthesis of a variant form of the classical membrane carrier for MTX and related compounds. Hence, for parental CCRF-CEM cells, the glycoprotein carrier appeared on western blots (from 4–10% gels) as a 117-kDa band; in CEM/MTX cells, the carrier migrated as a 98-kDa protein [19].

Although CEM/MTX cells were highly cross-resistant (162- to 300-fold) to other antifolate substrates for the classical transporter, including aminopterin, 10-ethyl-10-deazaaminopterin, ICI D1694, and 1843U89, they were only 3.6-fold resistant to (6*R*)-DDATHF. The latter is a potent inhibitor of GAR transformylase following its polyglutamation [5] and, likewise, is reported to compete with MTX for membrane transport [2, 14, 15]. Notably, only minor differences were observed in the levels of dihydrofolate reductase, thymidylate synthase, and GAR transformylase between parental and CEM/MTX cells. Disparate sensitivities to (6*R*,*S*)-DDATHF and MTX were described previously for the same CEM/MTX line by Jansen *et al.* [16] and by Sirotnak *et al.* [15] for two drug-resistant L1210 lines with impaired MTX uptake.

One explanation for the anomalous DDATHF sensitivity of the CEM/MTX line relative to the other antifolates involves the presence of an alternative transport system which facilitates DDATHF uptake and circumvents the defective MTX transporter. Although this role has been attributed to high affinity mFBPs [16, 17], Jansen *et al.* [42] were unable to detect significant [^{3}H]folic acid surface binding to either parental CCRF-CEM or CEM/MTX cells, nor were we able to detect mFBPs by western blotting. Although the possibility remains that different mFBP isoforms [17, 43] may present unique epitopes for antibody binding in our experiments, the use of *polyclonal* antibodies should obviate this concern. Hence, an mFBP-mediated uptake process is unlikely to account for the growth inhibitory effects of DDATHF on either line.

Rather, the inhibition profiles for [^{14}C]DDATHF

influx in parental CCRF-CEM cells closely resemble those for MTX uptake [44, 45] and further establish a shared high affinity transport system for a wide assortment of folates and antifolates, including DDATHF, MTX, 5-formyl tetrahydrofolate, 10-ethyl-10-deazaaminopterin, ICI D1694, and 1843U89. Conversely, folic acid competes poorly with both MTX and DDATHF for uptake by these cells. These data strongly support the earlier conclusions of Pizzorno *et al.* [14] and Sirotnak *et al.* [15] that DDATHF is actively transported by the classical folate carrier.

Direct competition for drug uptake was also observed between these assorted (anti) folates in CEM/MTX cells; however, the K_i values, calculated from their capacities to inhibit [^{14}C]DDATHF influx, were changed substantially. Indeed, the relative affinities for DDATHF, 5-formyl tetrahydrofolate, and folic acid were all *increased* (3- to 19-fold) from those in the parent line. Conversely, carrier affinities for MTX, 10-ethyl-10-deazaaminopterin, ICI D1694, and 1843U89 were *decreased* (approx. 1.5- to 7-fold). Whereas these results presumably reflect the structural alterations previously identified in the CEM/MTX classical transporter [19], the possibility that a separate uptake mechanism for certain of these compounds may exist cannot be discounted entirely. In any case, influx for both MTX and DDATHF was impaired severely.

It is the level of sustained synthesis of antifolate polyglutamates which most distinguishes MTX from DDATHF and likely contributes to the large disparity in their growth inhibitory effects on CEM/MTX cells. With MTX, the high K_m for binding to FPGS [7, 40, 46] limits its conversion to polyglutamates, even in cells with normal membrane transport, unless considerable unbound intracellular MTX is available. Not surprisingly, this effect becomes more acute for tumor cells characterized by impaired MTX influx and the generation of little or no free MTX substrate in excess of that bound to DHFR. Although CEM/MTX FPGS catalytic activity in cell-free homogenates was increased approximately 2-fold over the wild type with several antifolate substrates including MTX, MTX metabolism *in situ* remained exceedingly low at extracellular drug concentrations (i.e. 20 μM) sufficient to produce high levels of unbound intracellular monoglutamyl MTX. As previously suggested [47], an assortment of elements not detected under standard assay conditions (i.e. altered affinities for MgATP, glutamate, or mono- and polyglutamate substrates) can profoundly influence polyglutamate synthesis for poor FPGS substrates and may contribute to the low levels of MTX metabolism in intact CEM/MTX cells.

In contrast to MTX, the excellent substrate properties of DDATHF for FPGS [3, 7] facilitate its extensive polyglutamylation in CEM/MTX cells, even though intracellular monoglutamyl antifolate is present in extremely low levels. An apparent consequence of the lower levels of competing monoglutamyl substrate is the accumulation of longer chain length DDATHF polyglutamates in CEM/MTX than in parental cells [24, 47]. Total polyglutamate accumulations in the two lines were nearly identical after a 4-hr treatment with 2 μM DDATHF, offering a metabolic basis for their similar growth inhibitory sensitivities. The pharmacologic significance of DDATHF polyglutamate synthesis within this context involves the conversion of monoglutamyl antifolate to a highly retained form [8] capable of exerting up to 100-fold greater inhibition at GAR transformylase than the underivatized drug [5].

In light of these findings, the preservation of even a small difference in DDATHF sensitivities between the lines suggests that (i) intracellular events at 2 μM DDATHF may not *entirely* reflect those occurring at lower drug concentrations, and/or (ii) other elements unrelated to the levels of cellular GAR transformylase (i.e. differences in rates of monoglutamyl DDATHF efflux, intracellular folate pools, and/or inhibitor affinities for GAR transformylase) may also contribute to DDATHF pharmacologic activity in our growth inhibition assays. Although a recent report described identical rates of efflux for DDATHF and MTX in wild-type CCRF-CEM cells [14], in the present study, the low radiospecific activity of the [^{14}C]DDATHF preparation and the disproportionately high levels of DDATHF polyglutamates formed precluded direct assay of DDATHF first order efflux in the CEM/MTX subline.

In summary, our results offer a metabolic explanation for the divergent antitumor activities of DDATHF and MTX toward MTX-resistant tumor cells, characterized by a severe defect in drug transport. These findings are novel in that they suggest that if an antifolate is a sufficiently good substrate for FPGS, it can effectively circumvent transport-mediated resistance, even if its own uptake is quite impaired. An analogous effect to that seen with DDATHF might be expected for other antifolates that are polyglutamylated to high levels, and for other transport-impaired cells with adequate levels of drug uptake to sustain polyglutamate synthesis, and for which FPGS catalytic acitivity is largely intact.

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