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Marked suppression of the activity of some, but not all, antifolate compounds by augmentation of folate cofactor pools within tumor cells

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Abstract

Folates have been co-administered with some antifolates to diminish host toxicity; however, the extent to which this will reduce antitumor activity is not known. To further clarify this issue, studies were undertaken to characterize and quantitate the impact of alterations in intracellular folate levels on the activities of a variety of antifolates in L1210 murine leukemia cells. Intracellular folate cofactor levels increased almost in proportion to the increase in extracellular 5-formyltetrahydrofolate (5-CHO-THF) over a concentration range that encompassed physiological levels of 5-methyltetrahydrofolate. This resulted in a spectrum of increases in the ic_{50} values of antifolates upon continuous exposure to drugs [Lometrexol (DDATHF) (70x) > trimetrexate (TMQ) (30x), multitargeted antifolate, LY231514 (ALIMTA) (30x) > Raltitrexed, Tomudex (ZD1694) (10x), 6*R*-2′,5′-thienyl-5,10-dideazatetrahydrofolic acid (LY309887) (10x) > methotrexate (MTX) (6x) > (2*S*)-2-{*o*-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]benzamido}-4-(tetrazol-5-yl) butyric acid (ZD9331) (3x), N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-l-ornithine (PT523) (3x)]. Upon a 4-hr pulse exposure to drug, the ic_{50} values for DDATHF and ALIMTA were increased > 180- and 5-fold, respectively, with only a 2.5-fold increase in the extracellular 5-CHO-THF level within the physiological range. The reductions in drug sensitivities could be attributed to decreases in accumulation of polyglutamate derivatives of ALIMTA and DDATHF. Hence, in these studies, natural folates diminished the activity of agents that undergo polyglutamation by suppression of the formation of these active congeners at the level of folylpolyglutamate synthetase. For inhibitors of dihydrofolate reductase, the suppressive effect of endogenous folates appears to be due to competition between the antifolate and dihydrofolate at the level of the target enzyme. These data should be carefully considered in the design of regimens with antifolates, which incorporate co-administration of folates.

Keywords: Antifolate; Folate cofactors; Drug resistance; Polyglutamation

1. Introduction

Antifolates are competitive inhibitors of their target folate-requiring enzymes within cells. For the classical antifolates, MTX and aminopterin, dihydrofolate that builds up behind the block in DHFR is the basis for the competitive nature of the interaction among these agents, dihydrofolate, and their target enzyme [1]. For new generation antifolate inhibitors of thymidylate synthase and/or GAR transformylase that require formation of polyglutamate derivatives for activity, such as DDATHF, ALIMTA, and ZD1694 [2–4], the level of endogenous folate pools should modulate the rate and extent of formation of these congeners synthesized by FPGS within cells. Endogenous folate pools also have the potential for diminishing the activity of these agents due to competition at the level of their target enzymes. Low levels of endogenous folates in normal tissues are a determinant of the toxicity of antifolate inhibitors of GAR transformylase, and repleting these pools with added folate in animal models circumvents toxicity and may enhance therapeutic efficacy [5,6]. Conversely, enhanced accumulation of folate pools within cells that occurs by perturbations of folate transporters can result in markedly diminished antifolate activity [7,8]. Studies on drug resistance *in vitro*

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Abbreviations: ALIMTA, multitargeted antifolate, LY231514; 5-CH_3-THF, 5-methyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; DDATHF, Lometrexol, (6*R*)-5,10-dideazatetrahydrofolate; DHFR, dihydrofolate reductase; FPGS, folylpoly-γ-glutamate synthetase; GAR, glycinamide ribonucleotide; HBS, HEPES-buffered saline; MTX, methotrexate; RFC1, the reduced folate carrier; TMQ, trimetrexate; and ZD1694, Raltitrexed, Tomudex.

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usually utilize media in which the folate growth substrate is folic acid. This folate has a low affinity for the major folate transporter, RFC1, and enters cells largely by other mechanisms [9]. Recent studies utilizing 5-CHO-THF as the growth substrate demonstrated that ALIMTA or DDATHF activities can be preserved in cell lines resistant to MTX due to impaired RFC1-mediated transport [10–12]. This was due to selective, partial preservation of transport of these antifolates along with the contraction of the endogenous folate pool that accompanies a decrease in 5-CHO-THF transport [12,13].

This paper represents the first quantitative analysis of the relationship between levels of cellular folate cofactors and the activities of antifolates, using L1210 murine leukemia cells grown in 5-CHO-THF concentrations that include, and bracket, the physiological blood levels of 5-CH_3-THF. These data were then correlated with growth inhibitory activities upon continuous, or brief, exposure to antifolate agents with different enzyme targets, some of which do, while others do not, form polyglutamate derivatives. The data characterize the very critical role that the cellular folate pool can play as a determinant of antifolate activities, findings relevant to the design of clinical regimens in which the co-administration of folate supplements is considered to prevent toxicity.

2. Materials and methods

2.1. Chemicals

[3′, 5′, 7-^{3}H]-(6*S*)-5-CHO-THF was obtained from Moravek Biochemicals, and [3′, 5′, 7-^{3}H]-MTX was purchased from the Amersham Corp. [^{3}H]-ALIMTA (4.1 Ci/mmol), [^{3}H]-DDATHF (3.3 Ci/mmol), ALIMTA, DDATHF, and 6*R*-2′,5′-thienyl-5,10-dideazatetrahydrofolic acid (LY309887) were provided by Dr. Victor Chen (Eli Lilly). TMQ was a gift from Dr. David Fry (Warner-Lambert). ZD1694 and (2*S*)-2-{*o*-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-ylmethyl)-*N*-(prop-2-ynyl)amino]benzamido}-4-(tetrazol-5-yl) butyric acid (ZD9331) were obtained from Dr. Ann L. Jackman (ICI), and N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-l-ornithine (PT523) was a gift from Dr. Andre Rosowsky (Dana-Farber Cancer Institute). Tritiated chemicals were purified by high performance liquid chromatography before use.

2.2. Cell culture conditions

L1210 leukemia cells were maintained in complete RPMI-1640 medium containing 2.3 μM folic acid, 5% bovine calf serum (HyClone), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37° in a humidified atmosphere of 5% CO_2. In preparation for most studies, L1210 cells were transferred into, and grown for 1–3 weeks in, folate-free RPMI medium (HyClone) containing 5% dialyzed bovine calf serum (Life Technologies), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μg/mL) supplemented with different concentrations of 5-CHO-THF.

2.3. Growth inhibition

To assess growth upon continuous exposure to drugs, L1210 cells were conditioned by growth in medium containing different concentrations of 5-CHO-THF in 96-well plates (1×10^5 cells/mL) and then exposed to appropriate concentrations of the antifolates. After 72 hr, cells were counted using a hemocytometer, and viability was assessed by trypan blue exclusion. For brief exposures, 1 mL of cell suspensions (0.5×10^6 cells) in 10 or 25 nM 5-CHO-THF was incubated with different concentrations of antifolates at 37° for 4 hr. Cells were then pelleted by centrifugation, washed twice with drug-free medium, and grown in 96-well plates in this medium for 72 hr before cell numbers were determined.

2.4. Measurements of folate pools and accumulation of ALIMTA, DDATHF, and MTX

Cells (3×10^6) grown in complete RPMI-1640 were washed twice with folate-free RPMI and resuspended into the same medium supplemented with 0.64, 1.4, 4, 10, 25, or 62.5 nM [^{3}H]-5-CHO-THF (200 dpm/pmol). After 1 week at exponential growth, cells were harvested, washed twice with ice-cold HBS, and processed for intracellular tritium as described for transport studies (described below). For measurement of accumulation of ALIMTA, DDATHF, and MTX, cells grown in different concentrations of 5-CHO-THF were incubated with 50 nM [^{3}H]-ALIMTA, [^{3}H]-DDATHF, or [^{3}H]-MTX (~200 dpm/pmol), in the presence of 200 μM glycine, 100 μM adenosine, and 10 μM thymidine to circumvent the inhibitory effects of these agents. Cells were harvested after 3 days of exponential growth, washed twice with ice-cold HBS, and processed for determination of intracellular tritium [14].

2.5. HPLC analysis of DDATHF polyglutamates

L1210 cells grown in different concentrations of 5-CHO-THF were exposed to 50 nM [^{3}H]-DDATHF for 3 days in the presence of 200 μM glycine, 100 μM adenosine, and 10 μM thymidine; then the cells were harvested and washed three times with 0° HBS. One portion of the cell pellet was processed for dry weight and total tritum as described below. Another portion was processed according to a reported protocol [15]. Briefly, cell pellets were suspended in 50 mM phosphate buffer (pH 6.0) containing 100 mM 2-mercaptoethanol and boiled for 5 min. The precipitate was removed by centrifugation, and the supernatant containing radiolabeled DDATHF and its metabolites was separated on a

reversed-phase high performance liquid chromatography column (Waters Spherisorb, 5 μm ODS2 4.6 $\times$ 250 mm). Separation of the different polyglutamate derivatives was achieved by elution with 0.1 M sodium acetate (pH 5.5) for 5 min followed by two linear gradients of from 0–30 and 30–50% acetonitrile in 0.1 M sodium acetate over 35 and 20 min, respectively, and then 100% acetonitrile for 10 min. The flow rate was 1 mL/min, and 0.5-mL fractions were collected. Radioactive peaks of DDATHF and its polyglutamate derivatives were assigned by comparing their elution times with those of the non-radioactive standards up to the pentaglutamate. Standards for higher polyglutamates were not available, but three peaks that eluted before the pentaglutamate were detected and designated as the hexa-, hepta-, and octaglutamates.

2.6. Transport studies

Cells were harvested, washed twice with HBS (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4), and resuspended in HBS to 1.5 $\times$ 10^7 cells/mL. Cell suspensions were incubated at 37° for 20 min, following which uptake was initiated by the addition of [^{3}H]-MTX or [^{3}H]-5-CHO-THF, and samples were taken at the indicated times over which uptake was shown to be unidirectional. Uptake was terminated by injection of 1 mL of the cell suspension into 10 mL of ice-cold HBS. Cells were collected by centrifugation, washed twice with ice-cold HBS, dried, and digested with 1 N NaOH in 8-mL vials. Radioactivity was assessed in a liquid scintillation spectrometer.

3. Results

3.1. Relationship between the intracellular folate cofactor pool and the extracellular 5-CHO-THF level

L1210 murine leukemia cells were grown with 5-CHO-THF as the folate growth source over extracellular concentrations of from 0.64 to 62.5 nM, a range that encompasses from approximately one-tenth to two times the physiological levels of 5-CH_3-THF in humans [16]. As indicated in Fig. 1, the intracellular folate pool increased in near proportion to the increase in the extracellular folate level. The ratios of the intracellular to extracellular folate concentrations were very high: from 570:1 to 270:1 at extracellular 5-CHO-THF levels of 0.64 and 62.5 nM, respectively. This large gradient is due to the formation of reduced folate polyglutamates, which are retained within the cells [17].

3.2. Relationship between folate levels and growth inhibition by antifolates

Antifolates with different target enzymes and biochemical properties were chosen for study. Three are inhibitors of

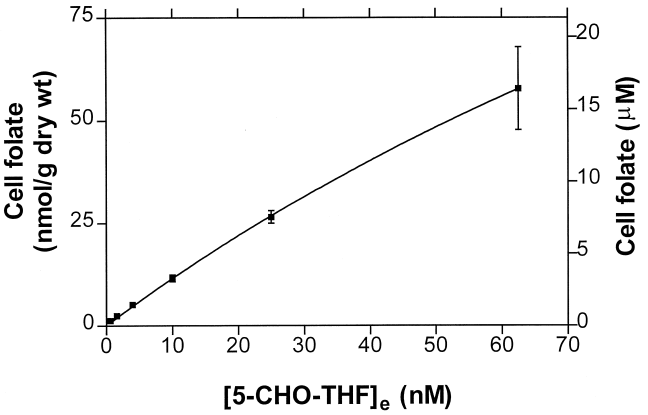

Fig. 1. Accumulation of intracellular folate cofactors as a function of the extracellular 5-CHO-THF concentration in the growth medium. L1210 cells (3×10^6) grown in complete RPMI-1640 medium were washed twice with folate-free RPMI-1640 and grown exponentially for 1 week in folate-free medium supplemented with different concentrations of 5-CHO-THF. The cells were then processed for intracellular folate, as described in "Materials and methods." Intracellular folate concentration in micromoles per liter of cell water was computed based on a ratio of intracellular water to dry weight of 3.5 in L1210 cells [14]. Data are the means ± SEM from three separate experiments.

DHFR (Table 1). MTX is a classic tight-binding inhibitor of this enzyme that forms polyglutamate derivatives in cells. TMQ is a lipid soluble, weaker inhibitor (K_i 10-fold that of MTX) of DHFR with a structure that precludes polyglutamate formation [19]. PT523 has an affinity for DHFR an order of magnitude greater than MTX with a γ-glutamate moiety that is blocked, also precluding polyglutamylation [21]. DDATHF and LY309887 target GAR transformylase; for both, cellular retention requires formation of polyglutamate derivatives [24–26]. Unlike DDATHF monoglutamate, LY309887 monoglutamate is a very potent inhibitor of the target enzyme [22]. ZD1694 and ZD9331 are inhibitors of thymidylate synthase [4,23]. The former requires polyglutamylation for tight binding to the target enzyme; the latter does not form polyglutamates and has a K_i for thymidylate synthase that is comparable to that of ZD1694 tetraglutamate [4,23]. ALIMTA is a multi-targeted agent that requires polyglutamylation for activity. In that form it is a very potent inhibitor of thymidylate synthase, a weaker inhibitor of GAR transformylase and in both its mono- and polyglutamyl forms an even weaker inhibitor of DHFR [3]. Except for TMQ, all these agents have high affinity for, and are transported by, RFC1 [23,27]. TMQ enters cells largely by passive diffusion.

The IC_{50} for growth inhibition by these agents was measured with a 3-day continuous exposure to the drugs, which followed at least 7 days over which cells were equilibrated with their respective extracellular level of 5-CHO-THF. At the lowest extracellular 5-CHO-THF level of 0.64 nM, the IC_{50} values for all drugs were less than 10 nM. The highest IC_{50} value was 8.3 nM for ZD9331, and the lowest value was 0.65 nM for ZD1694 (last column of Table 1). The upper

Table 1
Antifolate FPGS K_m or K_i for target enzymes

Antifolates	Target enzyme (K_i)	Murine liver FPGS (K_m)[a]	IC_{50}[b] (nM)
MTX	Human DHFR Mono- (4.1 pM)[c] Penta- (4 pM)[d]	166 μM	3.3 ± 0.4
TMQ	Human DHFR (43 pM)[c]		0.90 ± 0.06
PT523	rh DHFR (0.35 pM)[e]		1.2 ± 0.3
DDATHF	rm GAR transformylase Mono- (5.6 nM)[f] Hexa- (0.12 nM)[f]	9.3 μM	2.7 ± 0.6
LY309887	rm GAR transformylase Mono- (0.6 nM)[f]	6.0 μM (Hog liver)	4.2 ± 0.1
ZD1694	rm Thymidylate synthase Mono- (62 nM) Tetra- (1.0 nM)[g]	1.37 μM	0.65 ± 0.09
ZD9331	rm Thymidylate synthase (0.4 nM)[h]		8.3 ± 1.0
ALIMTA	rh Thymidylate synthase Mono- (109 nM), Penta- (1.3 nM)[i] rm GAR transformuylase Mono- (9300 nM), Penta- (65 nM)[i] rh DHFR Mono- (7.0 nM), Penta- (7.2 nM)[i]	0.80 μM	1.3 ± 0.3

rh is recombinant human, and rm, recombinant murine enzymes.
[a] [18].
[b] IC_{50} of the indicated antifolates under conditions of continuous exposure to 0.64 nM 5-CHO-THF. Values are means ± SEM of three separate experiments.
[c] [19].
[d] [20].
[e] [21].
[f] [22].
[g] [4].
[h] [23].
[i] [3].

panel of Fig. 2 indicates the ratio of the IC_{50} values obtained when cells were grown in the presence of higher 5-CHO-THF concentrations in the medium to the IC_{50} values at the lowest 5-CHO-THF level. It can be seen that there were relatively modest increases in the IC_{50} as the 5-CHO-THF concentration approached the physiological range. However, within the physiological range there were much greater increases, with considerable divergence, in the magnitude of these changes among the different antifolates. For instance, there was a 70-fold decrease in growth inhibition by DDATHF over the entire folate concentration range studied; this was the antifolate most sensitive to folate pool size. Next were TMQ and ALIMTA with a 30-fold decrease in growth inhibition; there was a 10-fold decrease in sensitivity to ZD1694 and LY309887 over this 5-CHO-THF concentration range. The IC_{50} for MTX was increased by a factor of 6. ZD9331 and PT523 were the least sensitive to changes in the folate level, with the IC_{50} increasing by factors of only 3 and 2, respectively (Fig. 2, lower panel). The inhibitory effect of 5-CHO-THF on the activity of DDATHF, MTX, and ZD1694 was less than reported previously [4,28], but in those studies a 5-CHO-THF concentration range (0.1 to 25 μM) was used that was one to three orders of magnitude higher than the physiological concentration of 5-CH_3-THF in the blood and much higher than the levels used in the present study.

To simulate the impact of cellular folate pools on drug activities under conditions in which exposure to the drugs was transient, as with an i.v. bolus or brief infusion, growth inhibition was determined after cells were exposed to drugs for 4 hr and then isolated and grown in drug-free medium. Growth inhibition was assessed at 10 and 25 nM 5-CHO-THF, concentrations within the physiological range of 5-CH_3-THF blood levels. With this brief exposure, IC_{50} values were substantially higher than those obtained with continuous exposure to the drugs (Table 2). At 10 nM 5-CHO-THF, the IC_{50} for MTX was increased by 390-fold, whereas IC_{50} values for LY309887, ALIMTA, DDATHF, and ZD1694 were ~8-, 14-, 35-, and 37-fold greater, respectively, in comparison to the values obtained with continuous exposure to the drugs (last column, Table 2). The impact of the increase in the intracellular folate pools under these conditions was also very different. There was a marked (>180-fold) increase in the IC_{50} for DDATHF in cells grown with 25 vs 10 nM 5-CHO-THF after a 4-hr exposure to the drug. There was an ~5-fold increase for ALIMTA, an ~3.5- and 2.5-fold increase for LY309887 and MTX, respectively, and only a 1.9-fold change for

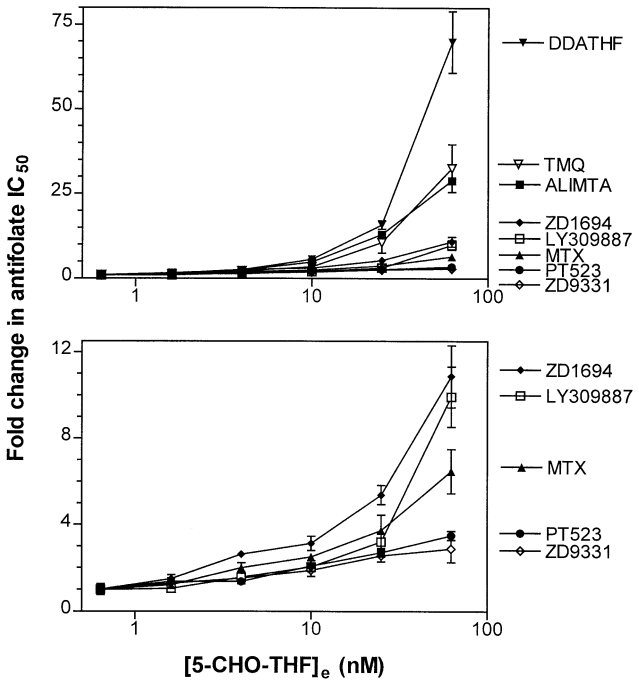

Fig. 2. Dependence of antifolate growth inhibition upon extracellular 5-CHO-THF with continuous exposure to drugs. L1210 cells grown in 96-well plates in medium containing different concentrations of 5-CHO-THF were exposed continuously to a range of drug concentrations for 3 days. Fold change in IC_{50} on the ordinate is the ratio of IC_{50} values at the indicated extracellular 5-CHO-THF level to the IC_{50} values at the lowest 5-CHO-THF concentration (0.64 nM) listed in Table 1. The bottom panel amplifies the data for the most potent inhibitors. Data are the means ± SEM from three separate experiments.

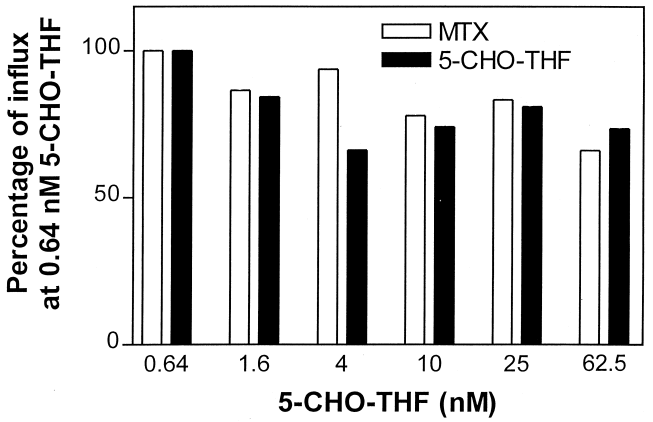

Fig. 3. Effects of folate levels on folate influx. L1210 cells grown in different concentrations of 5-CHO-THF were harvested, washed, resuspended in HBS, and incubated in the same buffer for 25 min. Transport was initiated by the addition of [^{3}H]-MTX or [^{3}H]-5-CHO-THF at a concentration of 1 μM and continued over an interval during which uptake was unidirectional. Data are the means from two separate measurements.

ZD1694 (next to last column, Table 2). Thus, agents that require polyglutamation, with the lowest sensitivity to alterations in the folate cofactor pools in cells upon continuous exposure, generally maintained that low sensitivity to folate pool size changes after a brief exposure to drugs. Agents that did not form polyglutamyl derivatives were not active after a 4-hr exposure to drug due presumably to their rapid efflux when cells were reconstituted in drug-free medium (data not shown).

3.3. Effect of differences in folate levels on folate influx in L1210 cells

Influx of either MTX or 5-CHO-THF (Fig. 3) was measured in cells grown for at least 1 week in medium containing from 0.64 to 62.5 nM 5-CHO-THF. These were the same conditions in which growth inhibition and other parameters were measured. There was a small decrease in folate influx that reached maximum suppression (~30%) at the highest 5-CHO-THF concentration. However, most of the influx decrease occurred over 5-CHO-THF concentrations of 0.64 to 4 nM. These small differences in transport could not explain the much greater decline in growth inhibition observed with the various antifolates.

Table 2
Comparison of antifolate growth inhibition, IC_{50} (nM), under conditions of continuous or transient exposure to drugs

Antifolate	IC_{50} (nM) Continuous exposure			IC_{50} (nM) Transient exposure			C/A
	10 nM 5-CHO-THF (A)	25 nM 5-CHO-THF (B)	B/A	10 nM 5-CHO-THF (C)	25 nM 5-CHO-THF (D)	D/C	
MTX	8.2 ± 0.2	12 ± 2	1.5	3200 ± 500	7900 ± 700	2.5	390
DDATHF	16 ± 2	43 ± 3	2.7	560[a]	> 100,000[a]	> 180	35
LY309887	8.5 ± 0.3	13 ± 2	1.5	65 ± 5	240 ± 30	3.7	8
ZD1694	2.0 ± 0.2	3.5 ± 0.3	1.8	74 ± 23	140 ± 31	1.9	37
ALIMTA	6.5 ± 0.5	17 ± 1	2.6	92 ± 13	500 ± 130	5.4	14

The IC_{50} values under continuous exposure conditions were obtained from Fig. 2 for the purpose of comparison. For transient exposure, cells grown in 10 or 25 nM 5-CHO-THF were incubated with a spectrum of different concentrations of each drug for 4 hr at 37°. Cells were washed twice with folate-free medium then resuspended in the same medium supplemented with 10 or 25 nM 5-CHO-THF, and allowed to grow for 3 days. Cell numbers were determined by homocytometer count. Data are the means ± SEM of at least three separate experiments.

[a] Four separate experiments were collated into a single graph, and an IC_{50} on the composite was determined.

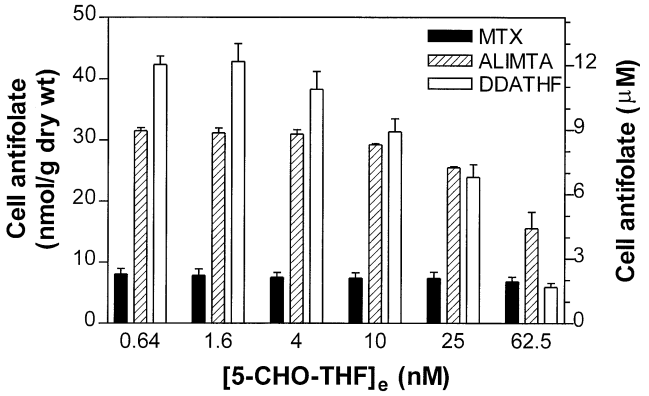

Fig. 4. Impact of folate levels on cellular accumulation of antifolates. L1210 cells grown in different concentrations of 5-CHO-THF were exposed to 50 nM [^{3}H]-MTX, [^{3}H]-ALIMTA, or [^{3}H]-DDATHF in the presence of 200 μM glycine, 100 μM adenosine, and 10 μM thymidine. After 3 days, cells were processed for determination of intracellular tritium. The antifolate concentration in micromoles per liter of cell water was calculated as described in the legend of Fig. 1. Data are the means ± SEM from three separate experiments.

3.4. Impact of folate levels on polyglutamation of antifolates

Cells were exposed continuously to 50 nM tritiated MTX, ALIMTA, or DDATHF for 3 days, following which total intracellular drug levels were measured. Total accumulation of DDATHF and ALIMTA reached 12 and 9 μM, representing a concentration difference of 240 and 180, respectively, in comparison to the extracellular drug level when extracellular 5-CHO-THF was 1.6 nM, consistent with the high FPGS substrate activity for these agents (Fig. 4). The intracellular concentration of MTX, a weaker substrate for FPGS, was considerably lower, 2 μM, of which about half was bound to DHFR. There was a marked decrease in DDATHF polyglutamate accumulation (~85%) when the 5-CHO-THF concentration was increased from 0.64 to 62.5 nM. The decrease in ALIMTA (~50%) was much greater than that observed for MTX (15%). For both DDATHF and ALIMTA, the decline in polyglutamation occurred primarily over an extracellular concentration range of 4 to 62.5 nM 5-CHO-THF, mirroring the marked increase in the IC_{50} values for these agents.

To assess the distribution of antifolate polyglutamates under these conditions, DDATHF and its congeners were extracted and analyzed by HPLC after cells were grown in 0.64, 4, or 62.5 nM 5-CHO-THF and then exposed to 50 nM [^{3}H]-DDATHF for 3 days. There was no detectable di-, tri- or tetraglutamates regardless of the concentration of 5-CHO-THF in the growth medium. Only penta- or higher polyglutamates were identified. There was no significant difference in the distribution of polyglutamates in cells grown in 0.64 or 4 nM 5-CHO-THF (Table 3). The heptaglutamate (~50%) was the major metabolite along with the hexa- (~20%) and octaglutamates (~25%). However, when cells were grown in 62.5 nM 5-CHO-THF, total antifolate accumulation decreased by a factor of ~7, and the major products were lower polyglutamate congeners. The hexaglutamate comprised ~54%, the pentaglutamate ~19%, and the heptaglutamate ~13% of total folate; negligible octaglutamate was detected, and a larger fraction of the total folate (11%) was monoglutamate. Thus, in addition to the substantial decrease in total DDATHF polyglutamates in cells when the 5-CHO-THF concentration was increased from 4 to 62.5 nM, there was an accompanying decrease in polyglutamate chain length.

4. Discussion

This report quantitates the impact that the level of endogenous folate cofactors can have in modulating the activities of antifolate drugs, in particular agents that in their polyglutamyl forms are direct inhibitors of GAR transformylase and/or thymidylate synthase. Increased cellular folate pools were associated with decreased levels of antifolate polyglutamates and diminished drug activities even when the folate pool was modulated within the physiological range. This effect was even more striking when exposure to drug was brief, as usually occurs in the clinical setting.

DDATHF was the agent most sensitive to the cellular folate cofactor pool size. In fact, at endogenous folate levels achieved when cells were grown in 25 nM 5-CHO-THF, an IC_{50} for this agent could not be reached after a 4-hr exposure to 100 μM DDATHF. Even when exposure to the drug was

Table 3
Dependence of DDATHF polyglutamate chain length on the extracellular 5-CHO-THF concentration in the growth medium

$[5\text{-CHO-THF}]_e$ (nM)	Total accumulation (nmol/g dry wt)	Monoglutamate (DDATHF) (%)	Pentaglutamate (%)	Hexaglutamate (%)	Heptaglutamate (%)	Octaglutamate (%)
0.64	38 ± 1	0.95 ± 0.25	2.9 ± 0.7	20 ± 2	51 ± 3	25 ± 5
4	31 ± 1	0.78 ± 0.01	2.0 ± 0.2	20 ± 1	48 ± 2	28 ± 2
62.5	5.2 ± 0.1	11 ± 0	19 ± 1	54 ± 7	13 ± 4	3.7 ± 2.7

L1210 cells were grown in folate-free medium supplemented with 0.64, 4, or 62.5 nM 5-CHO-THF and then exposed to 50 nM [^{3}H]-DDATHF for 3 days in the presence of 200 μM adenosine, and 10 μM thymidine. Cells were harvested and processed for determination of dry weight and HPLC analysis as described in section 2. All the intracellular radiolabel within cells after a 3-day exposure to radiolabeled antifolates can be accounted for on the basis of antifolate mono- and polyglutamates. Penta-, hexa-, hepta-, and octaglutamates of DDATHF have 4, 5, 6, and 7 additional glutamate moieties on the antifolate molecule, respectively. Data are the means ± SEM from three separate experiments performed on different days.

continuous, the IC_{50} increased by a factor of > 70 over the 5-CHO-THF range studied. This is likely due to the relatively high K_m for FPGS and a 10-fold difference in K_i for GAR transformylase between the mono- and hexaglutamate forms of this agent. Consistent with this was the lesser effect of endogenous folates on the activity of LY309887, which has a lower K_m for FPGS and a monoglutamate with a much lower K_i for GAR transformylase relative to DDATHF. There was a lesser, but nonetheless substantial impact on ALIMTA activity. While ALIMTA has a high affinity for FPGS, there is a two-order difference in affinity for thymidylate synthase and GAR transformylase between the mono- and pentaglutamyl forms, making the efficient formation of polyglutamyl derivatives particularly critical [3]. On the other hand, ZD1694 activity was less affected by the cellular folate level despite an FPGS $K_{m,}$ and mono- and polyglutamyl inhibitory activities similar to those of ALIMTA [4]. The basis for this difference is unclear but might be associated with the impact of the parallel inhibition of GAR transformylase by ALIMTA polyglutamates that is diminished as cellular folate pools are increased. Folate pool size had a negligible effect on ZD9331 activity. This agent does not form polyglutamyl derivatives, and its K_i for thymidylate synthase is only modestly lower, one-half and one-third, than those of ZD1694 tetraglutamate and ALIMTA pentaglutamate, respectively [23]. This observation suggests that the impact of the folate pool size within cells may be related largely to the inhibitory effect on the formation of antifolate polyglutamates by FPGS with a much lesser effect due to competition by 5,10-methylenetetrahydrofolate at the level of thymidylate synthase. Further studies would be required to determine if this might hold true for other inhibitors of thymidylate synthase and GAR transformylase that do not form polyglutamyl derivatives.

Beyond changes in total antifolate, there was a decrease in the DDATHF polyglutamate chain length at the highest level of 5-CHO-THF studied. This is consistent with the observation that high extracellular concentrations of folates decrease the chain length of natural folate polyglutamates [29]. The consequence of this alteration is uncertain. While the K_i for DDATHF polyglutamates is known to decrease as the chain length is increased up to the pentaglutamate [25], the significance of further lengthening of the molecule is unclear. The hexaglutamate, which has a very high affinity for GAR transformylase [22], was the major DDATHF metabolite at the highest 5-CHO-THF level employed. However, further studies are required to assess the inhibitory effects of the higher DDATHF polyglutamates and whether this or greater shifts in chain length are observed for other antifolates as well.

When exposure to the drugs was continuous, the effect of intracellular folate pool size on the activities of the inhibitors of DHFR appears to depend upon alterations in the interaction between the drugs and endogenous folate, in this case dihydrofolate, at the level of the target enzyme [30]. This is consistent with what is known about the mechanism of action of these agents. When DHFR is inhibited, there is an interconversion of tetrahydrofolate cofactors to 5,10-methylenetetrahydrofolate, which is then oxidized to dihydrofolate. Dihydrofolate, in turn, competes with antifolate for the very small amount ($<5\%$) of DHFR activity that is sufficient to sustain required levels of tetrahydrofolate cofactors within rapidly proliferating cells [30,31]. Further, net displacement by dihydrofolate of a small portion of MTX bound to DHFR by high levels of dihydrofolate that build up in cells results in rapid resumption of DHFR activity (within a few minutes) when free MTX exits the cells [1,32]. These are the factors that make polyglutamation of the drug so critical to its pharmacologic activity; the build-up of congeners results in high, sustained concentrations of inhibitor at the target enzyme since these derivatives do not efflux from the cells. PT523 is negligibly affected by the folate pool size; this is an agent that does not form polyglutamate derivatives, but its 10-fold higher affinity for DHFR than MTX [21] apparently effectively blunts the impact of high endogenous folates. On the other hand, TMQ, which is a much weaker inhibitor of DHFR than MTX, and also does not form polyglutamates, is highly susceptible to increases in the cellular folate level. This is attributed to more effective competition by endogenous dihydrofolate with TMQ at the level of DHFR.

Alterations in the level of folate pools have been associated with acquired resistance to antifolates. Resistance to DDATHF in an L1210 leukemia cell line maintained in medium in which the folate growth source was folic acid was shown to be due to two mutations in the reduced folate carrier, each of which markedly enhances carrier affinity for folic acid, resulting in enhanced accumulation of folate cofactors [7,33]. Impaired efflux of folic acid due to loss of energy-dependent exporters (likely members of the MRP family) led to the build-up of endogenous folates, resulting in resistance to DHFR inhibitors as well as agents that require polyglutamation such as DDATHF [8,34]. Finally, recent studies demonstrated that in some cell lines with acquired resistance to MTX, due to diminished transport via RFC1, sensitivity to DDATHF and ALIMTA could be preserved when cells were grown in 5-CHO-THF [12,13]. This was due to partial, preferential preservation of transport of the antifolates relative to MTX along with a concurrent, although lesser, decrease in transport of 5-CHO-THF. The latter resulted in contraction of the cellular folate cofactor pool that, while sustaining tumor cell growth, resulted in enhanced polyglutamation of the antifolate inhibitors with only a minimal decrease in the levels of these agents that accumulate in the MTX-resistant cells.

Cumulative toxicity has been a particularly difficult clinical problem with DDATHF [35]. Studies in animals indicate that the level of cellular folates in normal tissues is a critical determinant of host toxicity with this agent [36, 37]. This toxicity can be diminished by folate supplementation and, at least in some cases, this appears to be achieved while preserving or enhancing antifolate effects against tumor

cells [5,6]. This has led to the co-administration of folic acid or 5-CHO-THF in clinical trials with DDATHF in an attempt to assure adequate folate stores in susceptible host tissues [38–41]. However, while the prevention of host toxicity is readily demonstrated in clinical studies, preservation of antitumor activity is much more difficult to establish at the phase II level, and so the impact of folate supplementation on drug efficacy remains unclear. The current study indicates that the build-up of excessive folate levels in tumors by co-administration of folates in clinical regimens, or the self-administration of macrobiotics, will likely diminish antitumor activity for some antifolates. The relatively small effects of cellular folate levels on the activity of ZD9331 is a clear advantage for this agent in terms of the identification of dose levels that should be more reproducible and, therefore, safer among a patient population with variable endogenous folate stores.

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