

Sensitivity to 5,10-Dideazatetrahydrofolate Is Fully Conserved in a Murine Leukemia Cell Line Highly Resistant to Methotrexate Due to Impaired Transport Mediated by the Reduced Folate Carrier¹

Rongbao Zhao, Feng Gao, Solomon Babani, and I. David Goldman²

Departments of Medicine and Molecular Pharmacology, and the Albert Einstein Comprehensive Cancer Center, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT

A murine leukemia cell line was identified that is highly resistant to methotrexate (MTX), due to impaired transport, but fully sensitive to 5,10-dideazatetrahydrofolate (DDATHF). A valine-to-methionine substitution at amino acid 104 in the reduced folate carrier (RFC1) explains this disparity in drug resistance. Transfection of the V104M cDNA into an RFC1-deficient cell line markedly increased DDATHF influx (32×) but only modestly increased influx of MTX and 5-formyltetrahydrofolate (4- and 6-fold, respectively). The growth inhibition or growth requirements for these folates fell by factors of 18, 2, and 4, respectively, in the transfectant. Preservation of DDATHF influx in cells with V104M RFC1 resulted in even greater preservation (60%) of the exchangeable drug level. Another major element in the preservation of DDATHF activity was the impact of the mutated carrier on cellular folate pools. For folic acid, folate pools were essentially unchanged but DDATHF polyglutamate levels decreased in lines that express the V104M carrier. However, with 5-formyltetrahydrofolate as the growth source, there was a marked decrease in folate pools in the lines carrying the mutated carrier, and DDATHF polyglutamate levels were unchanged. Hence, DDATHF activity was preserved in cells with V104M RFC1 due to (a) relative conservation of DDATHF transport, and (b) depletion of cellular THF cofactors with diminishing folate cofactor competition at folylpolyglutamate synthetase and possibly glycinamide ribonucleotide formyltransferase. Hence, resistance to one antifolate, in this case MTX, because of a loss of

RFC1 transport activity need not exclude the subsequent utility of another antifolate that uses the same carrier.

INTRODUCTION

Folate cofactors play a key role in the biosynthesis of purine and thymidine precursors of nucleic acids, and this biochemical pathway has been a focal point for the development of antimetabolites that block folate-dependent reactions. MTX,³ the classical antifolate, achieves its pharmacological effects through depletion of cellular THF cofactors, resulting in cessation of THF cofactor-dependent processes. An important element in MTX activity is the formation of polyglutamyl derivatives that are retained and build up within cells to sustain inhibition of DHFR (1, 2). Polyglutamation of natural folates is also essential to their activity as one-carbon donors because these derivatives are retained in cells and are generally better substrates for folate-requiring enzymes than their monoglutamyl precursors (3). Over the past decade, a variety of new antifolates have been developed that achieve enhanced activity after formation of polyglutamate derivatives. In their polyglutamyl forms, these agents are very potent inhibitors of purine and/or thymidylate synthesis. DDATHF and LY309887 polyglutamates inhibit GARFT; ZD1694 polyglutamates inhibit thymidylate synthase (4–8). MTA polyglutamates are potent inhibitors of thymidylate synthase and to a lesser extent GARFT, and both mono- and polyglutamates are weak inhibitors of DHFR (9–11). All these agents are, or have been, in clinical trial; ZD1694 is approved for use in Europe (12–16).

These new antifolates are transported by RFC1, and resistance to each can be associated with loss of transport activity by this carrier (10, 17–19). Transport-related resistance to MTX has been studied in considerable detail, and with the cloning of RFC1 (20–25) it is now possible to better understand the molecular basis for loss of function in resistant cell lines. This laboratory has developed a panel of cell lines resistant to MTX due to loss of transport function associated with defined mutations in *RFC1* (26). Recently, studies were undertaken to determine the extent to which these cell lines might be cross-resistant

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² To whom requests for reprints should be addressed, at Albert Einstein College of Medicine Comprehensive Cancer Research Center, Chanin 2, 1300 Morris Park Avenue, Bronx, NY 10461. Phone: (718) 430-2302; Fax: (718) 430-8550; E-mail: igoldman@aecom.yu.edu.

³ The abbreviations used are: MTX, methotrexate; THF, tetrahydrofolate; DHFR, dihydrofolate reductase; DDATHF, (6R)-5,10-dideazatetrahydrofolate; LY309887, (6R)-2',5'-thienyl-5,10-dideazatetrahydrofolate; GARFT, glycinamide ribonucleotide formyltransferase; MTA, ALIMTA, multitargeted antifolate, LY231514; RFC1, the reduced folate carrier; TMQ, trimetrexate; 5-CHO-THF, 5-formyltetrahydrofolate; HPLC, high-performance liquid chromatography; GAT, 200 μM glycine, 100 μM adenosine, 10 μM thymidine; G418, Geneticin; HBS, HEPES-buffered saline; 5-CH₃-THF, 5-methyltetrahydrofolate.

to other antifolates; several lines had minimal or no cross-resistance to DDATHF. This report characterizes one such cell line and describes the basis for the preservation of DDATHF activity.

MATERIALS AND METHODS

Chemicals. [3',5',7-³H]-(6S)-5-CHO-THF was obtained from Moravек Biochemicals (Brea, CA), and [3',5',7-³H]MTX and [3',5',7,9-³H]folic acid were obtained from Amersham Corp. (Arlington Heights, IL). [³H]DDATHF (3.3 Ci/mmol), synthesized by Moravек Biochemicals, and unlabeled DDATHF and LY309887 were provided by Dr. Victor Chen (Eli Lilly, Indianapolis, IN). TMQ was a gift from Dr. David Fry (Warner-Lambert, Parke-Davis, Anna Arbor, MI). Tritiated chemicals as well as unlabeled MTX and 5-CHO-THF, (Ledlerle, Carolina, Puerto Rico) and folic acid (Sigma) were purified by HPLC before use.

Cells, Cell Culture Conditions, and Growth Studies.

Cells were grown in complete RPMI 1640 containing 2.3 μ M folic acid, 5% bovine calf serum (HyClone), 2 mM glutamine, 20 μ M 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μ g/ml) at 37°C in a humidified atmosphere of 5% CO₂. Cells were also grown in folate-free RPMI medium (HyClone) containing 5% dialyzed bovine calf serum (Life Technologies), 2 mM glutamine, 20 μ M 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μ g/ml) supplemented with 25 nM 5-CHO-THF. Prior to assessment of folate growth requirement, cells were maintained for 1–2 weeks in folate-free RPMI 1640 supplemented with 200 μ M glycine, 100 μ M adenosine, and 10 μ M thymidine (GAT) to deplete endogenous folates. For analyses of growth requirement for 5-CHO-THF or folic acid or inhibition by MTX, DDATHF, or LY309887, cells were grown in 96-well plates (1 \times 10⁵ cells/ml) and exposed continuously to appropriate concentrations of folates or antifolates. After 72–96 h, cell numbers were determined by hemocytometer count and viability was assessed by trypan blue exclusion. The L1210-G2 line was obtained after chemical mutagenesis followed by MTX selective pressure exactly as described previously (27). This cell line has been maintained in drug-free complete RPMI 1640 and has displayed a stable level of MTX resistance for more than 16 months.

Cloning of the Mutated Reduced Folate Carrier.

Poly(A)⁺ mRNA was purified using a Dynabeads mRNA DIRECT kit (Dyna) from L1210-G2 and L1210 cells. The first DNA strand synthesis was carried out with Superscript Reverse Transcriptase according to the manufacturer's protocol (Life Technologies). The RFC1 protein-coding sequence was amplified with *Pfu* polymerase (Stratagene) using oligonucleotide primers that flank the coding region of RFC1 (upstream primer at nt -46 from the translation start codon 5'-GCGGATCCTG-GAGTGTTCATCTTGG-3'; downstream primer at nt +82 from translation stop codon 5'-GCCTCGAGCTGGTTCAGGTG-GAGT-3'). Two 8-bp linkers were introduced into the primers so that both *Bam*HI and *Xho*I restriction sites were created in the PCR products to facilitate directional cloning. The PCR amplifications were performed for 35 cycles of 45 s at 95°C, 45 s at 60°C, and 3 min at 72°C. The 1682-bp-long predicted PCR product was purified on an agarose gel (Qiagen) and cloned into

a pCR-Blunt vector (Invitrogen); the sequence was determined on automated sequencer models 373A and 377 from Applied Biosystems in the DNA Sequencing Shared Resource of the Albert Einstein College of Medicine Comprehensive Cancer Center.

Transfections. RFC1-V104M and RFC1-G367E cDNAs were excised from their pCR-Blunt vectors (see above) by a double restriction with *Bam*HI and *Xho*I and recloned into pCDNA3.1(+) (Invitrogen) with the same restriction sites. MTX^rA cells (1 \times 10⁷) in which the endogenous RFC1 was mutated and not functional (28, 29) were electroporated (250 V, 330 microfarads) with 50 μ g of nonlinearized pCDNA3.1(+) harboring the mutated RFC1 cDNA in a final volume of 800 μ l of serum-free RPMI 1640. Cells were then diluted in 20 ml of complete RPMI 1640, allowed to recover for 48 h, adjusted to 2 \times 10⁵ cells/ml in medium containing G418 (750 μ g/ml of active drug), and then distributed into 96-well plates at \sim 4 \times 10⁴ cells/well. After transfectants were screened by Northern analysis, one clone with the highest RFC1 mRNA level was chosen for further study. This cell line, MTX^rA-V104M, was maintained in RPMI 1640 containing 750 μ g/ml G418 and displayed a stable DDATHF IC₅₀ over a period of more than half a year.

Northern Analyses. Total RNA was isolated from L1210-G2, MTX^rA-V104M, MTX^rA, and L1210 cells with the TRIzol reagent (Life Technologies). RNA (20 μ g) was resolved by electrophoresis on 1% agarose gels containing formaldehyde. Transfers and hybridizations were performed as described previously (30). Transcripts were quantitated by PhosphorImager analysis of the hybridization signals and normalized to β -actin.

Transport Studies. Influx measurements were performed in HBS [20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 5 mM glucose (pH 7.4)]. Cells were harvested, washed twice with HBS, and resuspended in HBS to a density of 1.5 \times 10⁷ cells/ml. After a 25-min incubation at 37°C, uptake was initiated by the addition of radiolabeled folate, and samples were taken at the indicated times. 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, and processed for measurement of intracellular radioactivity (30). For all folates and antifolates, initial rates were established over an interval in which cell uptake was linear as function of time with an extrapolated ordinate intercept at time 0 near the point of origin. For DDATHF efflux experiments, cells were loaded with [³H]DDATHF, separated by centrifugation, and resuspended in a large volume of drug-free HBS. Samples were obtained rapidly over the interval in which free drug exits the cells, and then later to monitor the nonexchangeable component, and processed as described above. Prior to transport studies with the transfectant, MTX^rA-V104 cells were grown for no longer than 5 days without G418 to ensure that expression of the mutated RFC1 was maintained.

Measurements of Folate Pools and DDATHF Accumulation. Cells (3 \times 10⁶) were grown in folate-free RPMI medium supplemented with either 2 μ M [³H]folic acid (30 dpm/pmol) or 25 nM [³H]5-CHO-THF (200 dpm/pmol). After 3 days at exponential growth, cells were harvested, washed twice with ice-cold HBS, and processed for intracellular tritium as described for transport studies. For measurement of DDATHF

Table 1 Growth inhibition by antifolates, and growth requirements for folates, in wild-type L1210 and L1210-G2 MTX-resistant cell lines

Growth inhibition by antifolates was determined in complete RPMI 1640 (2.3 μ M folic acid), and growth requirements were assessed after cells were grown in folate-free RPMI supplemented with GAT for 1 week to deplete endogenous folate pools. Data are the mean \pm SE of three experiments.

	L1210	L1210-G2	L1210/L1210-G2
Growth inhibition (IC_{50} , nM)			
MTX	10.0 \pm 1.5	217 \pm 17	22
TMQ	3.7 \pm 0.3	2.2 \pm 0.5	0.59
DDATHF	39 \pm 2	60 \pm 10	1.5 ($P < 0.2$)
LY309887	14 \pm 2	20 \pm 1	1.4 ($P < 0.15$)
Growth requirement (EC_{50} , nM)			
5-CHO-THF	1.7 \pm 0.6	15 \pm 4.5	8.8
Folic acid	173 \pm 17	360 \pm 23	2.0

accumulation, cells grown in complete RPMI medium containing 2.3 μ M folic acid or in folate-free RPMI 1640 supplemented with 25 nM 5-CHO-THF were incubated with 50 nM [3 H]DDATHF (300 dpm/pmol) and GAT, the latter to circumvent the inhibitory effects of DDATHF. Cells were harvested after 3 days of exponential growth, washed twice with ice-cold HBS, and processed for intracellular tritium, as described for transport studies, and polyglutamates (see below).

HPLC Analysis of DDATHF Polyglutamates. Cells exposed to [3 H]DDATHF were washed three times with 0°C HBS. One portion of the cell pellet was processed for dry weight and total tritium as described above. Another portion was processed according to a reported protocol (31). 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 with a reversed-phase HPLC column (Waters Spherisorb 5 μ M ODS2, 4.6 \times 250 mm) as reported previously with minor modification (30). 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. The polyglutamates were identified by comparison of elution times to those of unlabeled standards (gift from Dr. Richard Moran, Medical College of Virginia, Richmond, VA) and then normalized to units of nmol/g dry weight of cells.

RESULTS

Identification of a MTX-resistant, DDATHF-sensitive Murine Leukemia Cell Line. The MTX-resistant clonal cell line, L1210-G2, was isolated under MTX selective pressure (100 nM) with 25 nM 5-CHO-THF as the sole folate source after L1210 cells were treated with the mutagen ethylmethanesulfonate as reported previously (27). These cells exhibited a 22-fold increase in the MTX IC_{50} compared with the parental L1210 cells. There was no collateral resistance to TMQ; in fact, there was a small decrease in the IC_{50} in the L1210-G2 line (Table 1).

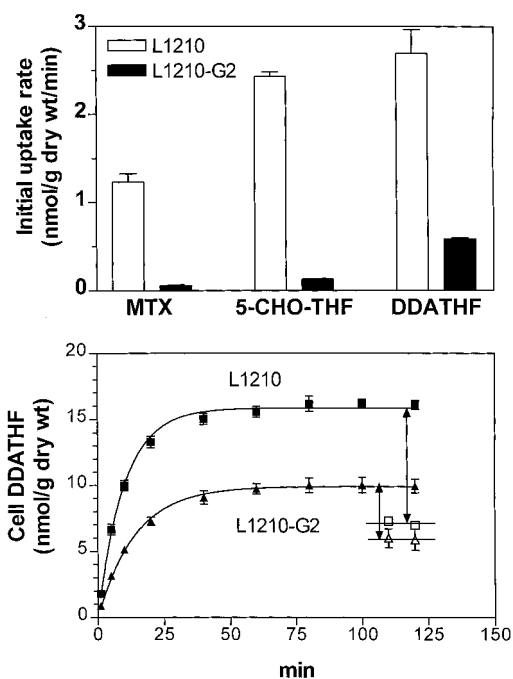


Fig. 1 Influx of MTX, 5-CHO-THF, and DDATHF (*top*), and the time course of net uptake of DDATHF (*bottom*) in L1210 and L1210-G2 cells. *Top panel*, cells grown in complete RPMI 1640 were incubated in HBS for 25 min at 37°C, and then exposed to 1 μ M tritiated MTX, 5-CHO-THF, or DDATHF, and influx was determined. *Bottom panel*, the time course of DDATHF uptake was monitored over 2 h (*closed symbols*), after which the cell suspensions were centrifuged and the cell pellets resuspended into a large volume of drug-free HBS for determination of exchangeable and nonexchangeable components (indicated by *double-headed arrows* and *open symbols*). Data are the mean \pm SE of three separate experiments.

L1210-G2 cells were not significantly cross-resistant to the GARFT inhibitors DDATHF or LY309887. The 5-CHO-THF growth requirement in this cell line was increased by a factor of \sim 9, but the folic acid growth requirement was increased by a factor of only 2.

Transport Properties of the L1210-G2 Cell Line.

DDATHF, as well as MTX and 5-CHO-THF, are excellent substrates for RFC1 and are transported in wild-type L1210 cells exclusively by this mechanism. As indicated in Fig. 1 (*top panel*), the initial uptake rate of both MTX and 5-CHO-THF was decreased by \sim 95% in L1210-G2 cells compared with L1210 cells. However, DDATHF influx was reduced by only 80%. Hence, DDATHF influx in this cell line was 10- and 5-fold greater than that of MTX and 5-CHO-THF, respectively, at a substrate concentration of 1 μ M. As indicated in Fig. 1 (*bottom panel*), when uptake was continued for 2 h and cells were at near steady state, the net level of DDATHF in L1210-G2 cells (10 nmol/g dry weight) was 60% that of L1210 cells (16 nmol/g dry weight). When cells were separated by centrifugation and resuspended into DDATHF-free buffer to allow efflux of exchangeable drug, it could be seen that the difference in net accumulation of DDATHF over this interval was due largely to a 2-fold decrease in exchangeable DDATHF in L1210-G2 cells with a smaller (\sim 20%) decrease in the level of nonexchangeable

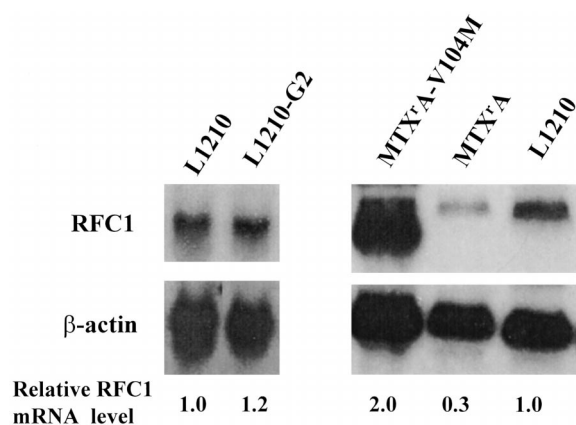


Fig. 2 Northern blot analysis of total RNA from L1210, L1210-G2, MTX^rA, and MTX^rA-V104M cells. Total RNA obtained from cells grown in complete RPMI 1640 was probed first with the full-length murine *RFC1* and then with β -actin cDNAs. The molecular size of the endogenous *RFC1* transcript in L1210 and MTX^rA cells (2.3 kb) was slightly greater than that of the transfectants (1.9 kb) derived from the expression vector. The numbers below the lanes are RFC1 mRNA levels relative to L1210 cells determined by PhosphorImager analysis from two separate experiments. The left and right panels are each representative of two separate experiments.

drug. Efflux of 90% of exchangeable drug from L1210-G2 cells, like wild-type L1210 cells, was characterized by a single first-order rate constant, excluding the possibility that a substantial fraction of intracellular DDATHF is loosely bound within the cells (data not shown). HPLC analysis confirmed that the exchangeable drug was the monoglutamate; the nonexchangeable fraction included the polyglutamates of DDATHF that did not decline during the efflux period. Hence, whereas influx of DDATHF mediated by RFC1 was decreased, albeit to a lesser degree than MTX or 5-CHO-THF, there was a much greater preservation of exchangeable DDATHF within the cells with little difference in the level of polyglutamates accumulated over this interval.

Characterization of the Amino Acid Composition of RFC1 in L1210-G2 Cells. The level of RFC1 mRNA in L1210-G2 cells was not significantly altered compared with L1210 cells (Fig. 2, left panel). cDNAs were isolated by reverse transcription-PCR, cloned, and sequenced. From eight randomly picked cDNA clones, two mutations, G443A and G1223A (GenBank U32469), were identified at a frequency of 5:3, respectively, in the RFC1 coding region. These two mutations were verified by a separate reverse transcription-PCR reaction. The former nucleotide change results in a methionine substitution for valine at position 104 (V104M), and the latter results in a glutamate for glycine substitution at position 367 (G367E).

Impact of V104M and G367E cDNA Transfections in RFC1 Null Cells on Transport and Sensitivity to Antifolates. Both mutated cDNAs were transfected separately into MTX^rA cells that lacked endogenous RFC1 activity due to a substitution of proline for alanine at amino acid 130 (29). Although RFC1 mRNA derived from the expression vector was detected in the G367E transfectants, none of the clones exhibited augmented influx or increased sensitivity to DDATHF compared with the recipient

MTX^rA cells. However, the sensitivity of V104M transfectants to DDATHF was markedly increased. Hence, the G367E mutation results in complete loss of RFC1 activity due to an intrinsic change in carrier function and/or impaired trafficking to the cell membrane; residual transport activity in the L1210-G2 line must be attributed solely to the allele containing the V104M substitution. One of the transfectants with the highest RFC1 mRNA level, identified as MTX^rA-V104M, was used for further studies to characterize the functions of the mutated carrier.

The RFC1 mRNA level in MTX^rA-V104M cells was 2-fold greater than that of L1210 cells, based on PhosphorImager analysis (Fig. 2, right panel). As indicated in Table 2, expression of the mutated carrier decreased the DDATHF IC₅₀ by a factor of 18 compared with MTX^rA cells, but this parameter was decreased by a factor of only ~2 for MTX. The 5-CHO-THF growth requirement in MTX^rA-V104M cells was decreased by a factor of only 3.6 compared with MTX^rA cells. Consistent with these changes, influx of DDATHF, MTX, and 5-CHO-THF was 32-, 3.8-, and 5.6-fold greater, respectively, in MTX^rA-V104M compared with MTX^rA cells. In addition, as observed in the parental L1210-G2 line, whereas DDATHF influx in MTX^rA-V104M was only one-third that of wild-type cells, the steady-state DDATHF concentration reached a level 70% that of L1210 cells (Fig. 3). Over a 2-h interval, net DDATHF uptake in MTX^rA cells reached only 10% that of L1210 cells. Hence, expression of MTX^rA-V104M alone in MTX^rA cells reproduced the transport phenotype and resistance pattern observed in L1210-G2 cells and substantially favored transport of DDATHF over MTX and 5-CHO-THF.

Influx Kinetic Parameters for the MTX^rA-V104 Transfectant. The kinetic basis for the changes in DDATHF influx mediated by RFC1-V104M were assessed (Table 3). In L1210 cells, the influx K_i for DDATHF was 0.49 μM , ~one-tenth that of MTX (27). In L1210-G2 cells and the transfected line, this parameter was increased by a factor of 7 and 16, respectively, consistent with a marked fall in the affinity of RFC1 for this substrate. However, the influx K_i for DDATHF mediated by the V104M carrier (~8 μM) was still comparable to the K_i for MTX influx mediated by the wild-type RFC1 in L1210 cells (~7 μM ; Ref. 27). There were smaller differences in the influx V_{max} mediated by the mutant carrier, although precise quantitation was not possible without a more reliable quantitative assessment of RFC1 protein expression at the cell membrane.

The influx K_i for other folates could not be determined accurately because of their apparent high levels and very low rates of transport. However, influx K_i s could be assessed based on inhibition of DDATHF transport and the measured influx K_i for this antifolate in L1210 and MTX^rA-V104M cells. As indicated in Table 4, the influx K_i s mediated by wild-type RFC1 for all of these folates were comparable to the values reported previously (27, 32). The influx K_i s mediated by RFC1-V104M for MTX and 5-CH₃-THF were increased by a factor of ~30; for 5-CHO-THF, there was a 20-fold increase, but the K_i for folic acid increased by a factor of only 2. Hence, there was a profound loss in carrier affinity for MTX and the reduced folates.

Impact of Folate Source on Growth Inhibition by MTX, DDATHF, and L309887 and Cellular Accumulation of Folate and DDATHF Polyglutamates. The folate source in the growth medium had a substantial effect on the activity of

Table 2 Characteristics of the MTX^rA-V104M transfectant compared with MTX^rA and L1210 cells

Growth inhibition by antifolates was determined in complete RPMI 1640 (2.3 μM folic acid). Growth requirements were assessed after cells were grown in folate-free RPMI medium supplemented with GAT for 1 week to deplete endogenous folate pools. Influx was determined over 1–2 min when uptake of drug was unidirectional. Data are the mean ± SE of three experiments.

Antifolate or Folate	MTX ^r A	MTX ^r A-V104M	MTX ^r A/MTX ^r A-V104M
Growth inhibition (IC ₅₀) or growth requirement (EC ₅₀)			
DDATHF	2280 ± 810	128 ± 3	18
MTX	500 ± 58	227 ± 37	2.2
5-CHO-THF	68 ± 13	19 ± 6	3.6
Influx (nmol/g dry wt/min)			
		MTX ^r A-V104M/MTX ^r A	
DDATHF	0.026 ± 0.006	0.84 ± 0.04	32
MTX	0.014 ± 0.006	0.052 ± 0.007	3.8
5-CHO-THF	0.022 ± 0.006	0.124 ± 0.015	5.6

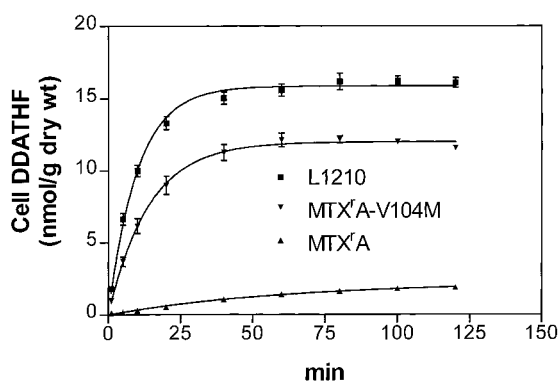


Fig. 3 Net uptake of DDATHF in L1210, MTX^rA, and MTX^rA-V104 cells. Cells grown in complete RPMI 1640 were incubated in HBS for 25 min at 37°C, and then exposed to 1 μM tritiated DDATHF; uptake was monitored over 2 h. Data are the mean ± SE of three separate experiments.

Table 3 DDATHF influx kinetics in L1210, L1210-G2, and MTX^rA-V104M cells

Influx parameters were obtained from nonlinear regression to the Michaelis-Menten equation. The concentration ranges were 0.25–4 μM for L1210 cells and 2–20 μM for L1210-G2 and MTX^rA-V104 cells. Data are the mean ± SE of three experiments.

Cell line	K _i (μM)	V _{max} (nmol/g dry wt/min)	V _{max} /K _i
L1210	0.49 ± 0.03	5.3 ± 0.8	11
L1210-G2	3.4 ± 0.4	3.8 ± 0.4	1.1
MTX ^r A-V104M	7.9 ± 0.6	10.9 ± 0.3	1.4

DDATHF. There were 2.4- and 5.3-fold decreases in the DDATHF IC₅₀ in the L1210-G2 and MTX^rA-V104M lines, respectively, when cells were grown in 5-CHO-THF versus folic acid, whereas the IC₅₀ in the L1210 cells was virtually unchanged (Fig. 4, top panel). This trend was also observed for MTX in L1210-G2 and MTX^rA-V104 cells, but to a much smaller extent (10 and 50%, respectively); there was no difference in L1210 cells (Fig. 4, bottom panel). In fact, the L1210-G2 and MTX^rA-V104M lines were somewhat more sensitive to DDATHF than L1210 cells (*P* < 0.1), whereas they were still 12- and 8-fold more resistant to MTX than L1210 cells

Table 4 Comparison of influx K_i values of MTX, 5-CHO-THF, 5-CH₃-THF, and folic acid in L1210 and MTX^rA-V104 cells

Influx K_i values were calculated from inhibition of [³H]DDATHF influx by folate/antifolate substrates based on the Michaelis-Menten equation, where DDATHF influx = V_{max}S/(S + K_i(1 + I/K_i)), and S and I are the [³H]DDATHF and inhibitor concentrations, respectively. The concentration of [³H]DDATHF was 1 μM, whereas nonlabeled MTX and 5-CHO-THF concentrations were 25 and 125 μM, 5-CH₃-THF was 10 and 50 μM, and folic acid was 250 and 1250 μM. The values shown are the mean ± SE of six determinations from three separate experiments.

Folate/antifolate	K _i (μM)		MTX ^r A-V104/ L1210
	L1210	MTX ^r A-V104	
MTX	6.0 ± 0.2	170 ± 18	28
5-CHO-THF	4.6 ± 0.5	91 ± 13	20
5-CH ₃ -THF	1.9 ± 0.1	60 ± 5	31
Folic acid	260 ± 30	590 ± 70	2.3

with 5-CHO-THF as the sole folate source. The pattern of LY309887 inhibition was similar to that of DDATHF except that this agent was more potent (data not shown).

Fig. 5 compares total folate accumulation when cells were grown with either 2 μM [³H]folic acid or 25 nM [³H]5-CHO-THF. There was an ~40% decrease in the folate level in wild-type L1210 cells grown with 5-CHO-THF compared with folic acid under these conditions. With [³H]folic acid as substrate, there was only a small (~10–20%) decrease in cellular folate accumulation in the L1210-G2 and MTX^rA-V104M lines compared with L1210 cells, consistent with the observation that only a small component of folic acid transport is mediated by RFC1 (33, 34). However, with [³H]5-CHO-THF, total folate accumulation was decreased to 19 and 22% that of L1210 cells, respectively, in these cell lines.

Fig. 6 indicates accumulation of DDATHF polyglutamates after a 3-day incubation with 50 nM [³H]DDATHF when cells were grown in folate-free medium containing either 2.3 μM folic acid or 25 nM 5-CHO-THF in the presence of GAT. With folic acid as substrate, total drug accumulation was decreased by ~30% in L1210-G2 and MTX^rA-V104M cells compared with L1210 cells. On the other hand, with 5-CHO-THF as the folate source, the DDATHF level was minimally increased in L1210 cells (~10%) compared with

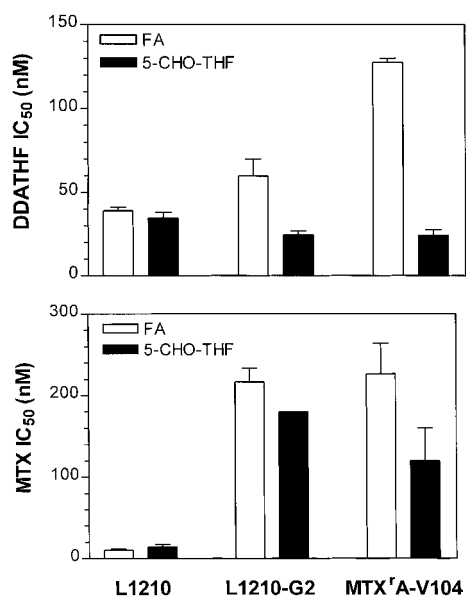


Fig. 4 Impact of folate source in medium on growth inhibition by DDATHF (*top*) and MTX (*bottom*) in L1210, L1210-G2, and MTX^rA-V104 cells. Cells were grown in folate-free RPMI medium supplemented with either 2.3 μ M folic acid (FA) or 25 nM 5-CHO-THF for 1 week, and then incubated continuously with different concentrations of the antifolates. After 72 h, cell numbers were determined. Data are the mean \pm SEM of three experiments.

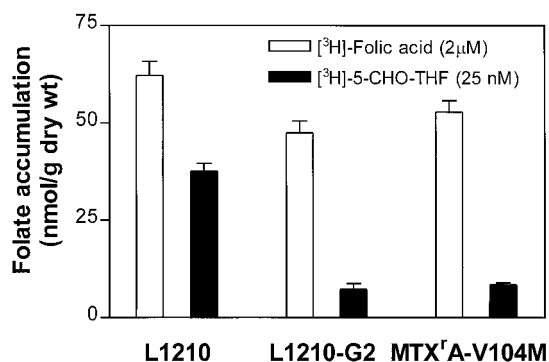


Fig. 5 Total folate cofactor accumulation in L1210, L1210-G2, and MTX^rA-V104M cells when grown with 5-CHO-THF or folic acid. Cells grown in complete RPMI 1640 were harvested, washed, and grown exponentially for 1 week in folate-free RPMI 1640 supplemented with 25 nM [³H]5-CHO-THF or 2 μ M [³H]folic acid. Cells were pelleted, washed twice with ice-cold HBS, and processed for determination of intracellular radioactivity as described in the "Materials and Methods" section.

growth in folic acid, and the levels in the L1210, L1210-G2, and MTX^rA-V104M lines were identical. On the basis of a ratio of intracellular water to dry weight (μ l/mg) of 3.6 in L1210 cells (30), intracellular DDATHF at 22 nmol/g drug weight (Fig. 6) corresponds to a concentration of 6 μ M, a level 120-fold greater than the extracellular concentration of 50 nM and represents, virtually entirely, polyglutamate derivatives of DDATHF as confirmed by HPLC analysis.

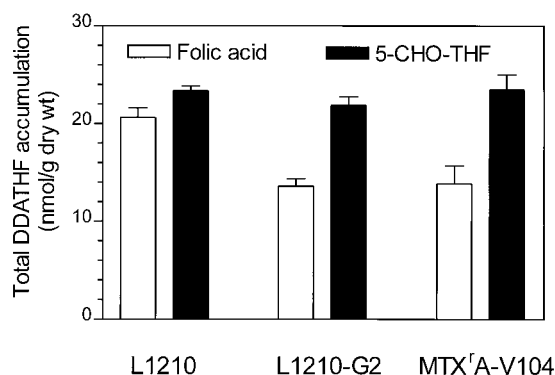


Fig. 6 Impact of folate source on DDATHF accumulation in L1210, L1210-G2, and MTX^rA-V104 cells. Cells were grown in complete RPMI medium (2.3 μ M folic acid) or in folate-free RPMI medium supplemented with 25 nM 5-CHO-THF for 1 week before incubation with 50 nM [³H]DDATHF and GAT. After 72 h, cells were harvested, washed twice with ice-cold HBS, and dissolved in 1 N NaOH for determination of intracellular radioactivity and DDATHF polyglutamate levels as described in the "Materials and Methods" section.

DISCUSSION

Previous studies from this laboratory using chemical mutagenesis under MTX selective pressure produced a panel of 26 L1210 leukemia cell lines resistant to MTX because of transport defects associated with defined point mutations in *RFC1* (26). Several of these mutant carriers have been characterized in detail and were found to be highly selective in discriminating among MTX and natural folates based on large differences in binding affinities and/or mobilities of the carrier loaded with substrate (27, 32, 35). This report extends this analysis to demonstrate that a mutant *RFC1* can also manifest selectivity among different antifolates and that resistance to one antifolate because of a loss of *RFC1*-mediated transport need not be accompanied by cross-resistance to all other antifolates that use the same carrier.

Transport of DDATHF and MTX are both mediated by *RFC1* in L1210 cells and both have a high affinity for this carrier; in fact, DDATHF is a much better substrate for the carrier with an influx K_t less than one-tenth that of MTX (27). Hence, it might have been expected that cells resistant to MTX because of a defect in *RFC1* would be collaterally resistant to DDATHF. This was not the case; marked (>20-fold) resistance to MTX was not accompanied by a significant decrease in sensitivity to DDATHF and LY309887. The data presented here suggest that a variety of factors can account for this phenotype. First, the mutated V104M carrier partially preserved DDATHF transport function relative to MTX. Hence, whereas only ~5% of influx activity remained for MTX in the L1210-G2 resistant line, ~20% of DDATHF activity was retained. Second, preservation of influx was associated with an even greater degree of preservation of the exchangeable cellular DDATHF level achieved—60% that of L1210 cells. The basis for this is not clear but might be due to decreased activity of DDATHF as a substrate for the folate exporter(s) (36–38). The functional characteristics of the mutated carrier in the L1210-G2 line were confirmed by transfection of the V104M cDNA into the trans-

port-deficient MTX^rA cells. Here, a level of influx ~30% that of L1210 cells translated into a larger increase in the steady-state level of DDATHF and a marked decrease in resistance to the drug. On the other hand, the mutated carrier had a much smaller impact on transport of MTX and 5-CHO-THF, and the IC₅₀s or EC₅₀s for these folates were only modestly decreased.

Another key factor, also related to the carrier mutation, that preserved sensitivity to DDATHF was the loss of transport activity for 5-CHO-THF, which resulted in an ~9-fold increase in the EC₅₀ for this folate substrate. The consequence of this transport loss was a marked (80%) decrease in the folate pool size when cells were grown in physiological concentrations of 5-CHO-THF. This will have at least two consequences: (a) The low folate pools should enhance DDATHF polyglutamation because of decreased competition at the level of folypolyglutamate synthetase. This is consistent with the observation that DDATHF accumulation in the L1210-G2 and transfected cell lines was comparable to the level in L1210 cells with 5-CHO-THF as the growth substrate but was depressed in folic acid, which is transported largely by an RFC1-independent mechanism (33, 34) and in its presence folate pools are unchanged. (b) The low folate pool should decrease competition between 10-formyltetrahydrofolate and the antifolate at the level of GARFT. Hence, the specific folate source in the medium will be a critical element in abrogating cross-resistance to DDATHF, and studies with folic acid *in vitro* are not relevant to *in vivo* conditions in which the physiological folate is 5-CH₃-THF, which has transport properties similar to 5-CHO-THF.

In this study, decreased folate cofactor pools preserved DDATHF activity. Other reports have demonstrated that increased folate pools suppress antifolate polyglutamation and result in DDATHF resistance. In a murine cell line selected for resistance to DDATHF, transport of DDATHF, 5-CHO-THF, and MTX was not significantly altered. However, I48F and/or W105G mutations in RFC1 markedly increased affinity for folic acid, augmented the cell folate pool, and depressed formation of DDATHF polyglutamates, rendering cells resistant to DDATHF when grown with folic acid. This was not observed when cells were grown in 5-CHO-THF (39). CEM/MTX-LF cells overexpress RFC1 and carry an E45K mutation that enhances carrier affinity for folic acid. The IC₅₀ for DDATHF in this cell line is increased by a factor of 270. Resistance disappears when this cell line is grown in 5 nM folic acid and cellular folate pools are depleted (40). Presumably, on the same basis, when mice are made folate-deficient, the toxicity of DDATHF is increased by three orders of magnitude (41).

In these and other studies, cells were selected for drug resistance in the presence of 25 nM 5-CHO-THF to mimic physiological conditions and to favor the acquisition of selective mutations that preserve reduced folate transport activity (27, 35). 5-CHO-THF transport was partially preserved relative to MTX, but in both the L1210-G2 and MTX^rA-V104M lines, the EC₅₀ for 5-CHO-THF was increased 10-fold to 15 nM. However, these cells continued to grow well with 25 nM 5-CHO-THF. Survival *in vivo* will, of course, depend on the folate blood level. For example, under normal conditions the blood folate level (largely 5-CH₃-THF) of ~20 nM (42) is 5- to 20-fold greater than the EC₅₀ of 5-CHO-THF for wild-type L1210 cells. At very low blood folate levels, tumor cells with impaired RFC1

function and/or low expression would be especially vulnerable to folate depletion. Although human tumors that become resistant to MTX could meet their folate requirements through possible mutations in RFC1 that selectively preserve transport of reduced folates, the data presented here explain how human leukemic lymphoblasts resistant to MTX because of decreased carrier protein expression are able to survive (43, 44). Hence, even cells with low RFC1 expression may retain sensitivity to antifolates such as DDATHF based on an accompanying loss of transport activity for reduced folates, resulting in depletion of cellular tetrahydrofolate cofactor pools.

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