The Mechanism of Transport of the Multitargeted Antifolate (MTA) and Its Cross-resistance Pattern in Cells with Markedly Impaired Transport of Methotrexate[1]

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ABSTRACT

MTA (LY231514) is an antifolate that targets multiple folate-dependent enzymes. In this report, MTA transport was characterized in wild-type L1210 cells and variants with impaired membrane transport or polyglutamation. MTA influx via the reduced folate carrier was somewhat faster (~30%) than that for methotrexate (MTX). Unlike MTX, MTA was rapidly polyglutamated in L1210 cells; hence, a folylpoly-γ-glutamate synthetase-deficient L1210 variant was used to assess net transport and efflux properties. The MTA transmembrane gradient for exchangeable drug was 2.5 times greater than the MTX gradient, attributable primarily to an efflux rate constant 40% that of MTX. No MTA was bound to dihydrofolate reductase. When grown with folic acid, MTX-resistant L1210 variants with mutations in the reduced folate carrier demonstrated cross-resistance to MTA, markedly reduced MTA accumulation, and only a slightly decreased intracellular folate cofactor pool as compared to L1210 cells. However, when 5-formyltetrahydrofolate was the growth substrate, these MTX-resistant cells were less resistant or negligibly resistant to MTA, accumulated more MTA, and had a lower folate pool as compared to L1210 cells. MTA activity and the intracellular folate pool in L1210 cells were inversely related. These data indicate that MTA polyglutamation in L1210 cells is favored by both the generation of high intracellular drug levels and high MTA affinity for FPGS relative to MTX. Cells resistant to MTX because of impaired transport may retain appreciable sensitivity to MTA because of a concurrent reduction in tetrahydrofolate cofactor transport resulting in cellular folate depletion, which diminishes endogenous folate suppression of MTA polyglutamation.

INTRODUCTION

The pyrrolopyrimidine-based agent MTA[3] (LY231514) is a unique new generation of antifolate that achieves pharmacological activity after conversion to its polyglutamyl derivatives within cells (1). This chemical transformation results in a marked increase in the affinity of these congeners for several THF cofactor-dependent enzymes. Hence, the pentaglutamate of MTA has a K_i (1.3 nM) for human TS nearly 2 orders of magnitude lower than that of the monoglutamate (109 nM) and a K_i for murine GARFT (65 nM) more than 2 orders lowers than the K_i of the monoglutamate (9.3 μM). On the other hand, the mono- and polyglutamates of this agent have comparable affinities for DHFR (~7 nM) at least 3 orders of magnitudes less than that of MTX (1). The pharmacological perturbations produced by MTA are complex and appear to represent a combination of suppression of both thymidyate and purine synthesis. Both physiological substrates are required to completely circumvent MTA cytotoxicity, but in cells with high expression of TS, a source of purine alone is sufficient to prevent drug activity (1, 2). MTA is a excellent substrate for FPGS, 2 orders of magnitude better than MTX and 1 order of magnitude better than DDATHF, a property that favors formation of its active derivatives in cells (3). In clinical studies, this agent appears to have activity against a variety of tumors, including non-small cell lung cancer, colorectal carcinoma, and mesothelioma (4–8).

Membrane transport of antifolates is a critical determinant of activity, and loss of transport is a frequent mechanism of resistance to MTX (9–15). The mechanism of transport of MTA has not been established, although the K_i for MTA inhibition of MTX influx suggests a high affinity for RFC1 (16), a member of the major facilitator superfamily of transporters (17). This agent also has a very high affinity for folate receptor α, a GPI-linked endocytotic pathway, that is comparable to the affinity of the preferred substrate for this route, folic acid (16). This report represents the first detailed assessment of the transport properties of this agent focusing on unidirectional fluxes and the level of concentrative transport achieved within the context of a comparison with MTX. The study also explores cross-resistance patterns to MTA in sev-

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[3] The abbreviations used are: 5-CH_3-THF, 5-methyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; DDATHF, (6*R*)-5,10-dideazatetrahydrofolate; DHFR, dihydrofolate reductase; FPGS, folylpoly-γ-glutamate synthetase; HBS, HEPES-buffered saline; MTA, multitargeted antifolate *N*-(4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo(2,3)pyrimidine-5-yl)ethyl]-benzoyl]-L-glutamic acid; MTX, methotrexate; PGA_1, prostaglandin A_1; RFC1, reduced folate carrier; THF, tetrahydrofolate; TS, thymidylate synthase.

eral cell lines with primary resistance to MTX because of impaired transport and demonstrates the critical role that the folate substrate in the growth medium plays in transport-related resistance phenomena.

MATERIALS AND METHODS

Chemicals. [3′,5′,7-^{3}H]-(6*S*)-5-CHO-THF was obtained from Moravek Biochemicals (Brea, CA); [3′,5′,7-^{3}H] MTX and [3′, 5′,7,9-^{3}H]folic acid were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). [^{3}H]MTA (4.1 Ci/mmol), unlabeled MTA, and MTA triglutamate were provided by the Eli Lilly Co. (Indianapolis, IN). Trimetrexate (TMQ) was a gift from Dr. David Fry (Warner-Lambert, Parke-Davis, Ann Arbor, MI). Tritiated chemicals, as well as unlabeled MTX, 5-CHO-THF (Lederle, Carolina, Puerto Rico), and folic acid (Sigma), were purified by high-performance liquid chromatography before use (18).

Cell Lines, Culture Conditions, and Growth Inhibition Assays. The MTX^{r}A line is an MTX-resistant L1210 murine leukemia variant obtained under MTX selective pressure with an A130P mutation in RFC1 that results in loss of carrier mobility (19). L1210-G1a cells were selected in the presence of MTX with 5-CHO-THF as the folate source and harbors an S46N mutation in RFC1 (20). L51 is a DDATHF-resistant L1210 variant isolated by chemical mutagenesis in this laboratory with a marked defect in FPGS activity (21). All cells were grown, unless otherwise noted, in complete RPMI 1640 containing 2.2 μM folic acid, 5% bovine calf serum (HyClone), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2. Cells were also grown in folate-free RPMI (HyClone) containing 5% dialyzed bovine calf serum (Life Technologies, Inc.), 2 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml) supplemented with 25 nM 5-CHO-THF. To assess MTA and MTX growth inhibition, cells were grown in 96-well plates (1×10^5 cells/ml) and exposed continuously to appropriate concentrations of the antifolates. After 72–96 h, cell numbers were determined by hemocytometer count, and viability was assessed by trypan blue exclusion.

Measurements of Folate Pools and MTA Accumulation. Cells (3×10^6) grown in complete RPMI 1640 were washed twice with folate-free RPMI and resuspended into the same medium supplemented with either 2 μM [^{3}H]folic acid (30 dpm/pmol) or 25 nM [^{3}H]5-CHO-THF (200 dpm/pmol). After 1 week of exponential growth, cells were harvested, washed twice with ice-cold HBS, and processed for intracellular tritium as described for transport studies. For measurement of MTA accumulation, cells grown in complete RPMI containing 2.2 μM folic acid or in folate-free RPMI 1640 supplemented with 25 nM 5-CHO-THF were incubated with 50 nM [^{3}H]MTA (200 dpm/pmol) in the presence of 200 μM glycine, 100 μM adenosine, and 10 μM thymidine to circumvent the inhibitory effects of this agent. Cells were harvested after 3 days of exponential growth, washed twice with ice-cold HBS, and processed for intracellular tritium and polyglutamates (see below).

High-performance Liquid Chromatography Analysis of MTA Polyglutamates. Cells exposed to 1 μM [^{3}H]MTA for 2 h were washed three times with 0°C HBS. One portion of the cell pellet was processed for dry weight and total tritium as described below. Another portion was processed according to a reported protocol (22). Cell pellets were suspended in 50 mM phosphate buffer at pH 6.0 containing 100 mM 2-mercaptoethanol and boiled for 5 min. The precipitate was removed by centrifugation, and the supernatant containing radiolabeled MTA and its metabolites, spiked with unlabeled MTA and MTA-triglutamate, was separated on a reversed-phase high-performance liquid chromatography column (Waters Spherisorb, 5 μm ODS2 4.6 × 250 mm) as reported previously with minor modifications (23). 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 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 1-ml fractions were collected. The level of MTA and its polyglutamate derivatives were normalized to units of nmol/g of dry wt of cells.

Folic Acid Binding Assay. Cells (2×10^7) were harvested and washed once with ice-cold acid buffer (10 mM NaAc, 140 mM NaCl, pH 3.5) and twice with cold PBS. The cells were incubated in 1 ml of PBS containing 5 nM [^{3}H]folic acid (26 Ci/mmol) at 4°C for 30 min, separated by centrifugation, and then washed twice with cold PBS and finally with the acid buffer (0.5 ml) to extract bound [^{3}H]folic acid. Tritium in the supernatant was assessed by liquid scintillation spectrometry, and the pellet was dried and weighed. The folic acid binding capacity was expressed in units of pmol/g of dry weight of cells.

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 1.5×10^7 cells/ml. For measurement of influx at pH 5.5, cells were washed once with unbuffered saline (140 mM NaCl, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$, 7 mM glucose) and then washed with and resuspended in 2-(4-morpholino)-ethanesulfonic acid-buffered saline (20 mM 2-(4-morpholino)-ethanesulfonic acid, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 5.5). Cell suspensions were incubated at 37°C for 20 min, after which uptake was initiated by the addition of [^{3}H]MTA or [^{3}H]MTX, 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, dried, and digested with 1 N NaOH in an 8-ml vial, and after fluor was added, radioactivity was assessed in a liquid scintillation spectrometer (23). When appropriate, uptake intervals were adjusted so that unidirectional conditions were sustained. For efflux measurements, cells were loaded with tritiated MTA or MTX, a small portion was taken for measurement of intracellular antifolate, and remaining cells were separated by centrifugation and resuspended into a large volume of drug-free buffer. Subsequently, samples were taken and injected into 10 ml of ice-cold HBS, separated by centrifugation, washed twice, and then processed for intracellular tritium as described above.

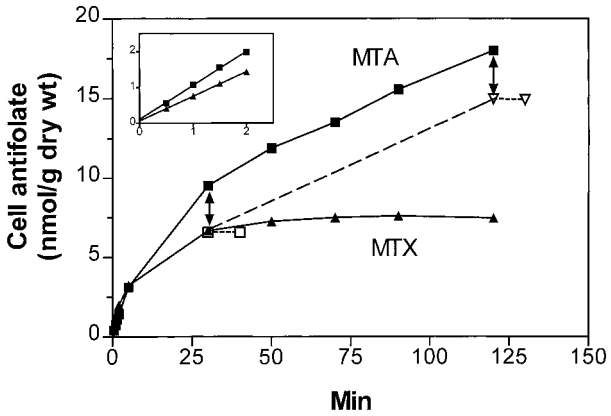

Fig. 1 Comparison of MTA and MTX influx and net uptake in L1210 cells. L1210 cells were harvested, incubated in HBS for 20 min at 37°C, and then exposed to 1 μM tritiated MTA (■) or MTX (▲), and uptake was monitored over 2 h. A portion of cells incubated with MTA for 30 min or 2 h was separated by centrifugation and resuspended in a large volume of MTA-free HBS for 30 and 40 min (□ and ▽, respectively) to determine nonexchangeable MTA. ↕, exchangeable MTA level. – – – –, projected increase in nonexchangeable drug. *Inset* compares initial uptake rates from the same experiment. Data are representative of three experiments.

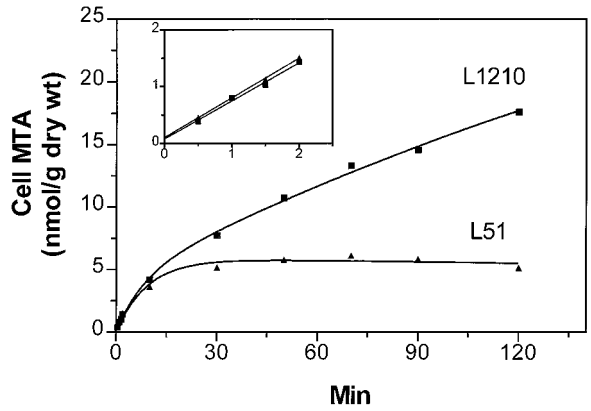

Fig. 2 Comparison of net MTA uptake in L1210 and L51 cells. L1210 and L51cells were incubated in HBS for 20 min at 37°C and then exposed to 1 μM tritiated MTA, and uptake was monitored over 2 h. *Inset*, expanded scale of the initial uptake. Data are representative of three experiments.

RESULTS

A Comparison of Cellular Uptake and Retention of MTA and MTX in Wild-type L1210 Cells. When L1210 cells were exposed to 1 μM tritiated MTX or MTA, the initial uptake of MTA was somewhat faster than that of MTX, and whereas intracellular MTX reached steady state within 30 min, net MTA uptake declined but continued at a substantial rate (Fig. 1). This continued net uptake of MTA was attributed to rapid accumulation of nonexchangeable radiolabel. Hence, when cells were separated by centrifugation after exposure to MTA for 30 or 120 min and resuspended into MTA-free buffer, a large nonexchangeable component was detected that increased in parallel (Fig. 1, – – – –) with the overall accumulation of the drug (——) and accounted entirely for the increase in net uptake of radiolabel over this interval. The level of exchangeable MTA did not change during this time, indicating that this intracellular MTA component was at steady state with extracellular drug.

A Comparison of the Transport Characteristics of MTA and MTX in an L1210 Cell Line with Markedly Diminished FPGS Activity. Rapid accumulation of a nonexchangeable component of MTA, presumably MTA polyglutamates (Fig. 1 and see below), precluded accurate assessment of MTA transmembrane gradients and efflux kinetics mediated by the membrane transport process(es) that govern translocation of this agent across the cell membrane. To eliminate the complication of polyglutamation, an L1210 leukemia cell line, L51, was used in which FPGS was mutated such that enzyme activity was negligible. The L51 cell line is 23-fold resistant to MTA as compared to L1210 cells upon continuous exposure to the drug (21). MTA influx in L51 cells was comparable to influx in L1210 cells (Fig. 2, *inset*). However, unlike L1210 cells, a MTA steady-state level of ~5 nmol/g dry wt was reached in L51 cells within 30 min (Fig. 2).

Net MTA and MTX uptake in L51 cells at an extracellular concentration of 1 μM are compared in Fig. 3. The steady-state levels for these antifolates were comparable under these conditions despite the fact that most intracellular MTX is tightly bound to DHFR. To determine whether MTA was also bound to DHFR, 10 μM TMQ was added to the transport buffer 15 min prior to the antifolates. This agent is a very potent inhibitor of DHFR and should block binding of both MTX and MTA to this enzyme without altering RFC1-mediated transport. It can be seen that addition of TMQ did not change the initial uptake rates for either MTA or MTX (Fig. 3, *inset*) but produced a 2-fold decrease in steady-state MTX. In contrast, the MTA steady-state level was not affected at all by TMQ (Fig. 3). When cells were resuspended into a large volume of drug-free buffer, 80–90% of both antifolates exited the cells rapidly. The exchangeable MTA level achieved (1.45 ± 0.02 μM) was ~2.5 times greater that that of MTX (0.61 ± 0.02 μM), with an MTA chemical gradient across the cell membrane of 1.5:1 based upon a ratio of intracellular water to dry weight of 3.5 μl/mg in L1210 cells (23).

Formation of MTA Polyglutamates in L1210 and L51 Cells. MTA and its polyglutamate derivatives were analyzed by high-performance liquid chromatography after incubation for 2 h with 1 μM radioactive drug in both L1210 and L51 cells (Fig. 4). Radioactive MTA and MTA-triglutamate were identified by comparing their elution times with those of nonlabeled standard compounds on a reverse phase column. Identification of MTA triglutamate, eluted at 43 min, and the monoglutamate, eluted at 57 min, permitted assignment of the diglutamate at 49 min. The two congeners that eluted more rapidly at 36 and 39 min were assigned as the penta- and tetraglutamates, respectively. In L51 cells, only a small fraction of total MTA was polyglutamated, as compared to L1210 cells, in which majority of drug was metabolized. The *bottom panel* of Fig. 4 compares the levels of MTA and MTA polyglutamates in the two cell lines per mg of dry weight. Total drug accumulation after a 2-h exposure to MTA in L51 cells was about 35% that of L1210 cells. Polyglutamates in L51 cells were about 12% that of L1210

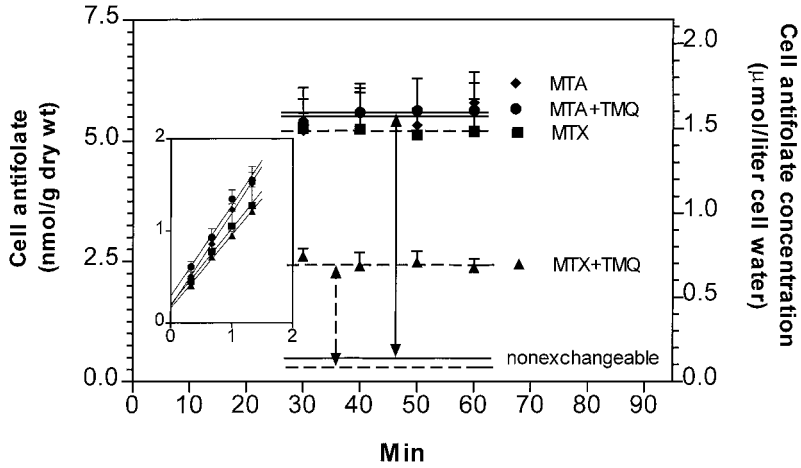

Fig. 3 Determination of exchangeable MTA or MTX in L51 cells. L51 cells were incubated in HBS for 20 min at 37°C and then exposed to 1 μM tritiated MTA or MTX. Initial uptake was assessed over 20–80 s; steady-state levels were monitored over 30–60 min. To study the effect of TMQ on MTA or MTX net uptake, 10 μM TMQ was introduced 15 min before tritiated MTA or MTX was added. A portion of cells at 90 min (only those with TMQ present) were centrifuged, suspended in a large volume of drug-free HBS, and incubated for 40 min to assess exchangeable and nonexchangeable MTA or MTX levels (—— and – – – –, respectively). *Right ordinate*, intracellular concentrations of antifolate in μmol/liter of cell water based upon a ratio of intracellular H_2O (μl) to dry weight (mg) of 3.5 in L1210 cells (23). *Vertical solid* and *dashed arrows*, exchangeable MTA and MTX levels, respectively. The data are the average of three separate experiments ± SE.

cells, whereas the MTA monoglutamate level was slightly greater (19%) than that of L1210 cells.

A Comparison of Influx and Efflux Kinetics for MTA and MTX in L51 Cells. Because the steady-state levels for free MTA and MTX differ by a factor of 2.5, this should be reflected in a comparable difference in the net unidirectional fluxes of these antifolates across the cell membrane. As indicated in Table 1, increased influx of MTA as compared to MTX was attributable to a 2-fold higher affinity for RFC1, an effect opposed by a lesser decrease in the influx V_{max} for this agent. This resulted in an influx V_{max}/K_t for MTA that was ~30% greater than observed for MTX. Efflux kinetics for these agents are also shown in Table 1. Here, L51 cells were loaded with either tritiated MTX or MTA, and the rate constants for the loss of exchangeable drug were measured. The rate constant for MTA efflux was 40% that of MTX. Hence, the small increase in MTA influx kinetics along with the much larger decrease in the efflux rate constant accounted for the enhanced concentrative transport of this agent relative to MTX.

Effect of Inhibition of Exit Pumps on Free MTX and MTA Levels in L51 Cells. Folate efflux is mediated by both RFC1 and unrelated energy-dependent exporter(s) (24–26). Because the influx V_{max}/K_t for MTA was increased but efflux was substantially lower than that of MTX, this might be attributed to a lower level of utilization by MTA of the exporter routes relative to MTX. If this were the case, inhibition of the exporters should augment exchangeable MTX to a greater extent than exchangeable MTA. This laboratory has reported that PGA_1, at a concentration of 7 μM, inhibits the exit pumps for MTX without a significant effect on RFC1-mediated influx, thus increasing free intracellular drug (27). As shown in Fig. 5, the exchangeable intracellular MTA level, when extracellular drug was 0.3 μM, approximated the exchangeable intracellular MTX level when extracellular MTX was 1 μM, consistent with the observation that MTA transport is more concentrative than MTX under these conditions. PGA_1 at 7 μM increased the steady-state exchangeable MTX level by ~90%, but exchangeable MTA was increased only ~30% in L51, a 3-fold difference. When cells were brought to the steady state at equimolar extracellular drug levels (1 μM), the addition of 7 μM PGA_1 increased the ratio of steady-state exchangeable MTX to MTA to 1.8 ± 0.1 (based upon the average of four separate experiments; data not shown). Hence, MTA appears to be a poorer substrate for the RFC1-independent exporters than MTX.

MTA Transport and Growth Inhibition in Cell Lines with Impaired RFC1-mediated Transport of MTX. A variety of cell lines have been developed in this laboratory that are resistant to MTX because of mutations in RFC1 that result in impaired transport (19, 20, 28–30). In the MTX^rA line RFC1 is mutated in the fourth transmembrane domain (A130P) with a marked loss of carrier mobility (19). The L1210-Ga line carries a mutation in the first transmembrane domain of RFC1, S46N, with a selective loss of carrier mobility (20). Both mutations result in substantial loss of 5-CH_3-THF and 5-CHO-THF transport, although in the latter case (S46N), the loss of reduced folate transport is less than that observed for MTX (20). Neither mutation results in a change in the affinity of carrier for these antifolates. MTA influx in L1210-G1a and MTX^rA cells was reduced to ~9 and 4% that of L1210 cells, respectively (Table 2), as compared to 1.0 and 2.6% for MTX influx (20). Associated with the decreased influx, the MTA IC_{50} was increased by factors of 5.8 and 22, and total MTA accumulation decreased by 74% and 90%, respectively, in L1210-G1a and MTX^rA cells as compared to L1210 cells when folic acid was the folate substrate in the growth medium. The IC_{50} for MTX was increased by factors of 12 and 71, respectively, in these cell lines (20). Thus, both RFC1 mutations resulted in impaired MTA transport and drug resistance, but to a lesser extent than for MTX.

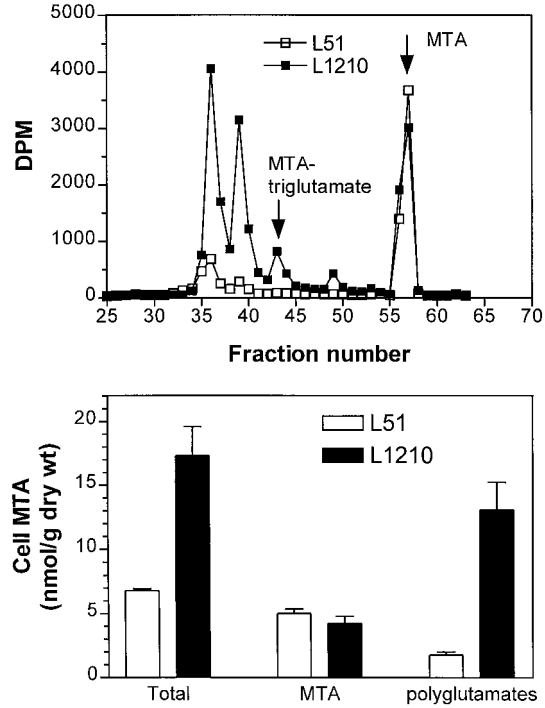

Fig. 4 High-performance liquid chromatography analysis of MTA and its polyglutamate derivatives in L1210 and L51 cells. *Top,* representative chromatograms of MTA and its polyglutamates separated by high-performance liquid chromatography. L1210 and L51 cells were incubated in HBS at 37°C for 20 min and then exposed to 1 μM [^{3}H]MTA for 2 h. MTA and its polyglutamates were extracted from cells and analyzed as described in "Materials and Methods." The MTA monoglutamate and triglutamate peaks, as identified by nonlabeled standards, are indicated by the *arrows*. *Bottom,* the levels of MTA and its polyglutamates normalized to dry weight of the cell pellet. The data are the mean ± SE of three separate experiments.

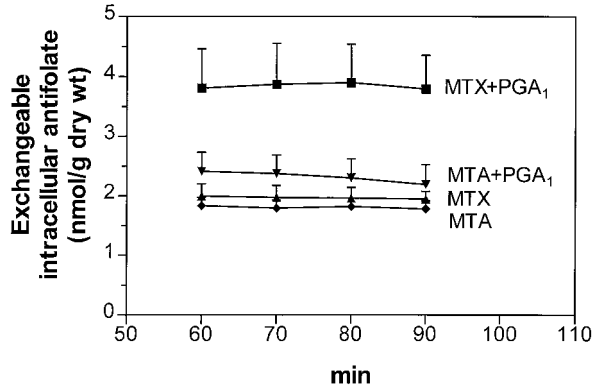

Fig. 5 Impact of PGA_1 on exchangeable intracellular MTA or MTX in L51 cells. After cells were incubated in HBS for 5 min and with 10 μM TMQ for additional 15 min, they were exposed concurrently to 0.3 μM [^{3}H]MTA or 1 μM [^{3}H]MTX at time 0, and incubation was continued for 30 min. Half of the cell suspensions were separated and incubated with 7 μM PGA_1 for another 30 min. Steady-state levels in the presence and absence of PGA_1 were then monitored every 10 min. The data are the mean ± SE from four separate experiments.

Table 1 Comparison of MTA and MTX influx and efflux kinetics

Influx kinetic parameters were obtained from nonlinear regression to the Michaelis-Menten equation in L1210 cells. Efflux was assessed by loading L51 cells with 2 μM [^{3}H]MTA or 2 μM MTX for 30 min, after which, cells were separated by centrifugation and resuspended into a large volume of drug free HBS, and cell antifolate levels were measured to determine initial efflux rates and the nonexchangeable component. The efflux rate constant was the slope of the line delineated by the log of the exchangeable cell antifolate level as a function of time after efflux was initiated. The data are the mean ± SE of three separate experiments.

	MTA	MTX	MTA/MTX
Influx K_t (μM)	3.7 ± 0.4	7.6 ± 0.4	0.48
Influx V_{max} (nmol/g dry weight/min)	7.4 ± 1.4	11.7 ± 1.5	0.63
Influx V_{max}/K_t	2.0	1.5	1.3
Efflux rate constant (10^2 min^{-1})	1.8 ± 0.2	4.4 ± 0.6	0.41

The same parameters were evaluated in cells grown in 25 nM 5-CHO-THF, a natural occurring folate that shares the properties of the physiological folate, 5-CH_3-THF, present in the blood of man and rodents. Although the 5-CHO-THF EC_{50} in MTX^{r}A cells was ~100 nM, obtained after cells were depleted of endogenous folates (20), MTX^{r}A cells grew with 25 nM 5-CHO-THF at only a slightly reduced rate. MTA influx under these conditions was decreased by ~40% in L1210 cells, slightly increased in the MTX^{r}A line, and unchanged in L1210-G1a cells. Most striking was the impact of the folate source on growth inhibition. Hence, as compared to wild-type L1210 cells, the MTA IC_{50} in MTX^{r}A cells increased by a factor of only 2.4, as compared to a 22-fold increase when folic acid was the growth source, and the change in IC_{50} was about one-half that observed with folic acid in the L1210-G1a line (Table 2). MTX resistance was also decreased in the MTX^{r}A line, but to a lesser extent than MTA, and was about the same in L1210-G1a cells when the folate source was shifted from folic acid to 5-CHO-THF. Accompanying the decrease in MTA resistance in L1210-G1 and MTX^{r}A cells was a ~2 or 3-fold increase, respectively, in MTA accumulation when 5-CHO-THF was used as folate source.

MTA influx in the resistant lines could not be attributed to transport via folate receptors despite the high affinity of this drug for this transporter (16). Hence, as indicated in Table 3, folic receptor expression, as assessed by surface binding, was quite low in all of the cell lines. Folate receptor expression actually decreased in the L1210-G1 line and was essentially unchanged in MTX^{r}A cells, as compared to expression in wild-type L1210 cells. Folate receptor expression was minimally increased (1.5–2 fold) when cells were grown in 5-CHO-THF *versus* folic acid, consistent with the lack of difference in MTA influx in the L1210-G1a line and a very minimal change in influx in the MTX^{r}A cells under these conditions (Table 3). There was no significant change in MTA influx measured at pH 5.5 in cells growing in 5-CHO-THF *versus* folic acid (data not shown). Thus, the low-pH folate transport system in L1210 cells (31, 32) did not contribute to alterations in MTA accumulation or activity when MTX-resistant cells were grown in 5-CHO-THF as compared to folic acid.

Table 2 Relationships among MTA influx, growth inhibition, and intracellular accumulation in RFC1-defective L1210 variants

Cells were grown in complete RPMI 1640 containing 2.2 μM folic acid or were transferred to, and grown in, folate-free medium supplemented with 25 nM 5-CHO-THF for at least 1 week, but no more than 3 weeks, before measurements were made. For MTA accumulation, cells were exposed to 50 nM [^{3}H]MTA for 3 days in the presence of 200 μM glycine, 100 μM adenosine, and 10 μM thymidine. All data are the mean ± SE of three separate experiments.

	MTA influx		MTA IC_{50}		MTX IC_{50}		Total MTA accumulation	
Cell line	nmol/g dry weight/min	%[a]	nM	Fold Δ[b]	nM	Fold Δ[b]	nmol/g dry weight	%[a]
Folic acid as the sole folate source								
L1210	1.7 ± 0.1	100	12 ± 1	1	7[c]	1	25 ± 2	100
L1210-G1a	0.16 ± 0.01	9.4	70 ± 8	5.8	85[c]	12	6.6 ± 0.5	26
MTX^TA	0.072 ± 0.002	4.2	260 ± 30	22	500[c]	71	2.6 ± 0.2	10
5-CHO-THF as the sole folate source								
L1210	0.98 ± 0.03	100	13 ± 2	1	14 ± 3	1	28 ± 3	100
L1210-G1a	0.17 ± 0.00	17	42 ± 3	3.2	150 ± 17	10	13 ± 1	46
MTX^TA	0.11 ± 0.01	11	31 ± 6	2.4	160 ± 10	11	8.0 ± 0.9	29

[a] Percentage of the level in L1210 cells.
[b] Fold change as compared to L1210 cells.
[c] From Ref. 20.

Table 3 Comparison of folic acid binding to cells grown with 2.2 μM folic acid or 25 nM 5-CHO-THF

Folic acid binding was assessed in cells grown in complete RPMI 1640 containing 2.2 μM folic acid or folate free-RPMI 1640 supplemented with 25 nM 5-CHO-THF. The LL1 cell line, which was adapted for the growth in 0.4 nM 5-CHO-THF and which stably overexpresses folate receptor α, was used as positive control (48). The folic acid binding capacity in LL1 line was 1300 ± 180 pmol/g dry weight. The data are the mean ± SE from three separate experiments.

Cell line	Folic acid bound to cells grown in 2.2 μM folic acid (pmol/g dry weight)	Folic acid bound to cells grown in 25 nM 5-CHO-THF (pmol/g dry weight)	Fold change, 5-CHO-THF/ folic acid
L1210	5.8 ± 0.4	11 ± 1	1.9
L1210-G1a	1.9 ± 0.2	3.6 ± 0.6	1.9
MTX^TA	8.5 ± 1.3	14 ± 2	1.6

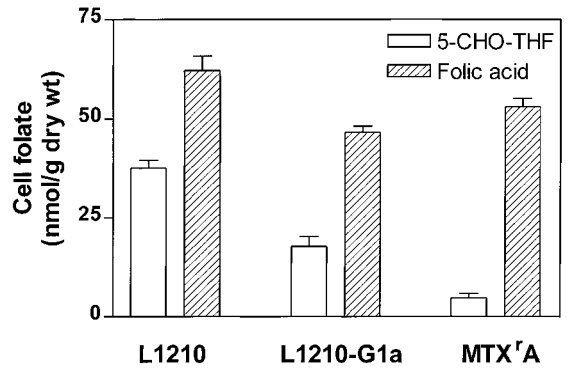

Fig. 6 Total folate cofactor accumulation in L1210, L1210-G1a, and MTX^rA cells when grown with 5-CHO-THF or folic acid. Cells grown in complete RPMI 1640 were harvested, washed, and grown exponentially for 1 week in folate-free RPMI 1640 supplemented with 25 nM [^{3}H]5-CHO-THF or 2 μM [^{3}H]folic acid. The cells were then processed for determination of intracellular radioactivity as described in "Materials and Methods." The data are the mean ± SE from three separate experiments.

Folate Cofactor Pools in the MTX-resistant L1210-G1a and MTX^rA Cell Lines. When cells were grown in 2.0 μM [^{3}H]folic acid, folate accumulation in L1210-G1a and MTX^rA cells was 75 and 85% that of L1210 cells, respectively, consistent with the observation that transport of folic acid under these conditions is mediated largely by a mechanism other than RFC1 (Fig. 6; Ref. 33). However, if 25 nM [^{3}H]5-CHO-THF was the folate source, the folate pool size in L1210-G1a and MTX^rA cells was reduced to 47 and 13% that of L1210 cells, respectively. Thus, the 8-fold drop in the MTA IC_{50} in MTX^rA cells was associated with a 3-fold increase in the accumulation of the drug (Table 2) and an 11-fold decrease in the natural folate pool (Fig. 6) when the folate source was changed from folic acid to 5-CHO-THF.

Growth Inhibition by MTA in L1210 Cells as a Function of the Extracellular 5-CHO-THF Concentration and Intracellular Folate Pool. As illustrated in Fig. 7, the level of intracellular folates rose as extracellular 5-CHO-THF was increased, whereas MTA sensitivity fell in an inverse relationship. Hence, as extracellular 5-CHO-THF was increased 39-fold from 0.64 to 25 nM and the intracellular folate pool increased 20-fold from 0.35 to 7.5 μM, the MTA IC_{50} increased by a factor of 13, from 1.3 to 17.3 nM. There was also an increase in the MTX IC_{50} but to a lesser extent, 3.5-fold. These data demonstrate the considerable impact the intracellular level of THF cofactors play in modulating the cell growth-inhibitory potency of MTA.

DISCUSSION

MTA is a promising new nonclassical antifolate that achieves its pharmacological effects by direct inhibition of enzyme sites downstream from DHFR (1). The current study was designed to broaden our understanding of the cellular pharmacology of this agent: its transport properties and polyglutamation in cells. In addition, this study assessed the consequences on MTA growth-inhibitory activity of alterations in tumor cell THF cofactor pool size and impaired transport via RFC1 in cell lines selected for resistance to MTX.

Polyglutamylation of MTA not only ensures cellular reten-

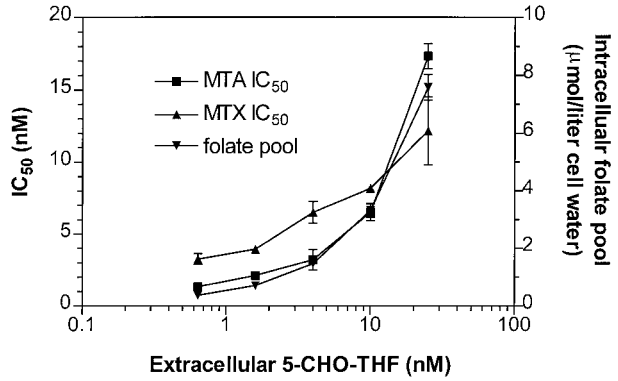

Fig. 7 Relationships among MTA or MTX $IC_{50,}$ intracellular folate pool size, and extracellular 5-CHO-THF concentration in L1210 cells. L1210 cells were grown in folate-free RPMI 1640 supplemented with different concentrations of 5-CHO-THF for at least 1 week before MTA or MTX IC_{50}s were determined. Intracellular folate pools were measured after cells were grown exponentially for 1 week in folate-free medium supplemented with different concentrations of [^{3}H]5-CHO-THF. The data are the mean ± SE from three separate experiments.

tion of the drug with the build-up of high levels of inhibitor within cells, but the addition of glutamate moieties markedly increases affinity of MTA for its two target enzymes, TS and GARFT. On the other hand, the affinity of MTA for DHFR is orders of magnitude lower, and is unchanged, when the drug is polyglutamated (1). Data in this paper indicate that there is essentially no binding of MTA to DHFR when the drug is present in cells at a concentration of 1.5 μM, a level more than 2 orders of magnitude greater than the K_i of MTA for this enzyme. Although some suppression of DHFR might be possible under conditions in which high levels of MTA polyglutamates accumulate within cells, there is considerable evidence to suggest that this is not of pharmacological importance. Hence, in MCF-7 breast and H630 colon human carcinoma cells lines that overexpress TS, the addition of hypoxanthine completely reverses the cytotoxicity of MTX, an effect that would not be possible if MTA depletes THF cofactor pools (2). Further, when cell are incubated with MTA at a concentration that totally suppresses cell growth, dihydrofolate does not accumulate as occurs if DHFR activity is eliminated, as observed with MTX (34–36).

MTA was very rapidly polyglutamated in wild-type L1210 cells under conditions in which there was essentially no polyglutamation of MTX; there was a profound difference in this property between the two antifolates. Enhanced polyglutamation of MTA is attribution both to high FPGS activity (3) for this agent and higher free MTA substrate levels relative to MTX in these cells. Polyglutamation was markedly reduced in an L1210 cell line, L51, in which FPGS activity is negligible but RFC1-mediated transport was unchanged relative to wild-type L1210 cells. This cell line proved to be a very useful model system for studying MTA transport properties that are complicated by rapid metabolism, such as efflux kinetics and steady-state transmembrane gradients. These studies documented the greater concentrative capacity for MTA relative to MTX and excluded the possibility that the higher levels of exchangeable intracellular MTA could be attributable to rapidly reversible binding to DHFR. Nor is it possible that there was appreciable reversible binding of the monoglutamate to TS or GARFT because the affinities of the monoglutamate for these enzymes are lower than that for DHFR (1).

The free levels of folates and antifolates in L1210 cells are governed by RFC1, a bidirectional anion exchanger, which creates small transmembrane folates gradients and is opposed by ATP-coupled exporters (23, 37, 38), likely to be members of the cMOAT family (39). Recent studies suggest that efflux of antifolates can be mediated by multidrug resistant proteins (MRP1 and MRP2; Ref. 40). Hence, influx of MTA and MTX is mediated by RFC1 alone; efflux is mediated by RFC1 and distinct exporters. In most cases, alterations in concentrative transport of MTX correlate with alterations in the RFC1-mediated entry process without significant change in efflux parameters in MTX-resistant lines (13).

In exploring the basis for enhanced concentrative transport of MTA relative to MTX in L1210 cells, the data suggested that this could not be attributed to differences in transport mediated by RFC1 alone. Hence, the MTA influx V_{max}/K_t was only ~30% greater than that of MTX. On the other hand, the MTA efflux rate constant was 40% that of MTX (Table 1), suggesting a large difference in transport mediated by the energy-coupled exporters. This conclusion was supported by the greater augmentation of free MTX levels relative to MTA by the exporter inhibitor, PGA_1, as would occur if a much lesser fraction of MTA efflux was mediated by this transporter under physiological conditions. Careful characterization of MTA *versus* MTX efflux mediated by energy-dependent exporters will require further study with an inside-out membrane vesicle system.

These studies demonstrate clearly that the THF cofactor pool size plays a critical role in modulating the growth-inhibitory effect of MTA. As the intracellular THF cofactor level increased with an increase in the extracellular 5-CHO-THF concentration encompassing, in part, the physiological range, there was a substantial increase in the MTA $IC_{50.}$ This is consistent with studies in mice in which a folate-deficient diet markedly increased MTA activity (41). Likewise, the activity and toxicity of DDATHF is highly sensitive to the folate status of animals (42). The level of natural folate pools within cells might modulate MTA activity by at least two mechanisms. First, as indicated as the current study, intracellular folates compete with and inhibit MTA polyglutamation at the level of FPGS. Consistent with this is the observation that the augmentation of the cellular folate pool, as a result of acquired mutations in RFC1 that enhanced carrier affinity for folic acid, markedly suppressed polyglutamation of DDATHF, rendering cells resistant to this agent (43). High folate pools may also compete with antifolates at the level of their target enzymes. For instance, expansion of folate pools increased the MTX $IC_{50,}$ but to a lesser extent than MTA, probably attributable to an increased build-up of dihydrofolate behind the block at DHFR as cellular THF cofactors are interconverted and then oxidized at TS, resulting in increased competition with MTX for the small fraction of DHFR required to sustain THF synthesis (35, 36). A related phenomenon was observed in a cell line selected for high-level resistance to pyrimethamine, a drug that does not form polyglutamate derivatives. In this case, resistance was

attributable to loss of exporter activity with markedly increased THF cofactor pools. These cells were cross-resistant to DDATHF and, to a lesser extent, to MTX (44).

Impaired antifolate transport, which results from mutations in RFC1, leads to cellular "folate deficiency," even when the extracellular 5-CHO-THF concentration is in the physiological range. This is attributable to a concurrent, although not always comparable, decrease in transport of the reduced folates that use the same carrier (20). The folate pool was not, however, substantially decreased when cells were grown with folic acid as the folate source because folic acid is a poor substrate for RFC1 (45) and enters cells largely by other mechanisms (27, 33), one of which is a low pH route (46). Most commercial media contain folic acid rather than 5-CHO-THF, which resembles the physiological substrate, 5-CH_3-THF. With these media, transport-related resistance to MTA, as well as to related agents, will be overestimated because cellular folate pools are minimally perturbed. It should be noted that even when MTX^rA and L1210-G1a cells were grown with 5-CHO-THF, they maintained a reduced, but still high-level, resistance to MTX but much lower or negligible resistance to MTA. Thus, tumors resistant to MTX because of deficiencies in transport via RFC1 may still retain appreciable sensitivity to MTA because of compensatory depletion of cellular THF cofactors. It follows then that any loss of transport, even that attributable to low RFC1 expression, as might occur with a change in regulation (47), would not necessarily be accompanied by comparable cross-resistance to MTA or related agents that require polyglutamation in the cell for activation and/or act at sites downstream to DHFR. Hence, these agents may have specificity and utility for use against tumors with acquired or intrinsic resistance to MTX attributable to impaired membrane transport.

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