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METHOTREXATE AND γ-*TERT*-BUTYL METHOTREXATE TRANSPORT IN CEM AND CEM/MTX HUMAN LEUKEMIC LYMPHOBLASTS

JOEL E. WRIGHT,*† ANDRE ROSOWSKY,* CAROL A. CUCCHI, JENNIFER FLATOW and EMIL FREI III‡

Dana-Farber Cancer Institute and Departments of *Biological Chemistry and Molecular Pharmacology and ‡Medicine, Harvard Medical School, Boston, MA 02115, U.S.A.

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Abstract—In a continuing investigation of determinants of their 200-fold methotrexate resistance and their collateral sensitivity to γ-*tert*-butyl methotrexate, the ability of CEM/MTX cells to transport the two drugs was analyzed and compared with that of CEM cells. The K_m and V_{max} values for the influx of methotrexate into CEM cells did not differ significantly from those of CEM/MTX cells, and this was the case for γ-*tert*-butyl methotrexate as well. Surface binding and influx rates were proportional to cell surface area, but differences in efflux rates and methotrexate uptake were too large to be explained on this basis. Neither methotrexate nor trimetrexate competed with γ-*tert*-butyl methotrexate influx in CEM cells. However, both drugs perturbed the γ-*tert*-butyl methotrexate steady state in CEM cells, resulting in slightly less uptake than with γ-*tert*-butyl methotrexate alone. However, the major difference between the two cell types was in the methotrexate uptake plateau, which was much greater in the case of the parental cell line. A related observation was the more rapid efflux of methotrexate from CEM/MTX cells than from CEM cells. The poor uptake, the associated meager capacity to polyglutamylate methotrexate and the enhanced methotrexate efflux appear to be responsible for its decreased activity against CEM/MTX cells. Half-lives for γ-*tert*-butyl methotrexate efflux were the same in both cell lines, allowing the drug to accumulate to cytotoxic levels despite its inability to form polyglutamates.

For nearly five decades, clinicians and cancer biologists have struggled with the problem of methotrexate (MTX)§ resistance. Seeking to overcome this difficult problem, medicinal chemists have synthesized a large number of MTX analogues [1, 2] An important outcome of this effort was the development of nonclassical lipophilic antifolates that can overcome MTX resistance caused by transport defects [3]; well-known examples of such compounds are trimetrexate (TMQ) [4, 5] and piritrexim [6, 7]. A different class of lipophilic antifolates are the MTX derivatives in which the γ-position of the glutamate side chain is esterified [8–11]. The rationale for preparing these compounds was that decreased negative charge and increased lipophilicity would improve cellular penetration. Modification of the γ-position was found to be appropriate because, unlike α-esterification, high dihydrofolate reductase (DHFR) binding affinity was maintained [8]. These considerations led to the synthesis of γ-*tert*-butyl methotrexate (TBM), which proved to be active against several MTX-resistant murine and human tumor cell lines [11]. Due to the sterically hindered nature of the *tert*-butyl group, TBM is stable in the presence of cellular esterases, in contrast to primary and secondary MTX esters. Moreover, since the γ-carboxyl group is blocked, TBM is not a substrate for folylpolyglutamate synthetase (FPGS) [12], and its effect on cell growth is not dependent on the level of intracellular FPGS activity.

Previously reported cytotoxicity assays with CEM human leukemic lymphoblasts and a derived subline, CEM/MTX, showed that the latter cells were about 200-fold resistant to MTX. The respective IC_{50} values were 0.032 and 6.6 μM for 48 hr of MTX exposure. In parallel determinations with TBM, the corresponding values were 0.62 and 0.50 μM. Thus CEM/MTX cells showed 24% collateral sensitivity to the monoester [11]. Several studies of MTX uptake in CEM and CEM/MTX cells revealed that diminished drug accumulation was an important determinant of MTX resistance in the latter cells [13–15]. Our preliminary analysis of TBM and MTX uptake in CEM and CEM/MTX cells suggested that TBM could overcome this defect by adopting an alternative transport route [16]. In the present work, we sought additional information on the transport kinetics of TBM and MTX in CEM and CEM/MTX cells, and the competition of TBM with MTX or TMQ for uptake. Our objective was to examine, in greater detail, the cellular mechanisms responsible for the 200-fold MTX resistance of CEM/MTX cells and their collateral sensitivity to TBM.

† Corresponding author: Dr. J. E. Wright, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115. Tel. (617) 632-3123; FAX (617) 632-2410.

§ Abbreviations: MTX, methotrexate, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid; DHFR, dihydrofolate reductase, 5,6,7,8-tetrahydrofolate: NADPH oxidoreductase (EC 1.5.1.3); TBM, γ-*tert*-butyl methotrexate; [^{3}H]MTX, [3′,5′,7-^{3}H]methotrexate; [^{3}H]TBM, γ-*tert*-butyl [3′,5′,7-^{3}H]methotrexate; TMQ, trimetrexate, 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl] quinazoline; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; and IME, Richter's improved folate-free medium.

METHODS

Drugs and radiochemicals. γ-*tert*-Butyl [3′,5′,7-^{3}H]methotrexate ([^{3}H]TBM) was prepared by our published method [17]. [3′,5′,7-^{3}H]Methotrexate ([^{3}H]MTX) was purchased from Moravek Biochemicals, Brea, CA. Radiochemical purity was monitored by HPLC. Significant radiochemical decomposition was found after about 8 weeks at −20°, requiring repurification by preparative HPLC on a Nova-Pak C_{18} cartridge (Waters, Marlborough, MA) using 0.01 M ammonium acetate, pH 7.9, with 3% ethanol (MTX) or 20% ethanol (TBM) as the eluent. Prior to the transport experiments, [^{3}H]MTX and [^{3}H]TBM were diluted with the corresponding non-labeled compounds to final specific activities of 5.1 and 6.8 Ci/mmol, respectively. For radioligand binding assays of DHFR, the specific activity of [^{3}H]-MTX was 20 Ci/mmol. TMQ was a gift from the Warner-Lambert Co., Ann Arbor, MI.

Cells. CEM and CEM/MTX cells for transport experiments were grown in suspension culture at 37° under 8% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Doubling times were determined by periodic hemocytometric measurement of cell density, starting at 1×10^5 cells/mL and continuing until a plateau was reached. Diameters of 5000 cells from each cell line were measured with a Coulter counter (model Z_f) [18]. Surface areas and volumes were calculated by assuming the cells to be spherical. For DHFR measurement, the cells were washed with phosphate-buffered saline (PBS) and resuspended at a density of 1×10^8 cells/mL in 0.15 M potassium chloride adjusted to pH 7.5 with potassium phosphate buffer. The suspensions were sonicated at 0–4°, using three 10-sec bursts at maximum miniprobe power and 20 sec of chilling between bursts. Three 20-μL aliquots were digested in 980 μL of 1 N sodium hydroxide for protein assay [19]; the remaining suspension was centrifuged at 0–4° for 15 min, and the supernatant was analyzed for DHFR content as described below.

DHFR analysis by radioligand binding assay. Intracellular DHFR content was determined by the method of Ohnoshi and coworkers [20] as modified by Drake and coworkers [21]. Thus, 800 μL of 100 μM reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) and 50 μL of 0.15 μM [^{3}H]-MTX (20 Ci/mmol) were incubated for 10 min at 20° with 0- to 150-μL aliquots of cytosolic extract in a total volume of 1.0 mL of 0.15 M potassium chloride in 0.01 M potassium phosphate buffer, pH 6.0. Non-bound radioligand was removed by addition of 500 μL of a 5% (w/v) aqueous suspension of activated charcoal (Fisher, Medford, MA) containing 1% dextran (Sigma, St. Louis, MO). The mixture was immediately filtered through a 0.45 μM Millex HA filter (Millipore, Bedford, MA) using a disposable 3-mL syringe. Aliquots of 500 μL of filtrate were added to 20 mL of Ready-safe scintillation fluid (Beckman, Fullerton, CA) and counted in a Beckman LS7000 instrument. Radioligand binding, expressed in picomoles, was plotted as a function of volume of cytosolic extract. Least squares analysis indicated a linear relationship ($R^2 > 0.99$). DHFR concentrations (pmol/mL) in the cell extract were calculated from the slope of this curve. From these values, the original densities of the cell suspension (10^8/mL), and the protein content (mg/10^8 cells), the intracellular DHFR content was converted into pmol/10^8 cells and pmol/mg protein.

Transport. Prior to uptake or efflux experiments, dead cells and debris were selectively removed from aliquots of 1×10^8 trypan blue excluding CEM or CEM/MTX cells as previously reported [22]. The clarified cell suspensions were gently pelleted and resuspended in Richter's improved folate-free medium (IME) (GIBCO, Grand Island, NY) supplemented with 10% dialyzed fetal bovine serum, 2 mM L-glutamine, and either [^{3}H]MTX or [^{3}H]-TBM at 37°. For determination of surface binding, parallel measurements were performed at 0–4°. Timing was begun immediately, and 1.0-mL aliquots were removed at 0.5-min intervals over the first 3 min for determination of MTX and TBM influx rates or at longer intervals from 1 to 20 min to obtain TBM uptake curves. Uptake of 0.5 or 1.0 μM [^{3}H]-TBM in the presence of simultaneously added equimolar concentrations of non-labeled MTX or TMQ was measured at appropriate time intervals from 1.0 to 120 min.

For efflux measurements, 1×10^8 trypan blue excluding cells from each line were gently pelleted and resuspended in growth medium containing 50 μM [^{3}H]MTX or [^{3}H]TBM, and were then incubated at 37° for 2 hr. They were then re-pelleted, washed with ice-cold PBS, and resuspended in 20 mL of IME in a water bath at 37°. Aliquots of 1.0 mL were removed at 2-min intervals between 0 and 12 min and at 10-min intervals from 20 to 50 min. In both the uptake and efflux experiments, when intervals between samplings were 5 min or more, the cells were returned to the incubator.

Aliquots from all the transport experiments were quenched in 14 mL of ice-cold PBS, and the cells were pelleted, washed twice with ice-cold PBS and re-pelleted. The final pellet was dissolved in 1.0 mL of 1 N NaOH overnight, and the radioactivity in an aliquot of each digest was determined by scintillation counting in Dimiscint (Dupont NEN, Billerica, MA), an alkali compatible fluid. The remaining volume of cell digest was used for protein assay [19]. Replicate experiments were performed twice on different days.

Influx rates determined over the first 3 min of uptake were calculated by the linear least-squares method, and were plotted as a function of the external concentration of MTX (five data points, 0.5 to 4 μM) or TBM (six data points, 0.5 to 10 μM). Four replicate experiments were performed on different days. K_m and V_{max} values were obtained by least-squares analysis at Lineweaver–Burk plots, and apparent first-order rate constants (k') for efflux were calculated using a kinetic data analysis program [23]. Differences between mean K_m and V_{max} values were tested for significance by Student's *t*-test [24].

RESULTS

A comparison of the uptake of 1.0 μM TBM by

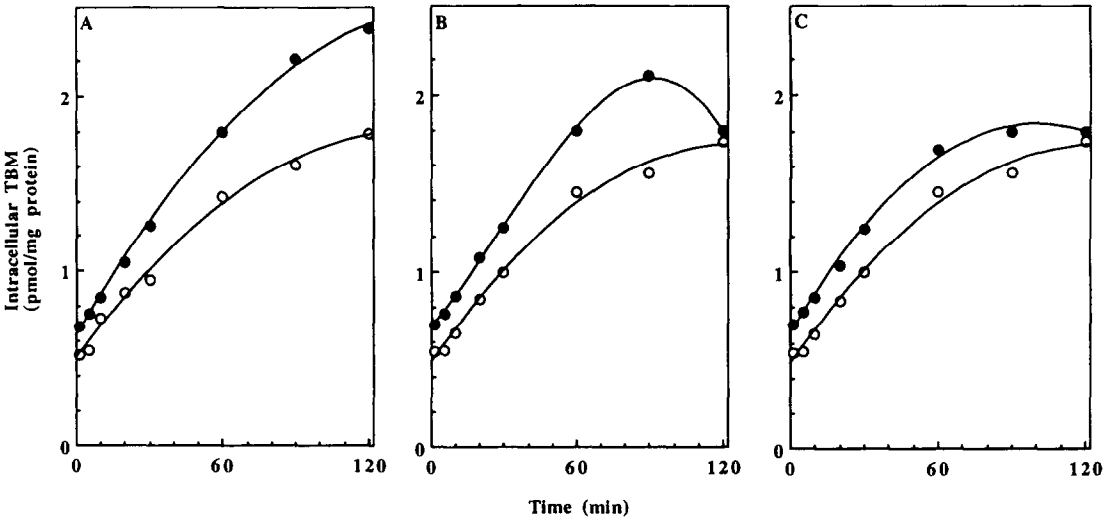

Fig. 1. Intracellular TBM versus time of exposure to external 1.0 μM [^{3}H]TBM. Key: (●) CEM; and (○) CEM/MTX cells. (A) [^{3}H]TBM alone; (B) [^{3}H]TBM + equimolar MTX; (C) [^{3}H]TBM + equimolar TMQ. Each data point is the mean of two separate determinations. The range of values for individual points was within 7% of the mean.

CEM and CEM/MTX cells is presented in Fig. 1A. Uptake was linear for at least 20 min, but slow accumulation continued even after 120 min. The steady-state level in CEM cells, calculated from the progress curve by first-order kinetic analysis, was 2.6 pmol/mg protein above the nonspecific surface binding level. The corresponding value in CEM/MTX cells was 1.7 pmol/mg protein. On this basis, TBM uptake in CEM/MTX cells was only 35% less than in the parental line. In units of pmol/10^8 cells, however, CEM/MTX cells took up 60% less TBM than the parental cells. Data obtained with 0.5 μM TBM were qualitatively similar (data not shown). Uptake of TBM in CEM cells differed from that of MTX, which had a steeper initial slope and was linear for only the first 4–6 min over a range of concentrations [13].

Lineweaver–Burk analysis of the initial slopes of TBM and MTX uptake curves yielded the kinetic constants in Table 1. The K_m values for MTX influx into CEM and CEM/MTX cells were 4.7 ± 1.0 and 7.4 ± 2.3 μM, respectively, with identical V_{max} values of 0.4 ± 0.06 pmol·min^{-1}·mg protein^{-1} for both cells. The differences in K_m were not statistically significant at $P = 0.05$. For TBM, the K_m values for CEM and CEM/MTX cells were 3.4 ± 1.0 and 3.2 ± 1.0 μM, respectively and the corresponding V_{max} values were 0.06 ± 0.006 and 0.05 ± 0.006 pmol·min^{-1}·mg protein^{-1}. Differences in K_m and V_{max} for TBM influx were also statistically insignificant, as previously reported [16]. However, the K_m for MTX efflux in CEM/MTX cells were significantly greater than that of TBM at $P < 0.05$. Surface binding of TBM, measured at 0–4°, corresponded very closely to the intercept of the 37° uptake data, estimated by extrapolation to zero time. When the external concentration of TBM was 1.0 μM, the nonspecific binding to CEM cells was 0.60 and the extrapolated intercept was 0.63 pmol/mg protein. Corresponding values for CEM/MTX cells were 0.48 and 0.50 pmol/mg protein.

Also compared in Table 1 are the mean diameter, surface area, volume, total protein and DHFR content for both cell lines. These parameters were evaluated because we had noted under the microscope that CEM/MTX cells were smaller. This being the case, we expected that CEM/MTX cells would show less surface binding of drugs, and might also contain less total protein per cell. The CEM/MTX cells were indeed found to have less surface area (314 vs 437 μm^2) and a smaller volume (524 vs 860 μm^3). The total protein content per cell (10.5 vs 17.3 mg/10^8 cells) was also smaller, but DHFR content of the two cell types was the same when the statistical overlap was considered (42 ± 5 vs 31 ± 6 pmol/10^8 cells). However, when the amount of DHFR was calculated in proportion to total protein per cell, it appeared to be slightly higher in the CEM/MTX cells (2.98 ± 0.16 vs 2.40 ± 0.20 pmol/mg protein). The doubling time of both cells was found to be 31 ± 0.5 hr.

The effects of non-labeled MTX and TMQ on [^{3}H]TBM uptake by CEM and CEM/MTX cells over a period of 2 hr are shown in panels B and C of Fig. 1. Neither MTX nor TMQ appeared to influence the initial uptake of TBM by the two cells over 20 min. However, there was a slight decrease in uptake after 90 min, presumably due to slow displacement of the TBM from DHFR or other possible intracellular binding sites by MTX and TMQ. As expected from the larger surface area of CEM cells in comparison with CEM/MTX cells, the amount of surface-bound MTX as well as surface-bound TBM, estimated by extrapolation to zero time, was greater in CEM cells. However, there was more surface-bound TBM than surface-bound MTX in both instances.

Efflux of MTX and TBM from either cell line

Table 1. TBM and MTX transport in CEM and CEM/MTX cells

	CEM	CEM/MTX
Cell characteristics		
Mean diameter (μm)*	11.8	10.0
Mean surface area (μm^2)	437	314
Mean volume (μm^3)	860	524
Protein content (mg/10^8 cells)†	17.3 ± 0.8	10.5 ± 1.7
DHFR (pmol/mg protein)†	2.40 ± 0.20	2.98 ± 0.16
DHFR (pmol/10^8 cells)	42 ± 5	31 ± 6
TBM influx‡		
K_m (μM)	3.4 ± 1.0	3.2 ± 1.0
V_{max} (pmol·min^{-1}·mg protein^{-1})	0.06 ± 0.006	0.05 ± 0.006
TBM efflux		
k' (min^{-1})	0.16	0.16
$T_{1/2}$ (min)	4.3	4.3
MTX influx		
K_m (μM)	4.7 ± 1.0	7.4 ± 2.3
V_{max} (pmol·min^{-1}·mg protein^{-1})	0.40 ± 0.06	0.40 ± 0.06
MTX efflux		
k' (min^{-1})	0.062 ± 0.030	0.42 ± 0.27
$T_{1/2}$ (min)	11	1.6

* Mean value for 5000 cells analyzed.
† Four replicates ± SD.
‡ Influx and efflux data for TBM and MTX represent two replicate experiments ± range.

obeyed first-order kinetics (Fig. 2). However, while MTX declined to steady state in CEM cells by 50 min, this level was approached in CEM/MTX cells after only 20 min. A high concentration of MTX was used deliberately in this experiment to ensure that the CEM/MTX cells still took up enough drug to permit efflux to be measured. At this concentration, CEM cells accumulated 80% more TBM than MTX during the preincubation. In CEM/MTX cells, accumulation of TBM was 90% greater. The MTX steady-state level after 50 min of efflux approximated that of cellular DHFR in both cells. However, efflux was much faster from the CEM/MTX cells, with a k' value of 0.42 ± 0.27 min^{-1} as compared with 0.062 ± 0.030 min^{-1} for the parental CEM cells. In contrast, the k' value for TBM efflux from CEM cells was the same as that from CEM/MTX cells (Table 1). Moreover, the difference between the

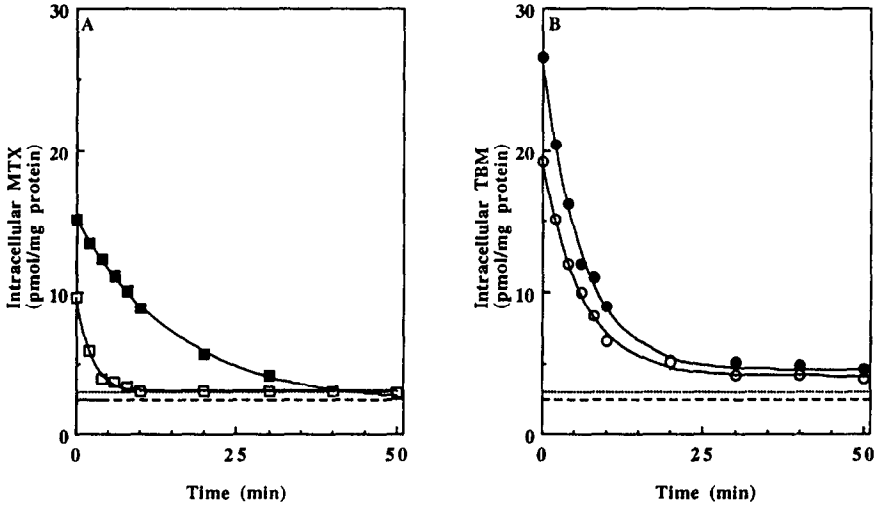

Fig. 2. Intracellular MTX or TBM versus time after resuspension into drug-free medium of CEM (■, ●) and CEM/MTX cells (□, ○) preloaded for 2 hr with 50 μM radioligand. (A) MTX efflux; (B) TBM efflux. Each data point is the mean of two separate determinations. The range of values for individual points was within 18% of the mean. DHFR levels: CEM (dashed line); CEM/MTX cells (dotted line).

amount of TBM in excess of the DHFR content (Fig. 2B) appeared to be greater in both cell lines at steady state than was the case with MTX.

DISCUSSION

Although the differences in K_m for MTX influx between CEM and CEM/MTX cells, discussed in our earlier report [16], were not statistically significant, the much higher uptake plateau in CEM cells [13] at all external concentrations of MTX was confirmed in the present work. No major difference in K_m or estimated steady-state concentration was observed with TBM over the concentration range 0.5 to 10 μM after surface binding was taken into account. This is consistent with our previous finding that CEM/MTX cells are not resistant to TBM [11], and supported the idea that TBM is taken up via a different pathway than the one used by MTX. The lower K_m for TBM as compared with MTX in CEM/MTX cells is noteworthy, considering that TBM is only a monocarboxylic acid. It is possible that the bulky *tert*-butyl ester group and lack of a negative charge at the γ-position prevent entry via the MTX/reduced folate pathway, and favor the use of an as yet unidentified alternative carrier. Binding of TBM to this carrier may be enhanced by its hydrophobicity.

Support for a separate influx pathway for TBM was also provided by the finding that initial uptake rates were not affected significantly by coincubation with equimolar MTX (Fig. 1B) or TMQ (Fig. 1C). While it could be argued that TBM uptake is unaltered by MTX or TMQ because TBM has higher affinity for the respective carriers of these drugs, we think this is unlikely because the same lack of effect was observed even in the presence of excess MTX or TMQ (data not shown). We conclude from these results that TBM, like TMQ in murine [4] and human [25] lymphoid cells, is not taken up by the same active transport pathway as MTX. However, our results also indicate that TBM does not share an active transport pathway with TMQ. This finding is consistent with the reported lack of cross-resistance between TBM and TMQ in murine P-388R cells, which are 22-fold resistant to TMQ by virtue of decreased influx and plateau accumulation [26]. The fact that displacement of TBM from CEM cells by MTX or TMQ was not observed for at least 90 min is of interest. Cellular TBM levels presumably start to decline when the MTX or TMQ levels become high enough to compete for binding to DHFR or other proteins. Our observation that TBM displacement from cells occurred relatively slowly in the presence of other tight-binding DHFR inhibitors like MTX and TMQ is consistent with more efficient uptake, and also suggests that dissociation of TBM from DHFR may be slow. It would be reasonable to speculate that the lipophilic γ-*tert*-butyl group in TBM would retard dissociation from the active site. However, detailed kinetic experiments on the interaction of TBM with DHFR would be needed to satisfactorily address this point.

The fact that MTX accumulation is greater in CEM than in CEM/MTX cells despite the similar kinetics of initial uptake indicated that the efflux rate would differ proportionately. The results shown in Table 1 and Fig. 2A demonstrate that this is indeed the case. The $T_{1/2}$ for MTX efflux was almost 7-fold longer for CEM cells than for CEM/MTX cells. This property, together with the diminished ability of CEM/MTX cells to polyglutamylate MTX [17], must contribute substantially to the observed resistance. It is likely that the two phenomena are linked since formation of slow effluxing MTX γ-di- and tri-glutamate species in CEM cells leads to enhanced plateau levels and CEM/MTX cells lack the ability to form significant amounts of these species [17]. When the incubation time was increased to 4 hr, we found evidence that substantial amounts of non-effluxing, longer chain MTX γ-polyglutamate species were formed. Thus, after 2 hr of incubation in the presence of 50 μM MTX followed by 50 min in drug-free medium, an efflux plateau very close to the DHFR content of both cells was observed, indicating that only stoichiometrically bound ligand was retained; however, after 4 hr in the presence of 50 μM MTX and then 80 min in drug-free medium, the efflux plateau in CEM cells was almost 2-fold greater than the DHFR level (data not shown). The rapidity with which MTX effluxes to steady state in CEM/MTX cells (Fig. 2A) even after 4 hr of preincubation is consistent with the previously reported polyglutamylation defect. In contrast to MTX, the efflux of TBM from both cells had the same half-life, maintaining a plateau somewhat higher than the DHFR level even after 50 min in drug-free medium. This suggests that an intracellular binding site other than DHFR may be enhancing retention of TBM, thereby contributing to its cytotoxicity.

Some of the differences between CEM and CEM/MTX cells appear to be related to the smaller cell diameter of the resistant subline. As shown in Table 1, the DHFR content in pmol/mg protein was 19% greater in CEM/MTX cells. However, when the molar amount of DHFR per cell was compared, the parental line was actually found to contain 35% more enzyme. The total protein content of CEM cells was about 64% greater than that of CEM/MTX cells, but again this should be adjusted for the difference in cell volume, which was likewise 64% greater in CEM cells. Thus, the total weight of protein per cell in the two lines was about the same. The finding of identical doubling times for the CEM and CEM/MTX cells is an important aspect of the model, since cytokinetic differences would make it difficult or impossible to correlate drug sensitivity with other resistance modalities.

Although CEM/MTX cells are about 200-fold resistant to MTX as compared with the parental cell line, TBM is more active against the MTX-resistant subline, indicating a degree of collateral sensitivity [16]. The ability of TBM to overcome MTX resistance in CEM/MTX cells is probably a result of several desirable features of the γ-*tert*-butyl ester group, including lipophilicity, and chemical stability which promote cellular accumulation of the drug with no requirement for polyglutamylation. Thus, its activity may remain undiminished in otherwise resistant cells with decreased cellular folylpolyglutamate synthetase activity.

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