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# Increased lysosomal uptake of methotrexate-polyglutamates in two methotrexate-resistant cell lines with distinct mechanisms of resistance

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MTX-PG, methotrexate polyglutamate

ABSTRACT

Methotrexate (MTX) resistance in mitoxantrone-selected MCF7/MX cells and in MTX-selected CEM/MTX cells is associated with reduced drug accumulation, albeit caused by different mechanisms. In addition, in both resistant cell lines the proportion of active long-chain MTX-polyglutamate (MTX-PG) metabolites is reduced relative to that in the respective parental cell line. Previous studies by others have implied that increased lysosomal uptake could affect the rate of MTX-PG hydrolysis, and hence the length distribution of the polyglutamate chains. However, in the two cell line pairs studied, the number of lysosomes per cell was not different between the corresponding parental and resistant cells. Instead, we observed a two- to three-fold increased facilitative uptake of MTX-$Glu_4$ by the lysosomes from these two independently derived MTX-resistant cell lines, compared to uptake by lysosomes from their corresponding parental cells. Enhanced lysosomal uptake of MTX-$Glu_4$ was reflected in an increased maximal uptake velocity, without a change in the apparent substrate affinity. In addition, the rate of MTX efflux from lysosomes from CEM/MTX cells was two-fold faster than from lysosomes from CEM cells. Consistent with this observation, the relative amount of short-chain MTX-$Glu_{1+2}$ species, as a fraction of the total amount of all MTX-$Glu_{1-4}$ species combined, was only half as large in lysosomes from CEM/MTX cells as in lysosomes from CEM cells. Together, these results suggest the possibility that increased lysosomal uptake, and hence enhanced sequestration of MTX-PGs in resistant cells, contributes to the development of high-level MTX resistance by decreasing the cytosolic levels of MTX-PGs.



## 1. Introduction

Methotrexate (MTX) is one of the most widely used drugs for the treatment of pediatric leukemias as well as breast cancer and sarcomas [1]. As for many other chemotherapeutic agents, the development of resistance to MTX therapy presents a severe challenge. Several resistance mechanisms have been described for MTX, including impaired cellular uptake [2–5]; increased efflux [6–9]; reduced binding to its target, dihydrofolate reductase (DHFR, E.C. 1.5.1.3) [2]; and decreased

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formation of MTX-polyglutamates (MTX-PGs), either through reduced synthesis by folylpolyglutamate synthase (FPGS, E.C. 6.3.2.17) [10,11] or through increased degradation by the lysosomal gamma-glutamyl hydrolase (gGH, E.C. 3.4.19.9) [12,13]. Recently, it was demonstrated that overexpression of the ABC transporter ABCG2 (also called Breast Cancer Resistance Protein, or BCRP) at the plasma membrane can confer MTX resistance [14]. For example, the human breast carcinoma cell line MCF7/MX, which expresses high levels of ABCG2 following continuous exposure to the structurally and functionally unrelated compound mitoxantrone, is highly cross-resistant to MTX [7,15]. Resistance in MCF7/MX cells is associated with increased ATP-dependent MTX efflux [7], and a lower proportion of long-chain MTX-PGs compared to the MCF7/WT parental cells [7,16]. A somewhat similar phenotype of reduced drug uptake, poor polyglutamylation, and enhanced drug efflux has also been described in the MTX-selected CEM/MTX cells [6,17,18]. However, while there was no evidence of reduced drug uptake in the MCF7/MX cells [7], reduced uptake appears to be the main mechanism of resistance in the CEM/MTX cells, and is caused by a mutation in the reduced folate carrier [19,20]. Thus, despite the somewhat similar resistance phenotypes of MCF7/MX and CEM/MTX cells, these cell lines' primary mechanisms of resistance appear distinct. Nevertheless, an interesting observation common to these two resistant cell lines was the reduced cellular amount of long-chain MTX-PGs, which could be caused either by a decreased production of MTX-PGs by FPGS, or an increase in the deglutamylation of MTX-PGs by gGH. However, at least in the MCF7/MX cells, neither the activities nor the protein levels of these enzymes differ from those of the parental cells [7]. Furthermore, it has previously been shown that ectopic overexpression of gGH in MCF7 cells does not confer resistance to short-term MTX exposure [21], implying that elevated gGH per se is insufficient to cause MTX resistance. Consistent with this conclusion is the hypothesis that the rate-limiting step in MTX-PG deglutamylation is the transport of MTX-PGs into the lysosomes, rather than the actual amount of gGH activity present [22]. Other investigators also proposed, based on theoretical considerations and mathematical modeling, that lysosomal MTX-PG uptake plays an important role in the metabolism of MTX and its polyglutamates [23]. However, although lysosomal uptake of MTX-PGs has been previously described and biochemically characterized in murine S180 cells [22,24,25], its role in MTX metabolism is poorly understood, and the molecular identity of the transporter has not been determined.

In the present study, we wished to determine whether the depletion of long-chain MTX-PGs in MTX resistant cells, relative to levels in the corresponding sensitive parental cells, could be caused, at least in part, by increased transport of MTX-PGs into lysosomes. To this effect, lysosomes were isolated from both parental and resistant cells, and the transport of MTX-$Glu_4$, as well as that of MTX, was characterized in vitro. The results show that facilitative transport of MTX-$Glu_4$ into lysosomes from the resistant cells is two- to three-times more efficient than transport into lysosomes from the sensitive cells, whereas there is little difference in the lysosomal uptake of MTX between sensitive and resistant cells.

## 2. Materials and methods

### 2.1. Materials

Radiolabeled [$^3$H]MTX and [$^3$H]MTX-$Glu_4$ were obtained from Moravek Biochemicals (Brea, CA). The purity of the drugs was checked by HPLC. Lysotracker Red was from Molecular Probes (Eugene, OR). All other reagents were from Sigma (St. Louis, MO).

### 2.2. Cell culture

The human breast carcinoma cell line MCF7/WT and its mitoxantrone-selected derivative MCF7/MX [26] were cultured in improved minimal essential medium (Richter's modification) containing 10% fetal bovine serum and penicillin/streptomycin at 37 °C in a 5% $CO_2$ atmosphere. The human T-cell leukemia cell line CCRF/CEM (abbreviated here as CEM) and its MTX-resistant derivative CEM/MTX [27] were received from Dr. Larry Matherly (Wayne State University, Detroit, MI) and were grown in RPMI1640 medium with 10% fetal bovine serum and penicillin/streptomycin at 37 °C in a 5% $CO_2$ atmosphere. All cultures were periodically tested for mycoplasma contamination and found to be negative.

### 2.3. Fluorescence microscopy: lysosome quantification with LysoTracker Red

MCF7/WT and MCF7/MX cells were cultured on coverslips in 35-mm dishes for 48–72 h, to approximately 70% confluence. The medium was removed and replaced with 100 nM LysoTracker Red for 1 h, prior to viewing under the fluorescent microscope. CEM and CEM/MTX cells were cultured in suspension to approximately $10^6$ cells/ml before adding 100 nM LysoTracker Red for 1 h. Cells were then spread on polylysine coated coverslips for analysis by fluorescent microscopy. Multimode DIC/fluorescence images were recorded on a DeltaVision workstation (Applied Precision, Issaquah, WA) with a $60 \times 1.4$ NA PlanApo lens. For quantification experiments, Z-series of live cells were recorded on a Nikon TE300 Quantum microscope (Nikon Instruments, Melville, NY), equipped with piezo Z-positioner (Physik Instrumente, Karlsruhe/Palmbach, Germany) and CoolSnap HQ CCD camera (Photometrics, Scottsdale, AZ). The system was driven by Isee software (Isee Imaging, Raleigh, NC). It took approximately 5 s to record a full Z-series through a cell (18 focal planes, with a stepsize of 0.5 μm) on our system. In addition to 3-D fluorescence, a single-plane phase-contrast image corresponding to a middle section of the cell was recorded for each data set. For determination of the number of lysosomes in individual cells, 3-D datasets were first deconvolved using SoftWorX 2.5 software (Applied Precision), and then maximal intensity projections were computed. Maximal intensity projections were superimposed on the corresponding phase-contrast images, and contours of individual cells were traced manually. Then, the number of lysosomes was determined automatically using algorithms available in Isee.

### 2.4. Preparation of lysosomes

The method used was adapted from that described by Storrie and Madden [28]. MCF7/WT or MCF7/MX cells were seeded and

grown to confluence on 150 mm × 25 mm plates. The cells from 10 plates were harvested by removal of the medium and scraping of the cells into phosphate-buffered saline. After centrifugation at 500 × *g* for 5 min at 4 °C, the cells were re-suspended in 10 ml of ice-cold TS buffer (0.25 M sucrose, 20 mM Tris–HCl (pH 7.4)) containing EDTA-free protease inhibitors (Roche, Indianapolis, IN), and were homogenized by nitrogen cavitation at a pressure of 125 psi for 20 min. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C to obtain the post-nuclear supernatant, which was then loaded on top of a Nycodenz/Percoll step gradient (35% Nycodenz/17% Nycodenz/6% Percoll) and centrifuged for 20 min at 20,000 × *g* at 4 °C. During this step, lysosomes became enriched at the 35%/17% interface. The fraction containing the lysosomes was collected and washed in 12 ml TS buffer, and then centrifuged for 20 min at 30,000 × *g* at 4 °C. The resultant pellet containing the lysosomes was re-suspended in 150–200 μl TS buffer, and the protein concentration was determined by the Bradford method [29]. CEM or CEM/MTX cells from 10 × T75 flasks were pooled and washed with PBS, followed by resuspension in TS buffer. Approximately 200 × $10^6$ cells were homogenized in 2–3 ml TS buffer using a Dounce homogenizer with 20 strokes in an ice-bath. The cell homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The resulting post-nuclear supernatant was then layered on top of a Nycodenz/Percoll step gradient (35% Nycodenz/17% Nycodenz/6% Percoll) and centrifuged at 20,000 × *g* for 30 min at 4 °C. The interface at 6% Percoll/17% Nycodenz contained the lysosomes, which were collected and washed with 12 ml TS buffer and centrifuged at 30,000 × *g* for 30 min at 4 °C. The pellets were re-suspended in 100–200 μl TS buffer, and the protein concentration was determined by the Bradford method [29].

### 2.5. β-Hexosaminidase activity

To assess the quantity and integrity (i.e., latency) of the lysosomes, we measured the activity of the lysosomal marker enzyme, β-hexosaminidase [30,31] by incubating between 4 and 12 μg protein for 30 min at 37 °C in a reaction mixture containing 100 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 9.6 mM *p*-nitrophenyl-*n*-acetyl-β-D-glucosaminide, and 50 mM citrate (adjusted to pH 4.6 with $Na_2HPO_4$) in the absence or presence of 0.25% (w/v) Triton X-100. The reaction was terminated by the addition of 1 ml of 0.4 M glycine (adjusted to pH 10.4 with NaOH). Standards of *p*-nitrophenol were prepared, and the absorbance of each of these and of the reaction products was measured at 405 nm. One unit of β-hexosaminidase activity corresponds to 1 nmol *p*-nitrophenol produced/(min mg protein). The relative latency was calculated as %latency = ([activity (+) TX-100 − activity (−) TX-100]/[activity (+) TX-100]) × 100. Latency of a typical preparation was between 50 and 70% for lysosomes from MCF7/WT and MCF7/MX cells, and between 30 and 50% for lysosomes from CEM and CEM/MTX cells. Total levels of β-hexosaminidase activity per mg protein were 400–500-fold higher in the isolated lysosomes than in the cell homogenate, indicating enrichment of lysosomes in the isolated fraction.

### 2.6. Drug uptake experiments

Unless stated otherwise, all drug uptake experiments were conducted in triplicate with freshly prepared lysosomes, using 5 μg lysosomal protein in a 25 μl reaction mixture containing 100 mM KCl, 5 mM $MgCl_2$, 20 mM MOPS (pH 7.2), 100 mM sucrose and [$^3$H]MTX or [$^3$H]MTX-$Glu_4$ (specific activity 100 pCi/pmol), essentially as described by Barrueco and Sirotnak [22]. All reaction mixtures were pre-warmed at 37 °C before the uptake was initiated by the addition of the equally pre-warmed lysosomes. Reactions were terminated by dilution into ice-cold wash buffer (0.25 M sucrose, 20 mM MOPS, pH 7.2) and rapid filtration through GF/F glass microfibre filters (Whatman, Maidstone, England). The filters were washed five times with ice-cold wash buffer, and the radioactivity remaining on the filter was counted the following day using a liquid scintillation counter. Unless indicated otherwise, results are expressed as pmol drug taken up per unit of β-hexosaminidase activity.

### 2.7. Time-dependent uptake assays

Lysosomes were incubated with 200 μM [$^3$H]MTX or [$^3$H]MTX-$Glu_4$ for various lengths of time ranging from 15 s to 10 min at 37 °C.

### 2.8. Osmotic sensitivity

Lysosomes from MCF7/MX cells were incubated with 200 μM [$^3$H]MTX or [$^3$H]MTX-$Glu_4$ for 10 min in the standard reaction buffer with increasing sucrose concentrations from 100 to 500 mM.

### 2.9. Determination of kinetic parameters

Drug concentrations ranging from 10 to 200 μM were used for the determination of $K_m$ and $V_{max}$ values. Reactions were incubated for 30 s, since uptake was demonstrated to be linear over this time period. The data were analyzed by non-linear regression, and $K_m$ and $V_{max}$ values were estimated according to Michaelis–Menten kinetics with the Prism4® (GraphPad, San Diego, CA) software package. Curves and best-fit parameters were compared by *F*-test, with a *p*-value of $p \leq 0.05$ considered as significant.

### 2.10. Hydrolysis of MTX-$Glu_4$ by lysosomes

Lysosomes (50 μg protein in 200 μl uptake buffer) from CEM and CEM/MTX cells were incubated with 200 μM [$^3$H]MTX-$Glu_4$ for 10 min at 37 °C. Lysosomes were then collected on GF/F membranes and washed five times with cold MOPS buffer (pH 7.2). The membranes containing the lysosomes were air dried, and then boiled for 5 min in 1 ml water. Aliquots were collected by centrifugation, lyophilized, reconstituted to 100 μl, and then analyzed for MTX-PG species by HPLC as described [12].

### 2.11. Efflux of MTX and MTX-$Glu_4$ from isolated lysosomes

Isolated lysosomes from CEM and CEM/MTX cells were incubated with 200 μM [$^3$H]MTX or [$^3$H]MTX-$Glu_4$ for 5 min at 37 °C, followed by 20-fold dilution into efflux buffer in the absence of any drug. The incubation was continued and

aliquots were then taken at various times from 0 to 10 min as indicated, and were analyzed for remaining drug as described above for the uptake studies.

## 3. Results

### 3.1. *Lysosomes in sensitive and resistant cells*

Increased intra-lysosomal drug sequestration in whole cells can be caused either by higher numbers of lysosomes per cell, as was observed in other anti-folate resistant cells [32], or by increased uptake per individual lysosome. Therefore, in order to assess the number of lysosomes per cell, we incubated the cells with the fluorescent lysosome-specific dye, LysoTracker Red, and counted the lysosomes. There was little difference in LysoTracker Red staining between sensitive parental and the corresponding resistant cells (Fig. 1). Parental MCF7/WT cells contained on average $102 \pm 16$ ($n = 5$) lysosomes per cell, whereas MCF7/MX cells contained $117 \pm 20$ ($n = 5$) lysosomes per cell; however, this difference was not statistically significant. In addition, the activity of the lysosomal marker enzyme β-hexosaminidase [30,31] was essentially identical in MCF7/WT and in MCF7/MX cells at $26.4 \pm 4.1$ nmol/(min mg protein) in the parental and $27.7 \pm 5.9$ nmol/(min mg protein) in the resistant cell homogenates. Similarly, there was no difference in the number of lysosomes per cell between sensitive CEM and resistant CEM/MTX cells ($96 \pm 18$ in CEM cells versus $105 \pm 25$ in CEM/MTX cells, $n = 11$ each, $p > 0.5$), or the activity of β-hexosaminidase ($620 \pm 140$ nmol/(min mg protein) in CEM homogenates versus $700 \pm 430$ nmol/(min mg protein) in CEM/MTX homogenates). These data suggest that it is unlikely that a difference in the number of lysosomes per se accounts for any differences in lysosomal drug uptake in whole cells.

### 3.2. *Drug uptake by lysosomes*

To determine whether MTX and its polyglutamylated analogue, MTX-$Glu_4$, accumulated differently in lysosomes from wild type and resistant MCF7 cells, we incubated isolated lysosomes with 200 μM of either drug species. Aliquots were

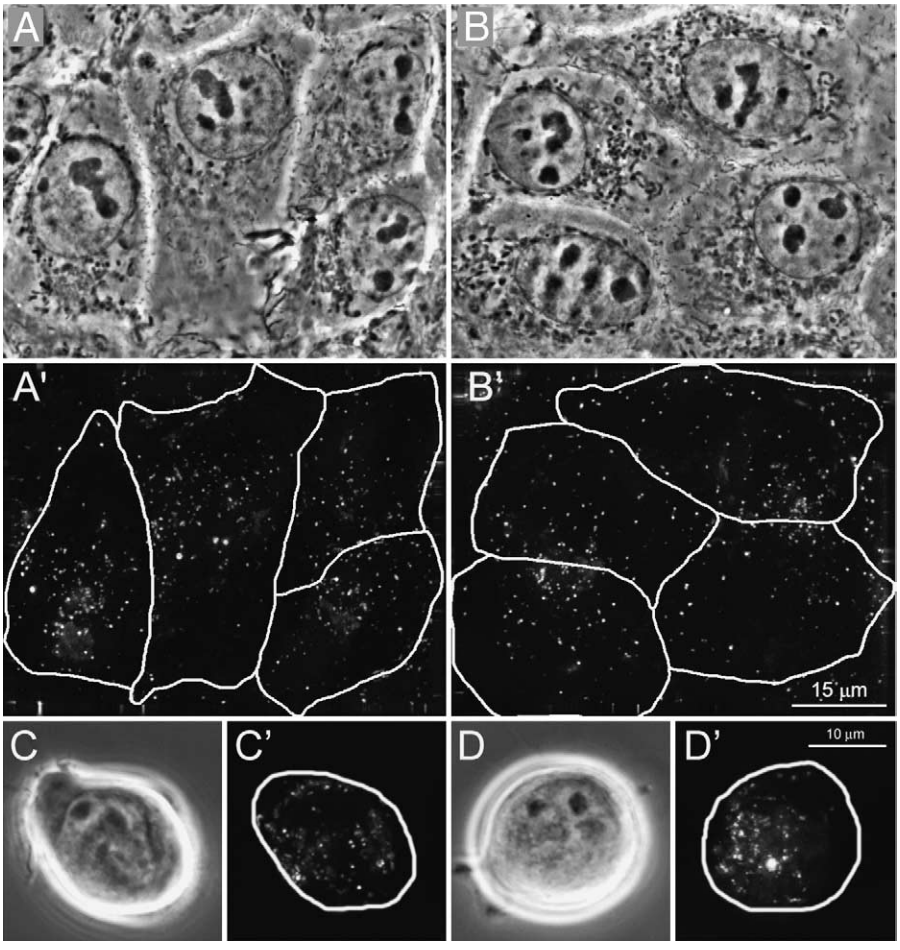


**Fig. 1 – LysoTracker Red staining of lysosomes in MCF7/WT and MCF7/MX, and CEM and CEM/MTX cells. The pattern and extent of lysosomal labeling by LysoTracker Red were determined by fluorescent microscopy of live confluent cultures of MCF7/WT (A, A′) and MCF7/MX cells (B, B′), and in CEM (C, C′) and CEM/MTX (D, D′) cells. Panels A–D show phase-contrast images of the cells; panels A′–D′ show LysoTracker Red staining within the manually traced cell boundaries (see Section 2). There was no significant difference in the number of lysosomes counted per cell between the corresponding parental and resistant cells.**

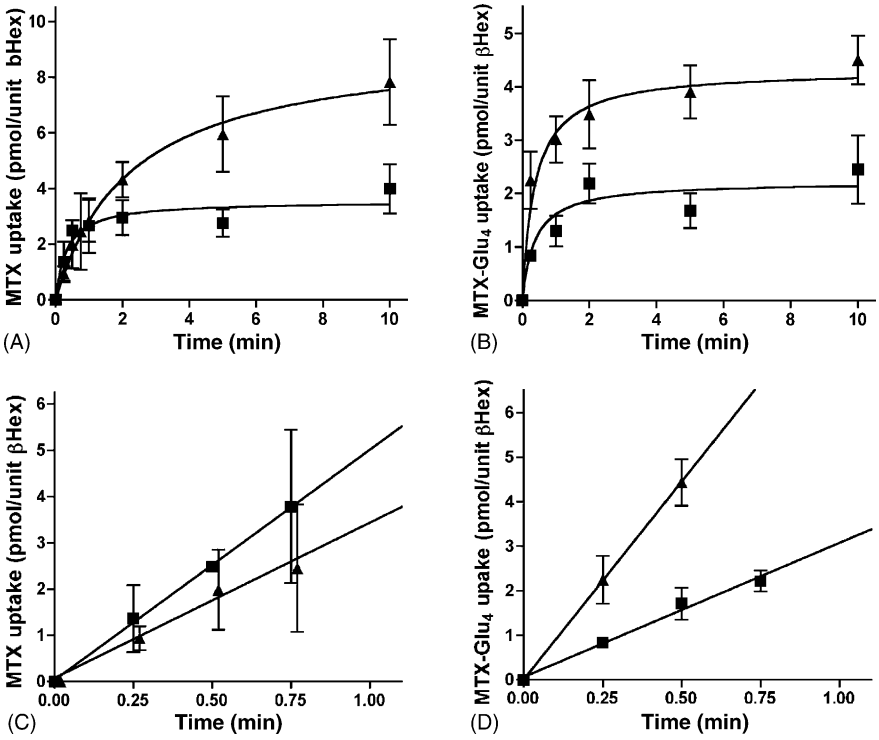


**Fig. 2 – Time-dependent uptake of MTX and MTX-$Glu_4$ by lysosomes isolated from parental MCF7/WT and resistant MCF7/MX cells. Time-dependent MTX (A) and MTX-$Glu_4$ (B) accumulation in lysosomes from MCF7/WT (■) and MCF7/MX (▲) cells was measured in the presence of 200 μM [$^3$H]MTX or [$^3$H]MTX-$Glu_4$. Data represent means ± S.E.M. of three to five independent experiments performed with separate lysosome preparations. Panels C and D show that uptake was linear for the first 30–45 s of drug incubation.**

removed and the amount of intra-lysosomal drug was measured at various time points. Fig. 2B shows the time-dependent accumulation of MTX-$Glu_4$ in lysosomes isolated from MCF7/WT and MCF7/MX cells. The initial rate of uptake by lysosomes from MCF7/MX cells was almost three times faster than the rate by lysosomes from MCF7/WT cells, ($8.87 \pm 0.02$ pmol/(min unit β-Hex) versus $3.02 \pm 0.23$ pmol/(min unit β-Hex); $p < 0.001$) (Fig. 2D), and the overall drug accumulation was higher in lysosomes from the MCF7/MX cells at each time-point. In contrast to the rates of MTX-$Glu_4$ uptake, the initial rates of MTX uptake were similar between lysosomes from parental and resistant cells (Fig. 2C). However, the total accumulation of MTX after 10 min in lysosomes derived from MCF7/MX cells was somewhat higher than in lysosomes from MCF7/WT cells (Fig. 2A).

In order to determine whether other MTX-resistant cells displayed a similarly increased lysosomal MTX-PG uptake, we isolated lysosomes from CEM cells and from their MTX-resistant derivative CEM/MTX cells. CEM/MTX resistant cells are similar to MCF7/MX cells in that they also exhibit increased MTX efflux and reduced MTX-PG formation (data not shown) [6]. However, one notable difference between these two cell lines is that ABCG2 protein was undetectable by Western blot in either the resistant CEM/MTX or the sensitive CEM cells (data not shown), whereas MCF7/MX cells exhibit very high levels of ABCG2 expression [14,15]. Consequently, the CEM/MTX cells are not collaterally mitoxantrone-resistant, nor are they sensitized by the ABCG2 inhibitor fumitremorgin C (data not shown). Fig. 3, panels B and D, shows that uptake of MTX-$Glu_4$ is both higher and faster by lysosomes from CEM/MTX cells than by those from parental cells, whereas there is only a small increase in lysosomal uptake of MTX in these resistant cells (Fig. 3A and C). Together, these results suggested that transport of MTX, especially that of its polyglutamate derivatives, into lysosomes is increased in these resistant cells.

### 3.3. *Osmotic sensitivity of drug transport*

In order to assess whether the uptake of MTX and MTX-$Glu_4$ by lysosomes was true transport as opposed to non-specific binding of drug, we altered the osmolarity of the uptake buffer by varying the sucrose concentration. As shown in Fig. 4, drug uptake was inversely proportional to the sucrose concentration, indicating that true transport into the lumen of the lysosomes was occurring. These results are in agreement with those reported by Barrueco and Sirotnak [22], who also found that maximum uptake of both MTX-$Glu_4$ and MTX occurred at 100 mM sucrose in the presence of 100 mM potassium chloride.

### 3.4. *Kinetics of drug uptake*

In order to further characterize the increased drug uptake by lysosomes from resistant cells, we measured short-term (30 s) uptake at increasing drug concentrations (Fig. 5A

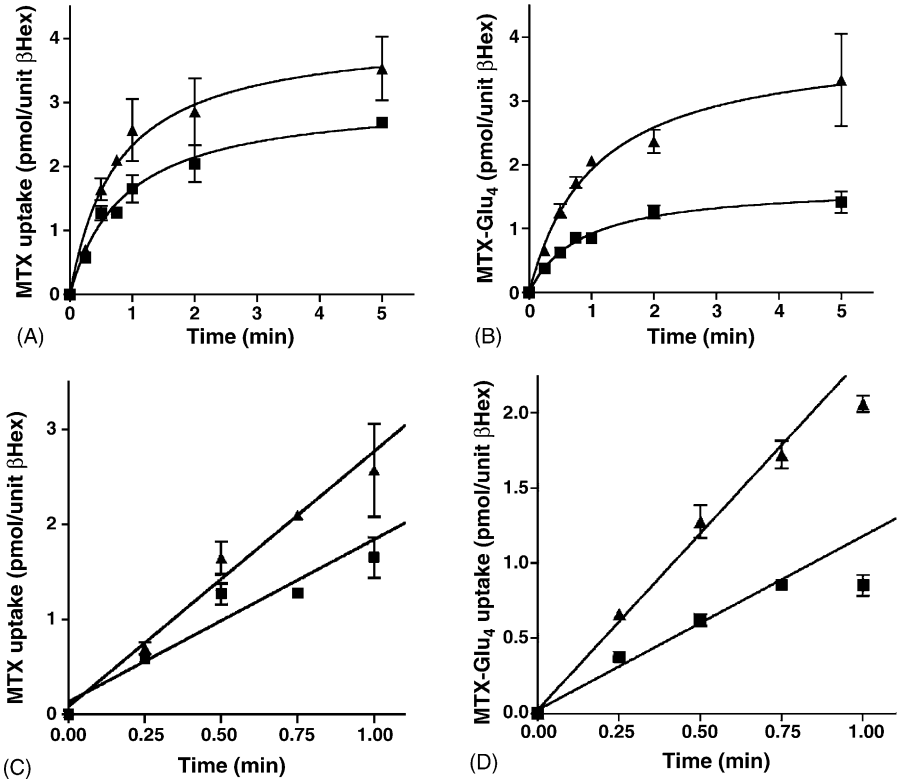


**Fig. 3 – Time-dependent uptake of MTX and MTX-$Glu_4$ by lysosomes isolated from parental CEM and resistant CEM/MTX cells. Time-dependent MTX (A) and MTX-$Glu_4$ (B) accumulation in lysosomes from CEM (■) and CEM/MTX (▲) cells was measured in the presence of 200 μM [$^3$H]MTX or [$^3$H]MTX-$Glu_4$. Data represent means ± S.E.M. of three independent experiments performed with separate lysosome preparations. Panels C and D show that uptake was linear for the first 30–45 s of drug incubation.**

and B), and determined the kinetic parameters, $K_m$ and $V_{max}$, for the transport of MTX and MTX-$Glu_4$ into isolated lysosomes (Table 1). Uptake of MTX-$Glu_4$ by lysosomes from both resistant cell lines was consistently higher than by lysosomes from the corresponding parental cells (Fig. 5A and B). Consequently, maximal velocities, i.e., $V_{max}$ values, for MTX-$Glu_4$ were also higher in lysosomes from the resistant cells than in those from the respective parental cells (Table 1). In contrast, the binding affinities for MTX-$Glu_4$, i.e., the apparent $K_m$ values, were not significantly different between lysosomes from the parental and lysosomes from the resistant cells. These characteristics together resulted in approximately two-fold higher transport efficiencies for MTX-$Glu_4$ in the resistant cells than in the corresponding parental cells, as shown by the higher $V_{max}/K_m$ ratios. In contrast, uptake of MTX was not significantly different between lysosomes from parental MCF7/WT and those from resistant MCF7/MX cells, and there were no differences in either the $V_{max}$ or $K_m$ values for MTX between lysosomes from the sensitive MCF7/WT cells and those from the resistant MCF7/MX cells. Overall similar results were obtained with lysosomes from CEM and CEM/MTX cells. Uptake of MTX-$Glu_4$ was higher by lysosomes from CEM/MTX cells than by those from CEM cells, whereas there was little difference in the uptake of MTX between the two types of lysosomes (Fig. 5B). Interestingly, however, the uptake of MTX by lysosomes from both the sensitive and resistant CEM cells was as high as that of MTX-$Glu_4$ by the lysosomes from the CEM/MTX cells. In contrast, uptake of MTX by lysosomes from either of the MCF7 cells was substantially lower than that of MTX-$Glu_4$. Nevertheless, these results together suggest that lysosomal transport of MTX-$Glu_4$ is elevated in the resistant cells.

### 3.5. *Lysosomal MTX-PG metabolism*

In order to determine whether there were differences in the intralysosomal degradation of MTX-PG, we incubated isolated lysosomes from CEM and CEM/MTX cells for 10 min with 200 μM [$^3$H]MTX-$Glu_4$, followed by analysis of the individual MTX-PG species by HPLC. Consistent with the overall higher uptake of MTX-$Glu_4$ by the lysosomes from the resistant cells, there were more total MTX-PGs in these lysosomes than in lysosomes from the sensitive cells (Fig. 6A). Furthermore, the amount of short chain MTX-PGs relative to the total amount of all MTX species combined was lower in the lysosomes from the resistant cells than in the lysosomes from the sensitive cells (17% versus 32% MTX-$Glu_{1+2}$) (Fig. 6B). This result suggested that the actual hydrolysis reaction was not elevated in the lysosomes from the resistant cells; rather, the observed differences in MTX-PG distribution may be due to altered efflux from the lysosomes.

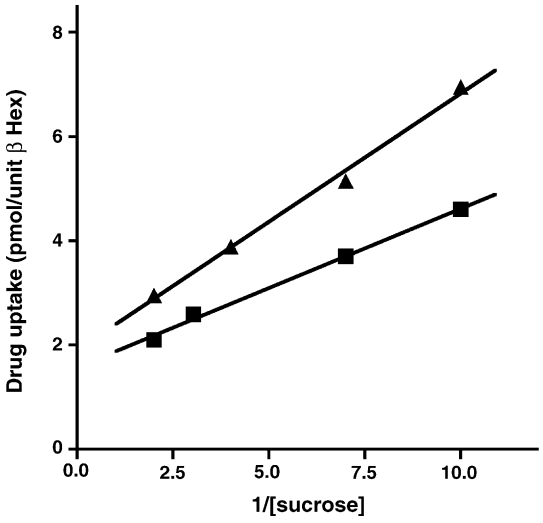


**Fig. 4 – Osmotic sensitivity of MTX and MTX-Glu$_4$ uptake. MTX (■) and MTX-Glu$_4$ (▲) uptake by lysosomes from MCF7/MX cells was measured in the presence of increasing glucose concentrations. Lysosomes were incubated for 10 min in the presence of 200 μM [$^3$H]MTX or [$^3$H]MTX-Glu$_4$.**

### 3.6. *Lysosomal MTX and MTX-Glu$_4$ efflux*

To determine whether MTX or MTX-PG efflux differed between lysosomes from sensitive and resistant cells, we incubated isolated lysosomes from CEM and CEM/MTX cells for 5 min with 200 μM [$^3$H]MTX or [$^3$H]MTX-Glu$_4$, followed by incubation in drug-free buffer, and measurement of the remaining MTX or MTX-Glu$_4$ at various times thereafter. Efflux was rapid, with a half-life for MTX-Glu$_4$ of approximately 1.3 min, and no difference was seen between the lysosomes from the resistant and those from the sensitive cells (Fig. 7B). In contrast, the rate of efflux of MTX from lysosomes derived from CEM/MTX cells was twice as fast as the rate from lysosomes derived from CEM cells, with respective half-lives for MTX efflux of 1.3 and 2.5 min ($p < 0.001$) (Fig. 7A). Thus, it is possible that the apparent relative depletion of short-chain MTX-PGs from lysosomes derived from resistant cells is the result of the more rapid efflux of these drug species from these lysosomes.

## 4. Discussion

The cellular uptake and subsequent fate of the anti-folate MTX are relatively complex and depend upon the interaction of several different reactions. In most cells, MTX enters via the reduced folate carrier [33]. Once inside the cell, MTX becomes polyglutamylated by FPGS, in a process that sequentially adds up to five additional glutamate residues to the parent compound and results in increased retention of the drug inside the cell [34,35]. Consequently, MTX is more likely to be cytotoxic to the cell since, in addition to the inhibition of dihydrofolate reductase, MTX-PGs also inhibit thymidylate synthase, as well as the purine biosynthesis enzymes glycinamide ribonucleotide formyltransferase and aminoimidazole carboxamide ribonucleotide formyltransferase [36–38]. Conversely, increased deglutamylation of MTX-PGs by gGH has been shown to reduce the cells' sensitivity to MTX [12]. Since deglutamylation occurs inside the lysosomes, the catabolic polyglutamate hydrolysis reaction is physically separated from the anabolic polyglutamate synthesis reaction.

It is well known that T-cell and some B-cell acute lymphoblastic leukemias that respond relatively poorly to MTX treatment accumulate lower levels of long-chain MTX-PGs than do sensitive B-cell leukemias [39]. The reason for this difference is not yet fully understood. However, based on a mathematical model, Panetta et al. concluded that "lysosomal transport of MTX-polyglutamates is likely to be important" [23]. A similar phenotype has also been observed in some drug-resistant cell lines [6,7]. For example, we have previously shown that MTX cross-resistant MCF7/MX cells accumulate proportionately less long-chain MTX-PGs than do drug-sensitive MCF7/WT cells. Similarly, MTX-resistant CEM/MTX cells exhibited a relative depletion of long-chain MTX-PGs, when compared to the parental CEM cells [18,20]. However, analysis of the activities of FPGS and gGH, respectively, did not reveal consistent changes in either enzyme that could account for the observed imbalance in MTX-PG distribution in these

**Table 1 – Kinetic parameters for uptake of MTX and MTX-Glu$_4$ by lysosomes derived from MCF7/WT, MCF7/MX, CEM, and CEM/MTX cells**

| | $K_m$ (μM) | | $V_{max}$ (pmol/(min unit β-hexosaminidase)) | | $V_{max}/K_m$ | |
|---|---|---|---|---|---|---|
| | MTX | MTX-Glu$_4$ | MTX | MTX-Glu$_4$ | MTX | MTX-Glu$_4$ |
| MCF7/WT | 54.7 ± 23.0 | 59.9 ± 22.8 | 1.62 ± 0.35 | 3.56 ± 0.72[b] | 0.030 | 0.059 |
| MCF7/MX | 51.0 ± 19.6 | 87.4 ± 27.7[ns] | 1.50 ± 0.29 | 7.59 ± 1.45[a] | 0.029 | 0.087 |
| CEM | 212.6 ± 78.2 | 273.5 ± 119.7 | 4.27 ± 1.24 | 2.87 ± 1.03 | 0.020 | 0.010 |
| CEM/MTX | 218.1 ± 57.6 | 180.9 ± 55.6[ns] | 5.38 ± 1.13[ns] | 3.89 ± 0.91[ns] | 0.025 | 0.022 |

$K_m$ and $V_{max}$ values were determined according to the Michaelis–Menten equation from the data presented in Fig. 5. One unit β-hexosaminidase activity corresponds to 1 nmol *p*-nitrophenol produced/(min mg protein). ns, not significant ($p > 0.1$) compared to corresponding values in the corresponding parental cell line.

[a] $p < 0.05$ vs. MTX-Glu$_4$ in MCF7/WT cell lysosomes and vs. MTX in MCF7/MX cell lysosomes.

[b] $p = 0.06$ vs. MTX in MCF7/WT cell lysosomes.

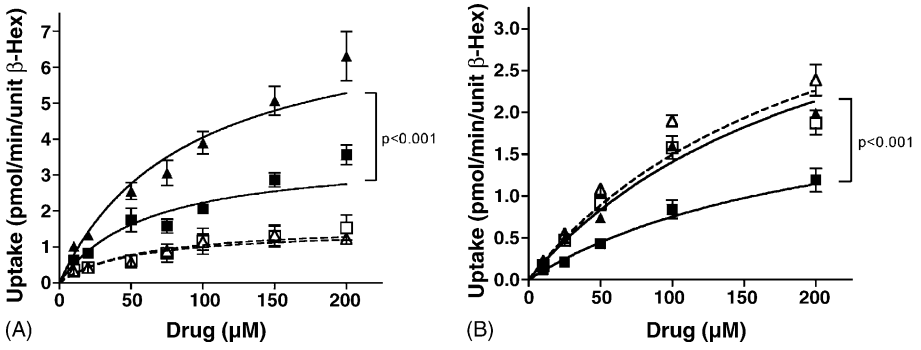


**Fig. 5 – Dose-dependent uptake of MTX and MTX-Glu$_4$ by isolated lysosomes. Lysosomes from (A) MCF7/WT (□, ■) and MCF7/MX (△, ▲) cells and (B) CEM (□, ■) and CEM/MTX (△, ▲) cells were incubated with various concentrations of [$^3$H]MTX (open symbols, dashed lines) or [$^3$H]MTX-Glu$_4$ (closed symbols, solid lines) for 30 s at 37 °C. Data represent means ± S.E.M. of three to five individual experiments performed with separate lysosome preparations, after individual values were corrected for non-specific binding of drug. Curves were fitted according to the Michaelis–Menten equation. The curves for MTX-Glu$_4$ were significantly different between MCF7/WT and MCF7/MX, and between CEM and CEM/MTX lysosomes, respectively, with p values of $p < 0.001$. The curve for MTX uptake by lysosomes from CEM cells is virtually identical to the curve for MTX-Glu$_4$ uptake by lysosomes from CEM/MTX cells and therefore cannot be distinguished.**

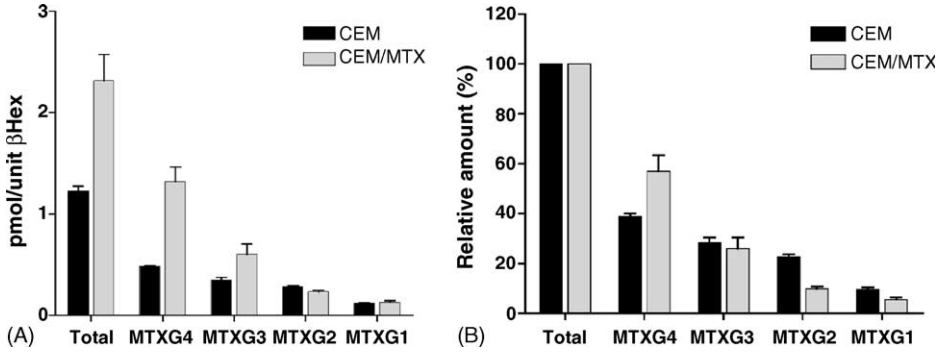


**Fig. 6 – Hydrolysis of MTX-Glu$_4$ in lysosomes from CEM and CEM/MTX cells. Isolated lysosomes were incubated with 200 μM [$^3$H]MTX-Glu$_4$ for 10 min, followed by analysis of individual MTX-PG species by HPLC. (A) Absolute amounts of each species; (B) amount of each species relative to the total amount of MTX and its polyglutamates. Results are the mean ± S.E.M. from three separate experiments.**

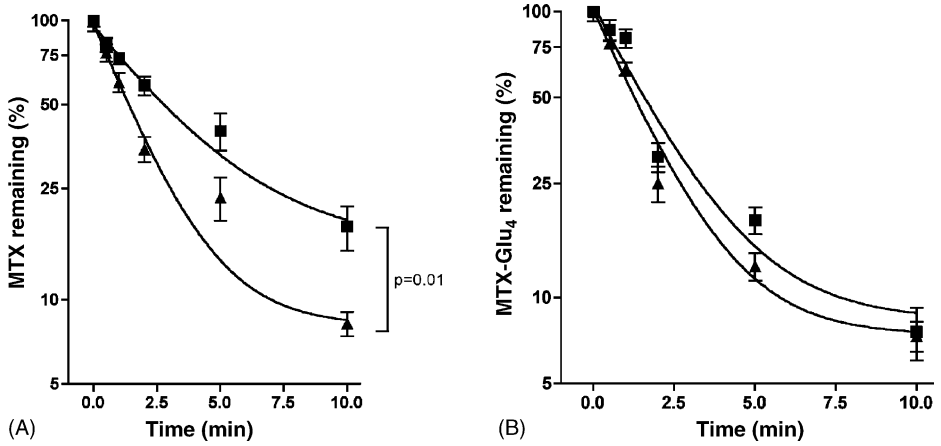


**Fig. 7 – Efflux of MTX and MTX-Glu$_4$ from isolated lysosomes from CEM and CEM/MTX cells. Lysosomes were incubated for 5 min with (A) 200 μM [$^3$H]MTX or (B) [$^3$H]MTX-Glu$_4$, after which time they were diluted 20-fold into efflux buffer and incubated for a further 0–10 min. At the indicted times aliquots were collected and analyzed for remaining drug. Data shown are the means ± S.E. from three separate experiments. Half-lives were calculated by linear regression from the normalized and log$_2$-transformed initial data points up to 2 min, the time period over which efflux was linear. CEM (■) and CEM/MTX (▲).**

two resistant cell lines [7,18,20]. Since gGH is a lysosomal enzyme, the MTX-PGs must traverse the lysosomal membrane before deglutamylation can occur. A facilitated lysosomal MTX-PG transport mechanism has been described and biochemically characterized in the murine S180 cell line, although the carrier responsible was not identified [22,24,25]. Furthermore, it was proposed that lysosomal uptake of MTX-PGs was the rate-limiting step for deglutamylation. However, to date little information exists concerning the possible role of this transport process in the regulation of cellular MTX-PG deglutamylation and/or anti-folate resistance. Here, we hypothesized that an increase in the actual uptake of MTX-PGs by lysosomes results in increased deglutamylation and hence contributes to the observed reduction in the accumulation of long-chain MTX-PGs.

In order to test this hypothesis, we measured the uptake of MTX-$Glu_4$, as well as that of MTX, by lysosomes isolated from two independent pairs of MTX sensitive and resistant cell lines. The data presented here indicate that uptake of MTX-$Glu_4$ by the lysosomes from the resistant cells was indeed increased when compared to uptake by the lysosomes from the respective parental cells. This difference was reflected in a higher maximum velocity for MTX-$Glu_4$ uptake with no apparent change in the apparent binding affinity, resulting in an almost two-fold higher transport efficiency ($V_{max}/K_m$). Both the maximal velocities and the binding affinities that we measured were similar to those reported previously by Barrueco and Sirotnak [22]. Surprisingly, however, we did not find significant differences between the apparent binding affinities for MTX and MTX-$Glu_4$. This lack of a difference is in contrast to the results reported in the literature for S180 cells. While the exact reasons for this discrepancy are not known, it may merely be due to experimental differences. Whereas Barrueco and Sirotnak inferred their binding affinities for MTX-$Glu_4$ from inhibition studies, the values presented here were obtained by direct measurements. Nevertheless, transport of MTX-$Glu_4$, was more efficient in the lysosomes from the resistant cells. Thus, the results presented here are consistent overall with the expected function of the proposed MTX-PG transporter in lysosomes. Furthermore, they support our hypothesis that increased activity of this as-yet unidentified transporter may contribute to the reduced levels of long-chain MTX-PGs observed in the resistant cells.

Lysosomes have been implicated in the sequestration of various anticancer agents. Sequestration reduces the drug's cytosolic concentration and its potential to be cytotoxic to the cells [40–48]. Furthermore, agents that inhibit lysosomal function have been shown to reduce lysosomal drug sequestration, and consequently, at least partially, to reverse drug resistance, in various cell lines [40,44–46]. By analogy, increased carrier-mediated uptake of MTX-PGs by lysosomes would reduce the cytosolic concentration of the drug (in addition to enhancing its deglutamylation), and could represent another component of the complex MTX-resistant phenotype. However, the extent of the contribution of this mechanism to the overall MTX metabolism and to resistance remains to be determined.

Interestingly, lysosomes from CEM/MTX cells exhibited not only an increased uptake of MTX and MTX-$Glu_4$, but also a more rapid efflux of MTX, but not MTX-$Glu_4$. This suggests that the overall turnover of MTX-PGs is also more rapid in these cells. Furthermore, it is possible that since MTX is the primary form that is transported out of the cell, when it is transported out of the lysosomes it is then directly moved to the plasma membrane for export from the cell, without being recycled through the cytoplasm and FPGS. The net result would be lower steady-state levels of MTX-PGs, and lower overall accumulation of total MTX in the cells, especially in conjunction with the overexpression of an ABC transporter such as ABCG2. While this is an attractive model, the fate of MTX after its deglutamylation in the lysosome is currently unknown, nor is the mechanism known which exports it from these organelles.

In summary, the work presented here documents for the first time that the facilitative transport of MTX-$Glu_4$ by lysosomes is increased in cells that are MTX-resistant. Increased lysosomal uptake of anti-folate polyglutamates could play a role in drug resistance by reducing the cytosolic concentrations of the drug and thereby limiting the drug's cytotoxic potential. Work is currently underway to identify the carrier responsible for the lysosomal uptake of MTX and its polyglutamates.

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