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# The Human Multidrug Resistance Protein MRP5 Transports Folates and Can Mediate Cellular Resistance against Antifolates

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## Abstract

Members of the multidrug resistance protein family, notably MRP1-4/ABCC1-4, and the breast cancer resistance protein BCRP/ABCG2 have been recognized as cellular exporters for the folate antagonist methotrexate (MTX). Here we show that MRP5/ABCC5 is also an antifolate and folate exporter based on the following evidence: (a) Using membrane vesicles from HEK293 cells, we show that MRP5 transports both MTX ($K_M = 1.3$ mmol/L and $V_{MAX} = 780$ pmol per mg protein per minute) and folic acid ($K_M = 1.0$ mmol/L and $V_{MAX} = 875$ pmol per mg protein per minute). MRP5 also transports MTX-glu$_2$ ($K_M = 0.7$ mmol/L and $V_{MAX} = 450$ pmol per mg protein per minute) but not MTX-glu$_3$. (b) Both accumulation of total [$^3$H]MTX and of MTX polyglutamates were significantly reduced in MRP5 overexpressing cells. (c) Cell growth inhibition studies with MRP5 transfected HEK293 cells showed that MRP5 conferred high-level resistance (>160-fold) against the antifolates MTX, GW1843, and ZD1694 (raltitrexed) in short-term (4 hours) incubations with high drug concentrations; this resistance was proportional to the MRP5 level. (d) MRP5-mediated resistance (8.5- and 2.1-fold) was also found in standard long-term incubations (72 hours) at low concentrations of ZD1694 and GW1843. These results show the potential of MRP5 to mediate transport of (anti)folates and contribute to resistance against antifolate drugs. (Cancer Res 2005; 65(10): 4425-30)

## Introduction

Multidrug resistance proteins (MRP1-9; ABCC1-6, ABCC10-12) are members of the ATP-binding cassette (ABC) superfamily of membrane transporters that mediate the ATP-dependent transport of various substrates across biological membranes (1–3). Although individual MRPs differ in the substrates they preferentially transport, most MRP substrates are organic anions, often conjugates of sulfate, phosphate, glucuronate, glutathione, or glutamate (2, 4, 5). MRPs are known for the broad spectrum of (anticancer) drugs that they transport out of cells, raising the possibility of their involvement in clinical multidrug resistance (6, 7).

We previously reported that MRP5 overexpression in human embryonic kidney (HEK293) cells results in low level resistance against several anticancer and antiviral drugs (e.g., 6-mercaptopurine and PMEA; refs. 8–10). Resistance is due to the active efflux of the monophosphorylated metabolites of these drugs (8, 10, 11). Other monophosphorylated nucleosides, such as 3′,5′-cyclic-GMP/AMP (5, 9, 12) and alaninyl-d4TMP (8), are also substrates and are actively excreted from cells by MRP5.

Here we extend the substrate spectrum of MRP5 to folic acid (FA) and several antifolates, the classic antifolate methotrexate (MTX) and two novel generations raltitrexed (Tomudex; ZD1694; ref. 13) and OSI-7904 (GW1843; ref. 14). ZD1694 and GW1843 are novel folate-based inhibitors of thymidylate synthase which were recently approved for clinical use. MTX is used for the treatment of various types of cancer and autoimmune disorders (15–18) and interferes with folate metabolism by inhibiting dihydrofolate reductase (DHFR) and, in its polyglutamate form, thymidylate synthase. (Anti)folates are mainly taken up by cells via the reduced folate carrier (RFC) after which intracellular polyglutamylation by folylpolyglutamate synthethase (FPGS) may occur (19, 20), a process important for cellular retention of (anti)folates. Both ZD1694 and GW1843 are much better substrates for FPGS than MTX, but GW1843 is not polyglutamylated beyond the diglutamate form (14). Potential antifolate resistance mechanisms include altered expression of target enzymes, altered metabolism, and decreased cellular accumulation, due to either reduced uptake by RFC or increased efflux by the ABC transporters MRP1-4 and breast cancer resistance protein (BCRP; refs. 21–26).

We show here that overexpression of MRP5 renders HEK293 cells highly resistant against ZD1694 and moderately resistant to GW1843 in standard growth cell inhibition tests. No significant resistance against MTX was observed under these conditions. However, in short-term (4 hours) incubations with high drug concentrations, resistance against MTX and ZD1694 was substantial with resistance factors of over 300-fold in MRP5 overexpressing cells. Using membrane vesicles prepared from MRP5-overexpressing HEK293/MRP5I cells, we found that FA, MTX, the diglutamylated form of MTX (MTX-glu$_2$), but not the triglutamate (MTX-glu$_3$), were transported by MRP5. Consistent with this substrate specificity, we found that the MTXglu$_2$ level was strongly reduced in MRP5-overexpressing HEK293 cells.

## Materials and Methods

**Materials.** [$^3$H]MTX, [$^3$H]MTX-glu$_2$, [$^3$H]MTX-glu$_3$, [$^3$H]FA, and [2,3-$^3$H]L-glutamic acid were from Moravek Biochemicals (Brea, CA). MTX-glu$_x$ and FA-glu$_2$-5 were from Schircks Laboratories (Jona, Switzerland), ZD1694 from Zeneca Pharmaceuticals (Macclesfield, United Kingdom), and GW1843 from GlaxoWellcome (Research Triangle Park, NC). Creatine

phosphate and creatine kinase were obtained from Roche (Almere, the Netherlands) and OE-67 membrane filters were from Schleicher and Schuell (Dassel, Germany). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell lines and culture conditions.** HEK293 cells, HEK293/5I (high overexpression), and HEK293/5E (moderate overexpression) MRP5-over-expressing cells were previously described (10, 11). Cells were routinely grown in DMEM (Invitrogen, Breda, the Netherlands) supplemented with 10% FCS (Invitrogen) and 100 units penicillin/streptomycin per mL (Invitrogen), at 37°C and 5% $CO_2$ under humidifying conditions.

**Vesicular transport assays.** The expression of the transporters was evaluated by Western blot as described previously (11), and activity was determined by measuring the ATP-dependent uptake of known substrates. Uptake of radiolabeled substrates by membrane vesicles was determined by the rapid filtration method as previously described (27). Briefly, vesicular uptake was done in phosphate-buffered medium [150 mmol/L sodium phosphate, 10 mmol/L $MgCl_2$, 10 mmol/L creatine phosphate, and 100 μg creatine kinase per mL (pH 7.4)] in the presence or absence of 4 mmol/L ATP. The ATP-dependent transport was calculated by subtracting the uptake in the absence of ATP from that in the presence of ATP (4 mmol/L). For inhibition studies, the ATP-dependent uptake in the presence of inhibitor was subtracted from that in its absence.

**Growth inhibition assays.** Cells were plated in 1 mL of culture medium in individual poly-D-lysine–treated wells of a 24-well plate (BD Biosciences, Alphen a/d Rijn, the Netherlands). The initial cell density was $1 \times 10^4$ cells/$cm^2$. One day after plating, drugs were added at various concentrations covering a three-log range and the cells were incubated in the presence of drug for either 4 or 72 hours. Following the 4-hour exposure, the medium was aspirated and cells were washed thrice with 2.5 mL of drug-free medium at 37°C. Thereafter, the cells were cultured in drug-free medium for an additional 68 hours. After 72 hours, cells were harvested and counted using a micro cell counter as described previously (21). Finally, drug concentrations inducing 50% cell growth arrest ($IC_{50}$) were determined.

We have noted that these experiments were sensitive to the exact culture conditions. After we finished the experiments presented here, we found that the use of RPMI, which has a lower folate level than DMEM, gave robust results, especially when cells were also spun down in the washing steps to avoid cell loss.

**[3H]methotrexate accumulation and polyglutamylation.** HEK293 cells and HEK293/MRP5I cells were cultured in 80-$cm^2$ flasks until 70% confluent. Subsequently, cells were exposed to 1 μmol/L [3H]MTX for either 4 or 24 hours. Thereafter, cells were washed twice with 10 mL of ice-cold HEPES-buffered saline, pH 7.4 (HBS). The cells were harvested and collected in 10 mL of ice-cold HBS. After centrifugation, cell pellets were resuspended in 1 mL of HBS, of which 10 μL aliquots were used for cell counting, 100 μL for radioactivity counting, and 890 μL for [3H]MTX-polyglutamate analysis by high-performance liquid chromatography as previously described (21).

**Folylpolyglutamate synthetase activity.** FPGS activity assays were carried out as described previously (23, 28). In short, cell pellets of $2 \times 10^7$ cells were suspended in 0.5 mL of extraction buffer containing 50 mmol/L Tris-HCl, 20 mmol/L KCl, 10 mmol/L $MgCl_2$, and 5 mmol/L DTT (pH 7.5). Total cell extracts were obtained by sonication (MSE Soniprep, amplitude 14 μm, 3 × 5 seconds with 10-second intervals, at 4°C). Cell debris was removed by centrifugation at 12,000 × $g$ for 15 minutes (4°C). The FPGS activity assay mixture contained 200 μg protein, 4 mmol/L [2,3-3H]L-glutamic acid, and 250 μmol/L MTX in a buffer consisting of 100 mmol/L Tris (pH 8.5), 10 mmol/L ATP, 20 mmol/L $MgCl_2$, 20 mmol/L KCl, and 10 mmol/L DTT (final volume, 250 μL). Following 2 hours of incubation at 37°C, the reaction was stopped by adding 1 mL of an ice-cold 5 mmol/L unlabeled L-glutamic acid (pH 7.5). Sep-Pak $C_{18}$ cartridges (Millipore, Waters Associates, Etten-Leur, the Netherlands) were used for the separation of free [3H]L-glutamate from MTX-[3H]diglutamate. Controls lacking MTX were included to correct for polyglutamylation of endogenous folates present in the cell extract.

## Results

**Antifolate resistance mediated by MRP5.** MRP5-mediated resistance against the DHFR inhibitor MTX and the thymidylate synthase inhibitors ZD1694 and GW1843 was determined for parental HEK293, HEK293/MRP5E (moderate MRP5 overexpression), and HEK293/MRP5I (high MRP5 overexpression) cells (Table 1). Growth of the cells was determined at high drug concentrations (μmol/L range) in short-term (4 hours) incubations and at low drug concentrations (nmol/L range) in standard long-term (72 hours) cell growth inhibition tests. In the long-term assays, we found substantial MRP5-mediated resistance against ZD1694 (8.4-fold), whereas resistance against MTX and GW1843 was much lower, 1.7- and 2.1-fold, respectively. In the short-term incubation experiments, resistance levels for all antifolates were much higher, reaching values of at least 1,600-fold for ZD1694, 270-fold for MTX, and 160-fold for GW1843 (Table 1). The levels of resistance to MTX, ZD1694, and GW1843 correlated with the cellular MRP5 level (9) and resistance found in previous studies: HEK293/MRP5I > HEK293/MRP5E > HEK293 (for details, see refs. 9–11).

**Vesicular transport of methotrexate by MRP5.** Transport of [3H]MTX by MRP5 was determined in vesicular uptake assays using inside-out membrane vesicles prepared from parental and HEK293 cells overexpressing MRP5 to a moderate (HEK293/MRP5E) and to a higher extent (HEK293/MRP5I; ref. 10). ATP-dependent transport was calculated by subtracting the transport determined in the absence of ATP by the transport found in the

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**Table 1.** Antifolate induced inhibition of cell growth in wild type and MRP5-overexpressing HEK293 cells

| | $IC_{50}$* (72-h exposure) | | | $IC_{50}$* (4-h exposure) | | |
|---|---|---|---|---|---|---|
| | MTX (nmol/L) | ZD1694 (nmol/L) | GW1843 (nmol/L) | MTX (μmol/L) | ZD1694 (μmol/L) | GW1843 (μmol/L) |
| HEK293 | 16 ± 0.2 | 7.4 ± 0.7 | 1.3 ± 0.5 | 1.8 ± 0.6 | 0.3 ± 0.1 | 0.8 ± 0.1 |
| HEK293/MRP5E | 19 ± 8 | 39 ± 10 | 2.4 ± 0.6 | 30 ± 2 | >500 | 89 ± 37 |
| HEK293/MRP5I[†] | 27 ± 7 | 62 ± 15 | 2.7 ± 0.6 | >500 | >500 | 129 ± 95 |

*Results are the mean ± SD from four to six separate experiments.
[†]5I and 5E refer to the clone number.

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presence of 4 mmol/L ATP. Replacing ATP with adenosine 5′-[γ-thio]triphosphate (ATP-γ-S), a nonhydrolyzable ATP analogue gave similar results as leaving out the ATP (data not shown), indicating that ATP hydrolysis is necessary for the MRP5-driven MTX transport. Uptake of [³H]MTX was proportional to the level of MRP5 overexpression (Fig. 1A); uptake increased with time for at
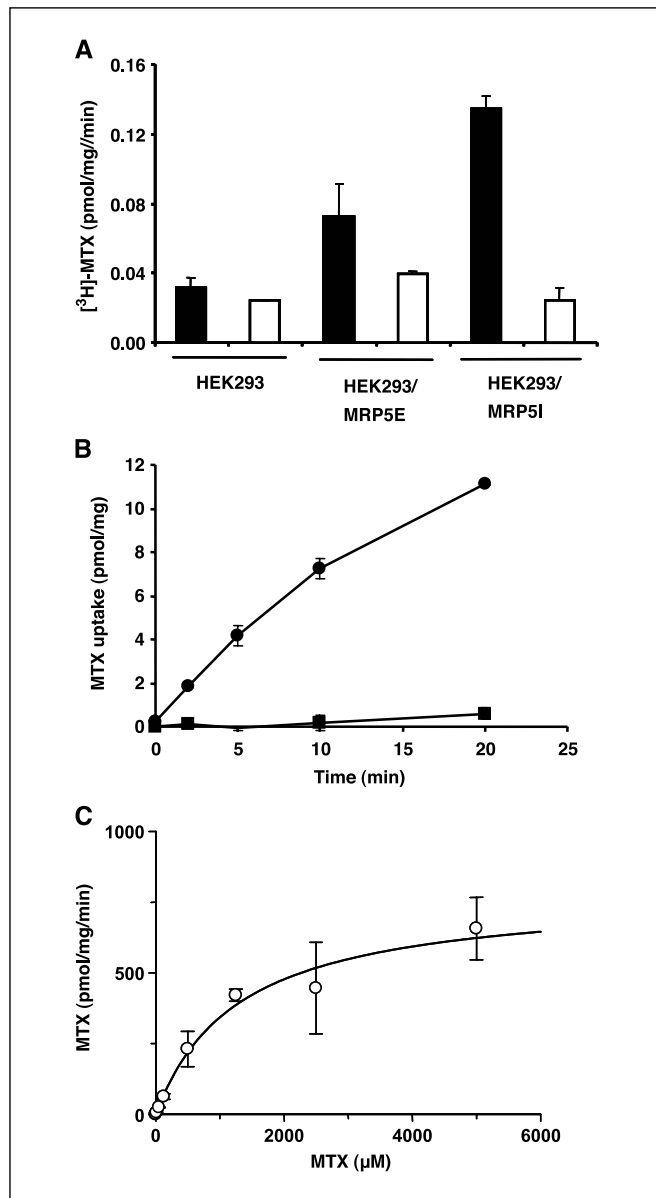


**Figure 1.** Transport of MTX and FA by MRP5 into membrane vesicles from HEK293 cells. *A,* uptake of [³H]MTX (0.3 μmol/L, 5 minutes at 37°C) was compared for vesicles (20 μg) prepared from parental HEK293 and its MRP5 overexpressing clones HEK293/MRP5E and HEK293/MRP5I cells in the presence (*black columns*) and absence (*white columns*) of 4 mmol/L ATP. *B,* time-dependent uptake of [³H]MTX (1 μmol/L) by vesicles prepared from parental HEK293 cells (■) and HEK293/MRP5I cells (●) was determined in the presence and absence of ATP and the ATP-dependent transport was calculated. Using a nonhydrolyzable ATP analogue (ATP-γ-S,) instead of ATP gave similar results as leaving out the ATP (data not shown). *C,* transport of MTX as a function of the MTX concentration by vesicles prepared from HEK293/MRP5I cells; ATP-dependent transport after 5 minutes was calculated as in (*B*). $K_M$ and $V_{MAX}$ values were estimated by fitting the data assuming Michaelis-Menten kinetics. *Points,* average of three independent experiments; *bars,* ±SD.
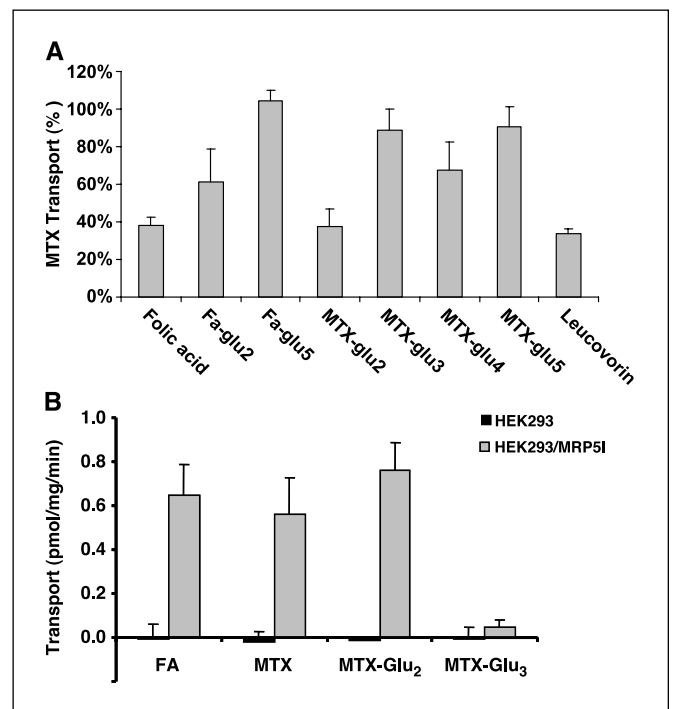


**Figure 2.** Inhibition of MTX transport by polyglutamylated (anti)folates. *A,* inhibitory effect of MTX, FA, leucovorin, and several of their polyglutamylated derivatives (all 1 mmol/L) was determined on the ATP-dependent transport of [³H]MTX (1 μmol/L) by MRP5I vesicles after 5 minutes of transport. *B,* ATP-dependent transport (5 minutes at 37°C) of [³H]FA, [³H]MTX-glu₂, and [³H]MTX-glu₃ (1 μmol/L) into vesicles prepared from parental HEK293 (*black columns*) and HEK293/MRP5I (*grey columns*) was determined and compared with the transport found for [³H]MTX (1 μmol/L; taken from Fig. 1C). *Columns,* average of three independent experiments; *bars,* ±SD.

least 20 minutes (Fig. 1B) and yielded a $K_M$ of 1.3 ± 0.3 mmol/L and a $V_{MAX}$ of 780 ± 70 pmol per mg per minute (Fig. 1C).

**Effect of polyglutamylation on MRP5 vesicular transport.** The finding that MRP5 conferred the highest level of antifolate resistance in short-term incubations suggested that MRP5 transports predominantly the parental (monoglutamylated) compound and/or the short-chain polyglutamate metabolites. We tested this by determining the inhibition of MRP5-mediated transport by various glutamylated forms of MTX, FA, and leucovorin (folinic acid). MTX, FA, and leucovorin and the diglutamate forms tested inhibited transport of [³H]MTX, whereas their higher glutamylated forms had little effect, suggesting that only the former are transported (Fig. 2A). This was confirmed in experiments with radiolabeled FA, MTX-glu₂, and MTXglu₃ (Fig. 2B). FA and MTX-glu₂ uptake continued for at least 20 minutes similar to the uptake of MTX in Fig. 1B, whereas MTX-glu₃ was hardly transported. FA and MTX-glu₂ were transported by the MRP5 vesicles with $K_M$ values of 1 ± 0.1 and 0.7 ± 0.1 mmol/L and $V_{MAX}$ values of 875 ± 75 and 450 ± 20 pmol per mg per minute, respectively (Fig. 3A and B). It is likely that some reduced FA metabolites are also transported by MRP5, as 1 mmol/L leucovorin inhibited MTX transport by ∼65% (Fig. 2A).

**Polyglutamylated methotrexate pools in HEK293 cells.** The results of the cytotoxicity and vesicular uptake experiments suggested that MRP5 decreases the accumulation of MTX. To test this, we determined the levels of the polyglutamylated forms of MTX, following 4 to 24 hours [³H]MTX (1 μmol/L) incubations of
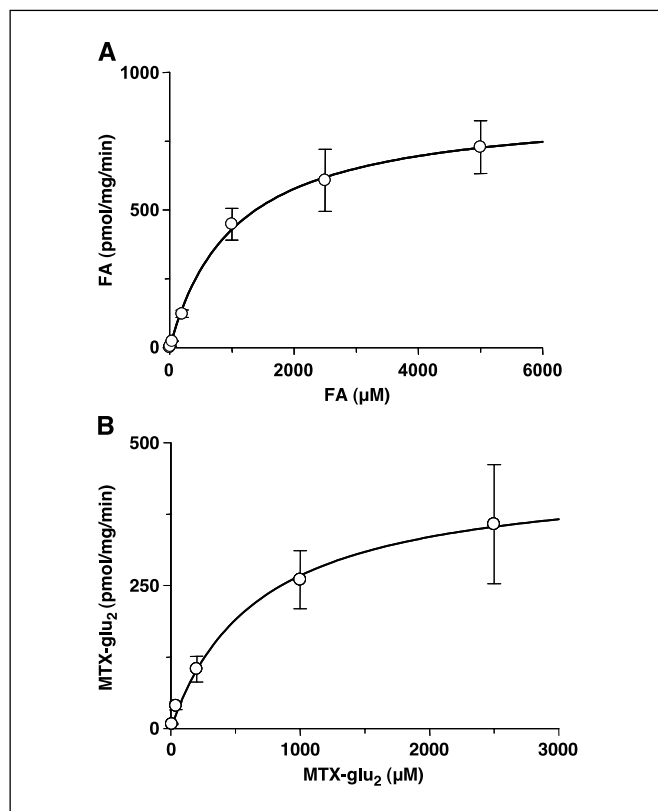
**Figure 3.** Transport of MTX and FA into membrane vesicles from HEK293 cells. The substrate concentration dependence of MRP5-mediated transport of (*A*) [$^3$H]FA and (*B*) [$^3$H]MTX-glu$_2$ were determined for HEK293/MRP5I vesicles after 5 minutes of uptake at 37°C, as described in Fig. 1*C* legend. *Points,* average of three independent experiments; *bars,* ±SD.

parental and HEK293/MRP5I cells (see Table 2). Under both conditions, HEK293/MRP5I cells accumulated 2- to 3-fold less total MTX-glu$_x$ than the HEK293 cells. In the HEK293 cells, the levels of MTX-glu$_2$ were consistently higher than the levels of MTX, whereas the reverse was found in the HEK293/MRP5I cells, strongly suggesting that MRP5 efficiently removes the MTX-glu$_2$ from the cells. Remarkably, MTX levels were somewhat increased in the MRP5I cells, which might be due to MRP5 driven intravesicular accumulation (see Discussion). To rule out the possibility that a

difference in FPGS activity was responsible for this difference, we tested the FPGS activity in both the HEK293 and HEK293/MRP5I cells. We found no significant difference: $1,040 \pm 160$ and $850 \pm 90$ pmol per hour per mg protein, respectively (mean $\pm$ SD; $P = 0.2$).

## Discussion

Published studies on the possible role of MRP5 in antifolate resistance have not been conclusive. Stark et al. (29) described down-regulation of MRP5, along with MRP1, in Chinese hamster ovary cells selected for resistance to the lipophilic antifolate pyrimethamine. This suggested that MRP5 does not play a role in antifolate resistance but rather in folate homeostasis (30). In contrast, Pratt et al. (31) reported that overexpression of MRP2 and MRP5 in HEK293 cells conferred resistance against a new polyglutamylatable antifolate Alimta. In standard long-term cell growth inhibition tests, Wijnholds et al. (10) initially did not detect MRP5-mediated MTX resistance in our transfected cells, but our current results show that MRP5 does give resistance, albeit only 1.8-fold (Table 1). The difference could be in details of the culture and assay conditions, which differed in Wijnholds et al. (10) and here. For instance, sensitivity to MTX is dependent on the folate level of the medium. MRP5 differs from MRPs 1 to 4 in being able to transport MTX-glu$_2$, a property it shares with another ABC-transporter, ABCG2 (BCRP; refs. 23, 32, 33). Like MRPs 1 to 4, MRP5 has a low affinity for MTX (Table 3).

Overexpression of MRP5 in HEK293 cells results in high-level resistance against short-term exposure to high concentrations of MTX and of the antifolates/thymidylate synthase inhibitors ZD1694 and GW1843 (refs. 13, 14; Table 1). In standard cell growth inhibition assays, these MRP5-overexpressing cells were also resistant against relatively low concentrations of GW1843 and ZD1694 but not of MTX. As expected from our vesicular transport experiments, MRP5 decreased the accumulation of MTX-glu$_2$ in cells incubated with [$^3$H]MTX (Table 2). Consequently, levels of the long-chain polyglutamate forms MTXglu$_{3-5}$ were also reduced (Table 2). Although the levels of long-chain MTX polyglutamates were still markedly lower in the MRP5 cells than in parental cells after exposure to MTX for 24 hours (Table 2), these levels are apparently sufficient for growth inhibition, explaining the sensitivity to MTX of the MRP5 cells after 72 hours of continuous exposure (Table 1). As reported before, a part of MRP5 in the MRP5I cells is present in intracellular vesicles (10, 11). This localization could result in intravesicular accumulation of MTX, which is the most likely

**Table 2.** Accumulation of [$^3$H]MTX and polyglutamylated forms of [$^3$H]MTX in HEK293 and HEK293/MRP5I cells

| | Incubation (h) | MTX (pmol/10$^7$ cells) | MTX-glu$_2$ (pmol/10$^7$ cells) | MTX-glu$_3$ (pmol/10$^7$ cells) | MTX-glu$_4$ (pmol/10$^7$ cells) | MTX-glu$_5$ (pmol/10$^7$ cells) | Total (pmol/10$^7$ cells) |
|---|---|---|---|---|---|---|---|
| HEK293* | 4 | $5.5 \pm 1.7$ | $15.6 \pm 2.7$ | $8.6 \pm 1.6$ | NA | NA | $30 \pm 6$ |
| HEK293/MRP5I | 4 | $7.8 \pm 0.1$ | $4.9 \pm 0.4$ | $2.4 \pm 0.8$ | NA | NA | $15 \pm 1.5$ |
| HEK293 | 24 | $6.2 \pm 2.7$ | $25.2 \pm 6.5$ | $27.1 \pm 3.9$ | $23.8 \pm 2.0$ | $7.5 \pm 2.2$ | $89 \pm 17.3$ |
| HEK293/MRP5I | 24 | $13.1 \pm 0.7$ | $7.6 \pm 0.4$ | $9.6 \pm 0.7$ | $5.5 \pm 0.3$ | $2.1 \pm 0.7$ | $38 \pm 2.7$ |

Abbreviation: NA, not attainable, below the detection limit.
*Cells were incubated with 1 μmol/L [$^3$H]MTX for 4 and 24 h after which the formation of the various intracellular [$^3$H]MTX polyglutamate forms was determined by high-performance liquid chromatography analysis (see Materials and Methods for details); Data are the average ± SD of three experiments.

**Table 3.** Transport kinetics determined for MTX transport by various ABC transporters

| ABC transporter | $V_{MAX}$ (pmol/ min/mg) | $K_M$ (mmol/L) | Reference |
|---|---|---|---|
| MRP1 (ABCC1) | 2,050 | 2.15 | Zeng et al. (38) |
| MRP2 (ABCC2) | 1,000* | 2.5-3 | Bakos et al. (40) |
| MRP3 (ABCC3) | 2,930 | 0.62 | Zeng et al. (38) |
| MRP4 (ABCC4) | 430 | 1.3 | Van Aubel et al. (41) |
| MRP4 (ABCC4) | 240 | 0.22 | Chen et al. (37) |
| MRP5 (ABCC5) | 780 | 1.3 | This study |
| BCRP (ABCG2) | 2,400 | 0.68 | Volk and Schneider (33) |
| BCRP (ABCG2) | 687 | 1.34 | Chen et al. (32) |

*Estimated by us from data in ref. (40).

explanation for the 2-fold increased MTX levels we find after 24 hours in the MRP5I cells (Table 2). However, the fact that the long-chain polyglutamate levels are decreased in the MRP5I cells can only be explained by a decreased cytosolic MTX level.

The low affinity of MRPs for MTX (Table 3) seems to argue against a potential role for these transporters in clinical MTX resistance. Although the ability of MRP5 to transport MTX-glu$_2$ in addition to MTX places it in a better position to cause MTX resistance than MRPs 1 to 4, the clinically observed MTX plasma concentration (34) during standard treatment schedules is in the range of 1 to 10 μmol/L, which is much lower than the $K_M$ of MRP5 for MTX (Table 3). Only in high-dose MTX treatments with MTX plasma levels of 0.1 to 1 mmol/L MRP5 might have an effect on MTX disposition, given the high capacity of MRPs for folate efflux (Table 3), particularly when compared with an ~100-fold lower capacity of the folate influx capacity of the RFC (18).

MRP5 could be more important, however, in resistance to the newer antifolates. Overexpression of MRP5 gives relatively high levels of resistance against ZD1694 and GW1843. Resistance against these drugs was also found for MRP1 and MRP2 (21), but the MRP5 resistance levels are much higher. For instance, MRP1 overexpression resulted only in 3-fold resistance to ZD1694 after 4 hours exposure (21), whereas we find 1,600-fold resistance in MRP5 cells (Table 1). The transport of the diglutamate forms of antifolates may contribute to this ability of MRP5 to confer higher levels of resistance than the other MRPs.

A definitive evaluation of the role of MRPs in clinical antifolate resistance is complicated by the fact that many MRPs also transport FA and reduced folate cofactors (18, 21, 32, 35–38). This may lower the concentration of intracellular folate, which competes with antifolates. A decreased intracellular folate concentration may even lead to hypersensitivity to antifolates (29). The contribution of MRPs to clinical antifolate resistance will therefore depend on the intracellular FA levels, the polyglutamylation rates and the presence of (anti)folate uptake proteins (e.g., RFC; refs. 18, 32, 37–39). Studies in patients will be required to assess whether MRPs in general and MRP5 in particular contribute to resistance to any antifolate in the clinic.

## Acknowledgments

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