

MOL #43661

Functional Role of Arginine 375 in Transmembrane Helix 6 of Multidrug Resistance

Protein 4 (MRP4/ABCC4)

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MOL #43661

Running title: Functional Role of Arg³⁷⁵ in TM6 of MRP4

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Abbreviations: MTX, methotrexate; cGMP, cyclic guanosine monophosphate; MRP, multidrug resistance protein; ABC, ATP-binding cassette; TM, transmembrane α -helices.

MOL #43661

ABSTRACT

Multidrug resistance protein 4 (MRP4) transports a variety of endogenous and xenobiotic organic anions. MRP4 is widely expressed in the body and specifically localized to the renal apical proximal tubule cell membrane, where it mediates the excretion of these compounds into urine. To characterize the MRP4 substrate-binding site, the amino acids Phe³⁶⁸, Phe³⁶⁹, Glu³⁷⁴, Arg³⁷⁵, and Glu³⁷⁸ of transmembrane helix 6, and Arg⁹⁹⁸ of helix 12, localized in the intracellular half of the central pore, were mutated into the corresponding amino acids of MRP1 and MRP2. Membrane vesicles isolated from HEK293 cells over-expressing these mutants showed significantly reduced methotrexate (MTX) and cyclic guanosine monophosphate (cGMP) transport activity compared to vesicles that expressed wild type MRP4. The only exception was substitution of Arg³⁷⁵ with serine, which had no effect on cGMP transport, but significantly decreased the affinity of MTX. Substitution of the same amino acid with a positively charged lysine returned the MTX affinity to that of the wild type. Furthermore, MTX inhibition of MRP4-mediated cGMP transport was non-competitive and the inhibition constant was increased by introduction of the R375S mutation. A homology model of MRP4 showed that Arg³⁷⁵ and Arg⁹⁹⁸ face right into the central aqueous pore of MRP4. We conclude that positively charged amino acids in transmembrane helices 6 and 12 contribute to the MRP4 substrate-binding pocket.

MOL #43661

Proteins belonging to the ATP-binding cassette (ABC) superfamily play a crucial role in human physiology, pharmacology, and toxicology, as well as numerous pathological conditions. To date 48 human ABC transporter proteins have been identified, including 9 multidrug resistance proteins (MRPs) belonging to the C subfamily (van de Water et al., 2005). MRPs are not only important for the tumor cell resistance they confer to chemotherapeutic drugs, but also for their endogenous expression in normal human tissues. MRP4/ABCC4 is widely distributed in epithelial tissue and blood cells and highest expression has been found in kidney, lung, prostate, liver, tonsils and bladder (Borst et al., 2007). In most epithelial cells MRP4 is located at the basolateral membrane, except for the renal proximal tubular cell where it is expressed apically (Van Aubel et al., 2002). Structurally, the human MRP4 protein consists of 1325 amino acids comprising two membrane-spanning domains each consisting of 6 transmembrane α -helices (TM), with two cytosolic ATP-binding domains. MRP4 mediates ATP-dependent transport of various organic anions from the intracellular to the extracellular side (Russel et al., 2008). Substrates include endogenous compounds like the cyclic adenosine (cAMP) and guanosine (cGMP) monophosphate (Van Aubel et al., 2002), folate (Chen et al., 2002), and uric acid (Van Aubel et al., 2005), as well as drugs like the antiviral nucleoside monophosphate analog PMEA (Schuetz et al., 1999) and the nucleobase analogs 6-mercaptopurine (Wielinga et al., 2002) and methotrexate (MTX) (Chen et al., 2002).

The mode by which MRPs transport their substrates has still to be identified. Among the different MRPs, the structure-function relationship of MRP1 has been investigated most extensively (Deeley et al., 2006). Several mutational studies have shown that polar amino acids in transmembrane helices 11 and 17 of MRP1 are involved in substrate binding (Ito et al., 2001a; Ito et al., 2001b; Zhang et al., 2001; Koike et al., 2004; Zhang et al., 2006). In a

MOL #43661

model most of their side chains were found to line the "pore" adjacent to the membrane cytosol interface. In contrast with MRP1, limited knowledge regarding the amino acids involved in substrate specificity of MRP2 (Ito et al., 2001a) and MRP3 (Zhang et al., 2003b) is available.

To date, no attempt has been made to characterize the MRP4 substrate-binding site(s). In the present study we investigated the role of Phe³⁶⁸, Phe³⁶⁹, Glu³⁷⁴, Arg³⁷⁵, and Glu³⁷⁸ present in transmembrane helix 6 (TM 11 in MRP1), and Arg⁹⁹⁸ in transmembrane helix 12 (TM 17 in MRP1) in transport of cGMP and MTX. These amino acids are highly conserved in MRP4 among different species (Chen and Klaassen, 2004), but there is a large variety within the MRP subfamily (Fig. 1) that might point to a specific mechanistic importance of these amino acids. When they were substituted by their analogues from MRP1/MRP2, most mutations completely abolished MRP4 transport activity, except for Arg³⁷⁵ in which cGMP transport was preserved whereas the affinity for MTX was reduced. We studied this mutation in more detail and developed a homology model of MRP4 to explain the results.

MOL #43661

Materials and Methods

Materials

[3',5',7'-³H(n)]-methotrexate sodium salt (51.5 Ci mmol⁻¹) and [8-³H]-guanosine-3',5'-cyclic phosphate NH₄ salt (7.7 Ci mmol⁻¹) were purchased from Moravek Inc. (Brea, CA). The Bac-to-Bac, Gateway system, culture medium, and Alexa Fluor 680 goat anti-rabbits IgG secondary antibody were purchased from Invitrogen (Breda, the Netherlands). Methotrexate, guanosine 3'5'-cyclic monophosphate, and GenElute Plasmid Mini-Prep kit were purchased from Sigma. Fetal bovine serum was purchased from MP Biochemicals (the Netherlands). Multiscreen HTS-HV 0.45 μm 96-wells filters were obtained from Millipore (Etten-Leur, the Netherlands). Protein concentrations were determined with an assay kit from Bio-Rad Laboratories (Veenendaal, the Netherlands). 8-Azidoadenosine 5'-triphosphate 2',3'-biotin-long chain-hydrazone (8N₃ATP-2',3'-Biotin-LC-Hydrazone) was purchased from Affinity labeling Technologies (Nicholasville, KY), and from Streptavidin horseradish peroxidase conjugate Amersham GE Healthcare (Buckinghamshire, UK)

Generation of Human MRP4 Baculovirus

Full-length of human MRP4 cDNA was generated as previously described (El Sheikh et al., 2007). Briefly, The Bac-to-Bac system, normally used for protein production in insect cells, was modified for protein expression in mammalian cells by introduction of the CMV promoter and Gateway destination elements (cassette that contains the chloramphenicol resistance gene and the ccdB gene flanked by attR1 and attR2 sites) in the pFastBacDual vector. In addition, the vesicular stomatitis virus G protein cDNA was cloned behind the P10 promoter of the pFastBacDual vector. The human MRP4 was cloned into the Gateway entry vector and transferred to the newly constructed Bac-to-Bac vector with the gateway LR

MOL #43661

reaction. Baculoviruses were produced as described in the Bac-to-Bac manual. As a negative control, the enhanced yellow fluorescent protein (EYFP) was also introduced into the Baculovirus expression system (Invitrogen).

Site Directed Mutagenesis

PCR was performed on a template pENTR-MRP4 vector using two primers (forward and reverse) produced by Biolegio (Nijmegen, the Netherlands) bearing one or more mismatched bases at the site of residues to be mutated. In this PCR reaction, PfuUltra II fusion HS DNA polymerase with the supplied reaction buffer, and dNTP mix 12.5 mM each completed to 50 μ l end volume were used. PCR amplification consisted of initial denaturation at 94 °C, followed by 20 cycles of denaturation at 94 °C, annealing at 58 °C, and elongation at 72 °C for 14 minutes. The PCR product was cut by DpnI for 2 hours at 37 °C and finally transformed into *E. coli* DH5 α cells. pENTR-MRP4 mutant plasmids were isolated using the GenElute Plasmid Mini-Prep kit (Sigma) from kanamycine resistant colonies. All mutations were confirmed by sequencing of the full-length MRP4/ABCC4 DNA. Twelve mutants of the human MRP4 were generated: FF/L- (F368L and F369-), ERE/SSQ (E374S, R375S, and E378Q), FFERE/L-SSQ (F368L, F369-, E374S, R375S, and E378Q), F368L, F369-, E374S, R375S, R375A, R375K, R375E, E378Q and R998A.

Transduction of MRP4 and Mutants Expression Vectors in HEK293 Cells

HEK293 cells were cultured in 182 cm² flasks till 40% confluency, after which the culture medium was removed and 3.5 ml fresh medium and 1.5 ml control EYFP, MRP4 or MRP4-mutant baculovirus were added. The cells were incubated for 15 min at 37°C, after which 20 ml medium was added. After 24 hours of transduction, sodium butyrate (5 mM) was added. Three days after transduction, the cells were harvested.

MOL #43661

Isolation of Membrane Vesicles and Protein Analysis

Cells were harvested by centrifugation at 3,000xg for 30 minutes. The pellets were re-suspended in ice-cold homogenization buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.4) supplemented with protease inhibitors (100 μ M phenylmethylsulfonyl fluoride, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 1 μ M pepstatin, 1 μ M E-64) and shaken at 4°C for 60 minutes. Lysed cells were centrifuged at 4°C at 100,000xg for 30 minutes, and the pellets were homogenized in ice-cold TS buffer (10 mM Tris-HEPES, 250 mM sucrose, pH 7.4) using a tight fitting Dounce homogenizer for 30 strokes. After centrifugation at 500xg at 4°C for 20 minutes, the supernatant was centrifuged at 4°C at 100,000xg for 60 minutes. The resulting pellet was re-suspended in TS buffer and passed through a 27-gauge needle for 30 times. Protein concentration was determined by Bio-Rad protein assay kit. Crude membrane vesicles were dispensed in aliquots, frozen in liquid nitrogen, and stored at -80°C until use.

Western Blotting

The membrane vesicle preparations (15 μ g protein) were solubilized in SDS-PAGE sample buffer and separated on SDS gels containing 7.5% acrylamide according to Laemmli (Laemmli, 1970). Subsequently they were blotted on nitrocellulose membrane using the iBlot dry blotting system (Invitrogen, CA, USA). Polyclonal anti-human MRP4 rabbit serum antibody (Van Aubel et al., 2002) was used to detect human MRP4 and MRP4 mutants. The secondary antibody used was the fluorescent Alexa Fluor 680 (Invitrogen). Signals were visualized with a fluorescent method, using Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, USA).

MOL #43661

Vesicular Transport Assays

Uptake of [³H]-MTX or [³H]-cGMP into membrane vesicles was performed using a rapid filtration technique. TSB buffer (TS buffer with 0.2 mg/ml bovine serum albumin) supplemented with a mixture of 4 mM ATP, 10 mM MgCl₂ was added to the membrane vesicles in a final volume of 30 μl. The reaction was started when the mixture was incubated at 37°C. After 15 minutes, reaction was stopped by placing samples on ice. After 1 minute, 150 μl ice cold TSB buffer was added to each reaction well. Diluted samples were filtered by a Multiscreen HTS-Vacuum Manifold filtration device (Millipore, Etten-Leur, the Netherlands) through 0.45-μm-pore 96-wells Millipore filters that were pre-incubated with TSB buffer. After adding 4 ml scintillation fluid to each filter and subsequent liquid scintillation counting, uptake of [³H]-MTX or [³H]-cGMP into membrane vesicles was studied by measuring radioactivity associated with the filters. In control experiments, ATP was substituted with AMP. Net ATP-dependent transport was calculated by subtracting values measured in the presence of AMP from those measured in the presence of ATP. Each experiment was performed in triplicate using three different batches of membrane vesicles.

Vesicular Inhibition Assays

To evaluate the inhibitory effects of MTX on [³H]-cGMP uptake in MRP4 and MRP4-R375S membrane vesicles, the previously mentioned transport assay was performed using 1, 10, and 100 μM cGMP, in the absence or presence of MTX concentrations ranging from 1 to 600 μM. Net MRP4- or MRP4 mutant-dependent transport was calculated by subtracting background values measured in EYFP-transfected control vesicles.

MOL #43661

Kinetic Analysis

All data were expressed as means \pm S.D. Curve-fitting of the resulting concentration-dependent transport curves was performed by nonlinear regression analysis using GraphPad Prism software version 4.03 (GraphPad Software Inc., San Diego, USA). Results of the inhibition assays were analyzed using Dixon's method, to estimate the inhibitory potency (K_i).

Immunoprecipitation and Photo-affinity labeling

Wild type MRP4, negative (EYFP) control and mutants (300 μ g protein vesicles) were incubated with 5 mM $MgCl_2$ and 100 μ M Azido ATP-Biotin (Schafer et al., 2001) for 5 minutes on ice. The mixture was photo-linked using UV light for 10 minutes. Immunoprecipitation of the photo-linked sample was performed using MRP4 polyclonal antibody (10 μ l/sample) (Van Aubel et al., 2002) linked to protein A immobilized on agarose (50% w/v, KemEnTec, Copenhagen, Denmark) (30 μ l/sample). The immunoprecipitated protein was blotted (see Western blotting) and visualized with Streptavidin horse radish peroxidase.

Molecular modeling of MRP4

The homology model of MRP4 was built with the YASARA molecular modeling program (Krieger et al., 2002). A common problem when building models of membrane proteins is the limited availability of experimental data. In case of MRP4, the related X-ray structure of MsbA appeared to be incorrect and was retracted, which left the structure of the bacterial ABC transporter Sav1866 from *S.aureus* solved at 3.0 Å resolution (Dawson and Locher, 2006) as the only available template for the transmembrane domain (PDB entry

MOL #43661

2HYD). The cytosolic ATP-binding domain is on the other hand well represented in the PDB. We therefore selected the more closely related ATP-binding domain from human MDR1 solved at 1.5 Å resolution (Ramaen et al., 2006), aligned it to Sav1866 with YASARA's MUSTANG module (Konagurthu et al., 2006) and then fused it with the Sav1866 transmembrane domain to create a hybrid-template. The alignment of the MRP4 sequence against this template was created with the T-Coffee multiple alignment algorithm (Notredame et al., 2000), using an additional 48 intermediate sequences to guide the alignment. These 48 sequences were randomly selected from Swissprot/TrEMBL, with the requirement that their transmembrane domains be closer to both the template and MRP4, than the template is to MRP4. The resulting alignment was manually tuned to account for single residue insertions and deletions in the membrane helices, which contain several Gly/Pro mediated deviations from ideality in the Sav1866 template. Loops were modeled by scanning a non-redundant subset of the PDB (>8000 structures) for fragments with matching anchor points, a minimal number of bumps and maximal sequence similarity. Side-chains were added with YASARA's implementation of SCWRL (Canutescu et al., 2003), then the model was subjected to an energy minimization with the YAMBER force field as described previously (Krieger et al., 2004; Krieger et al., 2006). Since no membrane was present during this minimization, the backbone of residues copied from the template was kept fixed. Validation of the model with WHAT_CHECK (Hooft et al., 1996) yielded an average quality Z-score of -2.2, which is better than the template (-2.9). A PDB file of the model and the alignment are available from the authors upon request.

MOL #43661

Results

Three mutants of human MRP4 were generated and expressed in HEK293 cells. In mutant FF/L- Phe³⁶⁸ and Phe³⁶⁹ in TM6 were replaced by their analogs of MRP1/MRP2 (Leu and nothing, respectively). In the second mutant ERE/SSQ Glu³⁷⁴, Arg³⁷⁵, and Glu³⁷⁸ in TM6 were also replaced by their analogs in MRP1/MRP2 (Ser, Ser, and Gln, respectively). The final mutant FFERE/L-SSQ was a combination of mutant FF/L- and mutant ERE/SSQ. Western blot analysis of membrane vesicles prepared from HEK293 cells over-expressing wild type MRP4 and the three mutants showed comparable expression levels (Fig. 2A). Often two bands are visible, of which the mutual ratio varies in different preparations. PNGase F treatment showed that the most intense band that runs at 170 kDa is the glycosylated MRP4 protein and the band that runs at 140 kDa, is the unglycosylated MRP4 band (data not shown). MRP4-mediated ATP-dependent transport of 0.5 μM [³H]-MTX or 1 μM [³H]-cGMP was 950 ± 60 and 114 ± 7 pmol/mg protein/minute, respectively. In figure 2B the transport activity of the mutants is compared to that of the wild type. Transport of both substrates in all mutants was significantly decreased compared to that of the wild type transporter. Moreover, the transport activity levels were comparable to that of the negative control.

To investigate the substitution of the amino acids of the previous mutants in more detail we constructed the single mutants F368L, F369-, E374S, R375S, and E378Q. Again the membrane vesicles of all mutants showed expression levels comparable to wild type MRP4 (Fig. 3A). The transport activity of both [³H]-MTX and [³H]-cGMP (0.5 μM and 1 μM , respectively) was significantly reduced in mutants F368L, F369-, E374S, and E378Q compared to wild type MRP4 (Fig. 3). Interestingly, cGMP transport activity of mutant R375S was 98 ± 2 % of wild type, whereas its MTX transport activity was only 55 ± 2 %.

MOL #43661

Next, we determined the transport activity of the mutants and wild type transporter at different substrate concentrations. Figure 4 shows the Michaelis-Menten plot for MTX and cGMP. Mutants F368L, F369-, E374S, and E378Q showed transport activity levels that were comparable to that of the negative control at all concentrations tested for both MTX and cGMP. The maximum transport rate (V_{max}) for the wild type and R375S mutant was 280 ± 60 and 270 ± 120 pmol/mg protein/min for MTX and 370 ± 30 and 270 ± 70 pmol/mg protein/min for cGMP, respectively. The apparent affinity (K_m) of wild type MRP4 and mutant R375S was 230 ± 90 and 720 ± 320 μ M for MTX and 610 ± 70 and 610 ± 80 μ M for cGMP, respectively. The K_m for cGMP was not influenced by substitution of Arg³⁷⁵ with Ser, whereas it was decreased three fold for MTX.

To test the interaction of MTX and cGMP in more detail we analyzed the possible inhibitory effect of MTX on [³H]-cGMP uptake for wild type and R375S mutant MRP4. A Dixon plot of net cGMP transport by wild type and R375S in the absence or presence of increasing MTX concentrations was constructed and analyzed by linear regression (Fig. 5). Remarkably, the intersection of the 3 lines representing MTX inhibition curves at different cGMP concentrations was at the x-axis for both wild type and R375S mutant, indicating a non-competitive inhibitory effect. The inhibition constant (intersection with the x-axis), K_i , for wild type MRP4 was 164 ± 4 μ M compared with 470 ± 70 μ M for mutant R375S.

At this moment it was, however, not clear whether this effect was due to the introduction of a polar serine or the deletion of the positively charged arginine. In the next experiment we replaced arginine with alanine, lysine, and glutamic acid. Furthermore, we also substituted the arginine present in TM12 for alanine. Figure 6A shows an equal level of protein expression of wild type as well as R375A, R375K, R375E, and R988A mutants. The MTX and cGMP transport activity of the wild type and mutants was measured and shown in figure 6B. Mutating the Arg³⁷⁵ residue into alanine or glutamic acid significantly decreased

MOL #43661

the transport activity for both MTX and cGMP, while mutating it into lysine retained the transport activity (96 ± 12 % for MTX and 97 ± 16 % for cGMP). In addition, the transport levels of R988A showed no significant difference from the negative control.

To determine the K_m values of the different mutants for MTX and cGMP concentration-dependent transport experiments were performed (Fig. 7). The transport activities of R375A, R375E, and R988A for MTX and cGMP did not differ from that of the negative control. The V_{max} values for MTX were 250 ± 20 and 190 ± 10 pmol/mg protein/min and those for cGMP were 420 ± 10 and 420 ± 20 pmol/mg protein/min for wild type and R375S mutant, respectively. The K_m values of wild type MRP4 and R375K were 230 ± 90 and 250 ± 20 μ M for MTX and 610 ± 70 and 640 ± 60 μ M for cGMP, respectively. Thus, R375K retained substrate affinity comparable to the wild type for both MTX and cGMP.

To determine whether the MRP4 mutants showed normal ATP binding, azido-ATP-biotin photolabeling of mutants was compared with that of wild-type and negative control (Fig. 8). The vesicles of MRP4 wild type and mutants showed increased 8-azido-ATP-biotin binding compared with the negative control. In all mutant preparations the 8-azido-ATP-biotin binding was comparable to that of the wild type. Moreover, the binding of 8-azido ATP-biotin was almost diminished when ATP was added.

To link the functional consequences of the mutations back to their structural basis, we built a homology model of MRP4 using the known X-ray structures of the bacterial ABC transporter Sav1866 from *S.aureus* (Dawson and Locher, 2006) and the ATP-binding domain of human P-glycoprotein/MDR1 as templates (Ramaen et al., 2006) (Fig. 9A). Sav1866 forms a dimer, while MRP4 encodes both copies in a single sequence. The predicted location of the mutated residues is shown in figure 9B. Based on this model, one can postulate that

MOL #43661

Arg³⁷⁵ and Arg⁹⁹⁸ face right into the pore and are thus very likely to interact directly with
MTX and cGMP.

MOL #43661

Discussion

To characterize the MRP4 substrate-binding site we investigated the influence of Phe³⁶⁸, Phe³⁶⁹, Glu³⁷⁴, Arg³⁷⁵, and Glu³⁷⁸ of transmembrane helix 6, and Arg⁹⁹⁸ of helix 12 on cGMP and MTX transport. These amino acids were substituted by their corresponding amino acids of MRP1 and MRP2. Most mutations completely abolished MRP4 transport activity. The only exception was substitution of Arg³⁷⁵ with serine, which had no effect on cGMP transport, but significantly decreased the affinity for MTX. Substitution of the same amino acid with a positively charged lysine returned the MTX affinity to that of the wild type. A homology model of MRP4 confirmed the crucial role of Arg³⁷⁵ and showed that it faced right into the central pore.

Wild type MRP4 and all mutants were equally expressed in vesicles isolated from transduced HEK293 cells. Nevertheless, several MRP4 mutants (FF/L-, ERE/SSQ, FFERE/L-SSQ, F368L, F369-, E374S, R375A, R375E, E378Q, and R998A) showed significantly diminished transport activity of either MTX or cGMP compared with that of wild type MRP4. This indicates that Phe³⁶⁸, Phe³⁶⁹, Glu³⁷⁴, Arg³⁷⁵, and Glu³⁷⁸, present in TM6, and Arg⁹⁹⁸, present in TM12, might comprise an important part of MRP4 substrate-binding site. Indeed, studies with LmrA, a bacterial ATP-dependent multidrug transporter, show that TM3, TM5 and TM6 (also TM9, TM11, and TM12) are involved in substrate binding (Ecker et al., 2004). These TMs have one face of the helix exposed to the pore, which forms a pathway for substrates through the membrane. Experimental evidence has shown that conformational changes of the nucleotide-binding domains (NBD) of P-glycoprotein/MDR1, leads to reorientation of the TM helices (Loo et al., 2007). TM6 is connected to NBD1 and changes in the position or orientation of TM6 resulting from mutations, may cause a conformational or positional change in NBD1 leading to diminished binding of ATP and

MOL #43661

transport activity (Zhang et al., 2004). We tested if the mutants were able to bind azido-ATP-biotin and observed no difference in binding between MRP4 and the mutants.

All the amino acids mutated in the present study are highly conserved in MRP4 among different species (Chen and Klaassen, 2004). There is, however, a big difference within the MRP subfamily that might point to a specific mechanistic importance of these amino acids. The two phenylalanines at position 368 and 369 in MRP4 are unique and absent in other MRPs. Replacement of these two amino acids by a single leucine (their counterpart in MRP1, 2, 3, and 5) resulted in the lack of MTX and cGMP transport. The Phe³⁶⁹, predicted to face outside the central pore and attach helix 6 to helix 3, most likely has a role in maintaining the structural integrity of the transporter. Phe³⁶⁸ faces into the pore and could mediate stacking interactions with the planar rings of cGMP and MTX. The negatively charged glutamic acid residues at position 374 and 378 of MRP4 correspond to a serine and glutamine, respectively, in MRP1, 2, and 6. The replacement of these two MRP4 amino acids by serine and glutamine also resulted in the lack of MTX and cGMP transport. Zhang et al. (Zhang et al., 2004) showed that replacement of MRP1 Ser⁶⁰⁴ (corresponding to E374S in MRP4) with alanine selectively increased 17 β -estradiol 17-(β -D-glucuronide) transport. In the model, Glu³⁷⁴ and Glu³⁷⁸ form salt bridges with Arg³¹⁷ and Arg²⁶², which points towards a role in maintaining the structural integrity of the transporter.

In this study we showed that the positively charged arginine residue at position 375 is important for MRP4-mediated MTX but not cGMP transport. The importance of positively charged amino acids to the binding and transport functionality was previously indicated for MRP1 (Zhang et al., 2003a). Deletion of the hydroxyl group of MRP1 residue 605 (S605A), corresponding to R375A in MRP4, decreased resistance to vincristine, VP-16, and doxorubicin (Zhang et al., 2004). Moreover, the Q359K/T360K mutation (corresponding to

MOL #43661

MRP4 Glu³⁷⁴ and Arg³⁷⁵) in the cystic fibrosis transmembrane conductance regulator (CFTR) protein causes cystic fibrosis (Quint et al., 2005).

In the present study, substitution of MRP4 Arg³⁷⁵ with serine resulted in a lower affinity for MTX, but the affinity for cGMP did not change (Table 1). Furthermore, the MTX inhibition constant for cGMP transport by mutant R375S was significantly lower than that of the wild type. When the arginine at position 375 of MRP4 was substituted with lysine, another positively charged amino acid, MTX and cGMP transport activities were similar to that of the MRP4 wild type. For MTX affinity the positive charge of MRP4 residue 375 is critical. When this charge is removed (R375S) the MTX affinity decreases or transport activity is absent (R375A and R375E). This positive charge is less important for cGMP transport, because in the presence of a hydroxyl group (R375S) the transport properties seem unchanged. As soon as this hydroxyl group is removed (R375A) or replaced with a (negatively charged) acidic group (R375E) transport of cGMP is absent. Our observation that the R375S mutation has a larger effect on MTX transport could be explained by the fact that MTX contains two negative charges that need to be compensated, while cGMP only has one. In any case our results show that lysine is equally well suited as arginine to mediate this interaction.

Photolabelling and mutational studies aimed at predicting regions of MRP1 protein that contribute to the substrate-binding sites have outlined the possible role of TM17 (Daoud et al., 2001; Deeley et al., 2006), which corresponds to TM12 in MRP4. In the present work, we mutated Arg⁹⁹⁸ located at MRP4 TM12 into alanine. Substitution of this positively charged amino acid resulted in nearly complete abolishment of transport activity for either MTX or cGMP. It was reported that the corresponding amino acid Arg¹²⁵⁷ in MRP2 showed decreased transport activity of glutathione-methylfluorescein when substituted with alanine (Ryu et al., 2000). Moreover, alanine substitution of Arg¹²⁴⁹ in MRP1 (also corresponding to

MOL #43661

R998A) impaired MRP1-mediated LTC₄ transport and reduced vincristine resistance (Ren et al., 2002). This indicates the participation of TM12 to the MRP4 binding pocket and emphasizes the importance of positively charged amino acids for MRP4 transport activity. Although mutagenesis studies confirm the functional requirement of a positive charge at Arg³⁷⁵, this has not been confirmed for Arg⁹⁹⁸. Glutamic acid (R998E) and lysine (R998K) mutants would be needed to strengthen this conclusion.

Complex substrate-transporter interactions have been described previously for ABC transporters like P-glycoprotein/MDR1 (Martin et al., 2000), MRP1 (Leslie et al., 2001), MRP2 (Zelcer et al., 2003), and MRP4 (Van Aobel et al., 2005). These complexities were attributed to multiple allosteric-binding sites. Here, we encountered similar complex transport inhibition patterns. Mutant R375S and wild type MRP4 possessed similar affinities for cGMP, but the MTX affinity of this mutant was nearly 3-fold lower than that of the wild type. This implicates that these two MRP4 substrates do not share the same substrate-binding site. This was also implied by the non-competitive inhibitory effect of MTX on MRP4-mediated cGMP transport. Therefore, we propose that MRP4 can bind two substrates at different regions within the aqueous cavity involving overlapping amino acids. This could partly explain the complex inhibitory and stimulatory kinetics that we encountered in previous studies (Van Aobel et al., 2005; El Sheikh et al., 2007). Our data are indicative; they are nonetheless based on a homology model and mutagenesis studies, both of which contain certain experimental caveats.

We conclude that amino acids in the transmembrane helices 6 and 12 may comprise a crucial part of the MRP4 substrate-binding site. The importance of a positive charge at residue 375 seems evident for MTX transport, but not for cGMP. This residue, which is predicted to be in the aqueous cavity of the inner leaflet of the membrane, clearly affects

MOL #43661

substrate specificity. Furthermore, a non-competitive inhibition between MTX and cGMP has been revealed, indicating separate MRP4 substrate-binding sites.

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MOL #43661

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MOL #43661

Legends for figures

Fig. 1. Alignment of amino acid sequence of TM6 (TM11) of MRP4 for multiple species and human MRP1-6.

Fig. 2. Western blot analysis (A), 0.5 μ M [3 H]-MTX (B), and 1 μ M [3 H]-cGMP (C) transport activity of wild type, FF/L-, ERE/SSQ, and FFERE/L-SSQ MRP4 transporter proteins. Upper panel (A) represents a Western blot of membrane vesicles isolated from HEK293 cells over-expressing MRP4 or MRP4 mutants FF/L-, ERE/SSQ, and FFERE/L-SSQ as well as a negative control detected by polyclonal anti-human MRP4 (representative of three). The MRP4 wild type transport activity for [3 H]-MTX (950 ± 60 fmol/mg protein/minute) and [3 H]-cGMP (114 ± 7 fmol/mg protein/minute) was set at 100%.

Fig. 3. Western blot analysis (A), 0.5 μ M [3 H]-MTX (B), and 1 μ M [3 H]-cGMP (C) transport activity of wild type, F368L, F369-, E374S, R375S, and E378Q MRP4 transporter proteins. Upper panel (A) represents a Western blot of membrane vesicles isolated from HEK293 cells over-expressing MRP4 or MRP4 mutants F368L, F369-, E374S, R375S, and E378Q as well as negative control detected by polyclonal anti-human MRP4 (representative of three). The MRP4 wild type transport activity for [3 H]-MTX (970 ± 80 fmol/mg protein/minute) and [3 H]-cGMP (106 ± 7 fmol/mg protein/minute) was set at 100%.

MOL #43661

Fig. 4. Concentration-dependent uptake of [3 H]-MTX and [3 H]-cGMP in membrane vesicles expressing human MRP4 mutants. Control (\blacktriangle), wild type (\blacksquare), F368L (\bullet), F369- (\square), E374S (\diamond), R375S (\circ), and E378Q (x) MRP4 membrane vesicles were incubated with [3 H]-MTX (upper graph) or [3 H]-cGMP (lower graph) concentrations indicated in the figure. ATP-dependent uptake was measured by subtracting uptake in the presence of AMP from that measured in the presence of ATP. Mean values \pm SE of three enzyme preparations are shown.

Fig. 5. Dixon plot showing the MTX inhibition of cGMP transport by human MRP4 and mutant R375S. Membrane vesicles were incubated with 1 μ M (open circle), 10 μ M (closed triangles), or 100 μ M (open squares) [3 H]-cGMP in the presence or absence of MTX concentrations ranging from 1 to 600 μ M. Specific uptake of MRP4 (upper graph) and mutant R375S (lower graph) was determined after subtraction of the negative control. The inhibition constant (K_i) can be estimated from the intersection point of the 3 lines with the x-axis. Mean values \pm SE of three enzyme preparations are shown.

Fig. 6. Western blot analysis (A), 0.5 μ M [3 H]-MTX (B), and 1 μ M [3 H]-cGMP (C) transport activity of wild type, R375A, R375K, R375E, and R998A MRP4 transporter proteins. Upper panel (A) represents a Western blot of membrane vesicles isolated from HEK293 cells over-expressing MRP4 or MRP4 mutants R375A, R375K, R375E, and R998A as well as negative control detected by polyclonal anti-human MRP4 (representative of three). The MRP4 wild type transport activity for [3 H]-MTX (980 ± 20

MOL #43661

fmol/mg protein/minute) and [³H]-cGMP (125 ± 13 fmol/mg protein/minute) was set at 100%.

Fig. 7. Concentration-dependent uptake of [³H]-MTX and [³H]-cGMP in membrane vesicles expressing human MRP4 mutants. Control (▲), wild type (■), R375A (●), R375K (○), R375E (◇), and R998A (x) MRP4 membrane vesicles were incubated with [³H]-MTX (upper graph) or [³H]-cGMP (lower graph) concentrations indicated in the figure. ATP-dependent uptake was measured by subtracting uptake in the presence of AMP from that measured in the presence of ATP. Mean values ± SE of three enzyme preparations are shown.

Fig. 8. Western blot of immunoprecipitated azido-ATP-biotin photolabeled wild type and mutant transporter proteins. 300 µg protein vesicles was labeled with 100 µM azido-ATP-biotin and the MRP4 proteins were immunoprecipitated with MRP4 polyclonal antibody linked to agarose protein A beads. The immunoprecipitated protein was blotted and visualized with streptavidin HRP. The addition of 5 mM ATP prevented photolabeling (data not shown).

Fig. 9. Homology model of human MRP4 (A). The first half of the protein containing transmembrane helices 1 to 6 and the first ATP-binding domain is colored with a gradient from blue over orange to cyan. The second half of the protein (corresponding to the second monomer in the dimeric Sav1866 modeling template) is shown in grey. A 180-

MOL #43661

degree rotation around the vertical axis would yield an almost identical picture of helices 7 to 12 and the second ATP-binding domain. Panel B shows a close-up of the channel formed by the 12 transmembrane helices, looking from the inside to the extra cellular side. The mutants described in this work are indicated. Graphics created with www.YASARA.org.

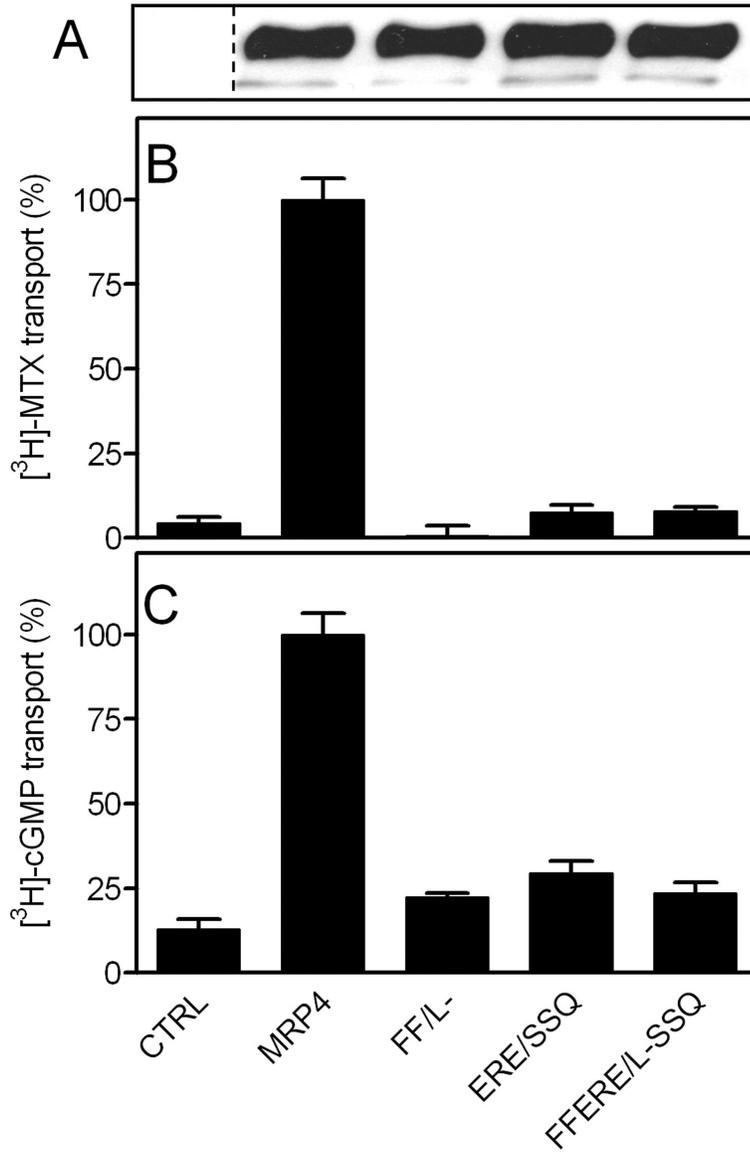
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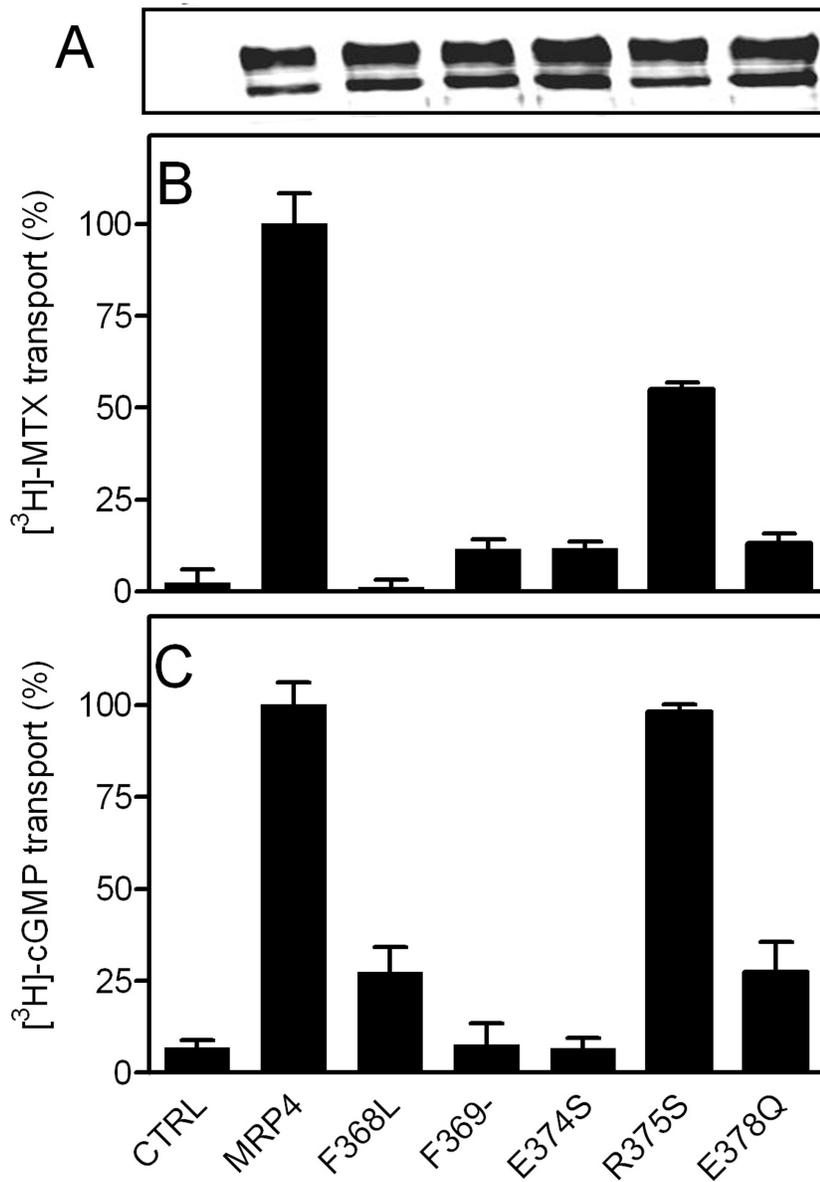
Table 1: Transporter kinetic values and inhibition constants.

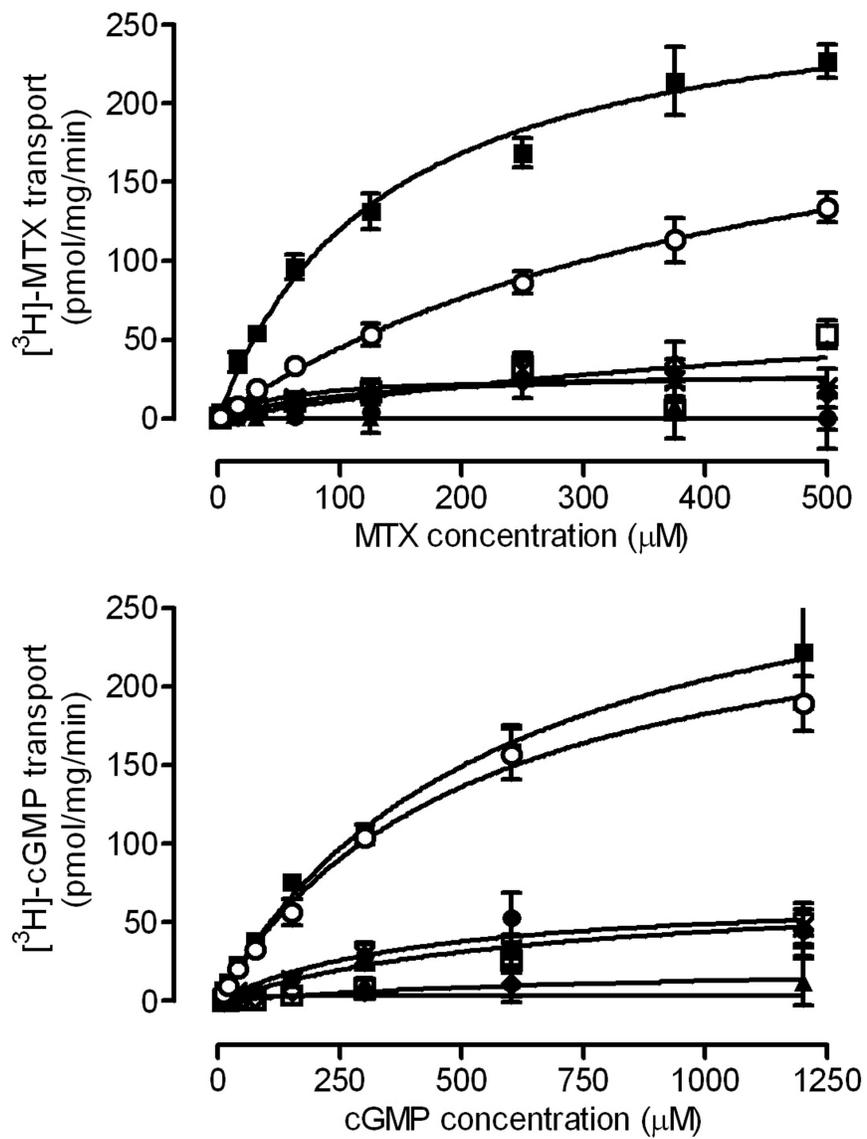
	MTX Km (μ M)	Vmax (pmol/mg/min)	cGMP Km (μ M)	Vmax (pmol/mg/min)	Ki (μ M)
Data from fig. 3 and 4:					
MRP4	230 \pm 90	280 \pm 60	610 \pm 70	370 \pm 30	164 \pm 4
R375S	720 \pm 320	270 \pm 120	610 \pm 80	270 \pm 70	470 \pm 70
Data from fig. 4:					
MRP4	230 \pm 90	250 \pm 20	610 \pm 70	420 \pm 10	
R375K	250 \pm 20	190 \pm 10	640 \pm 60	420 \pm 20	

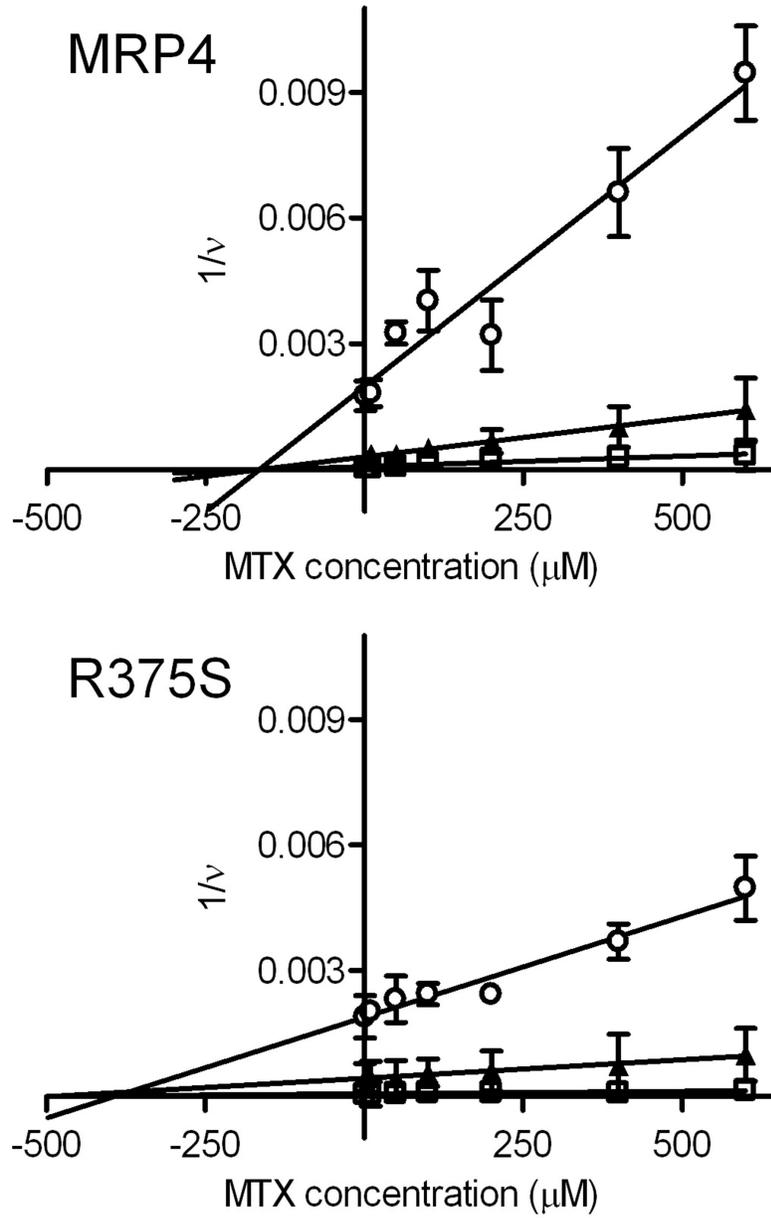
Figure 1

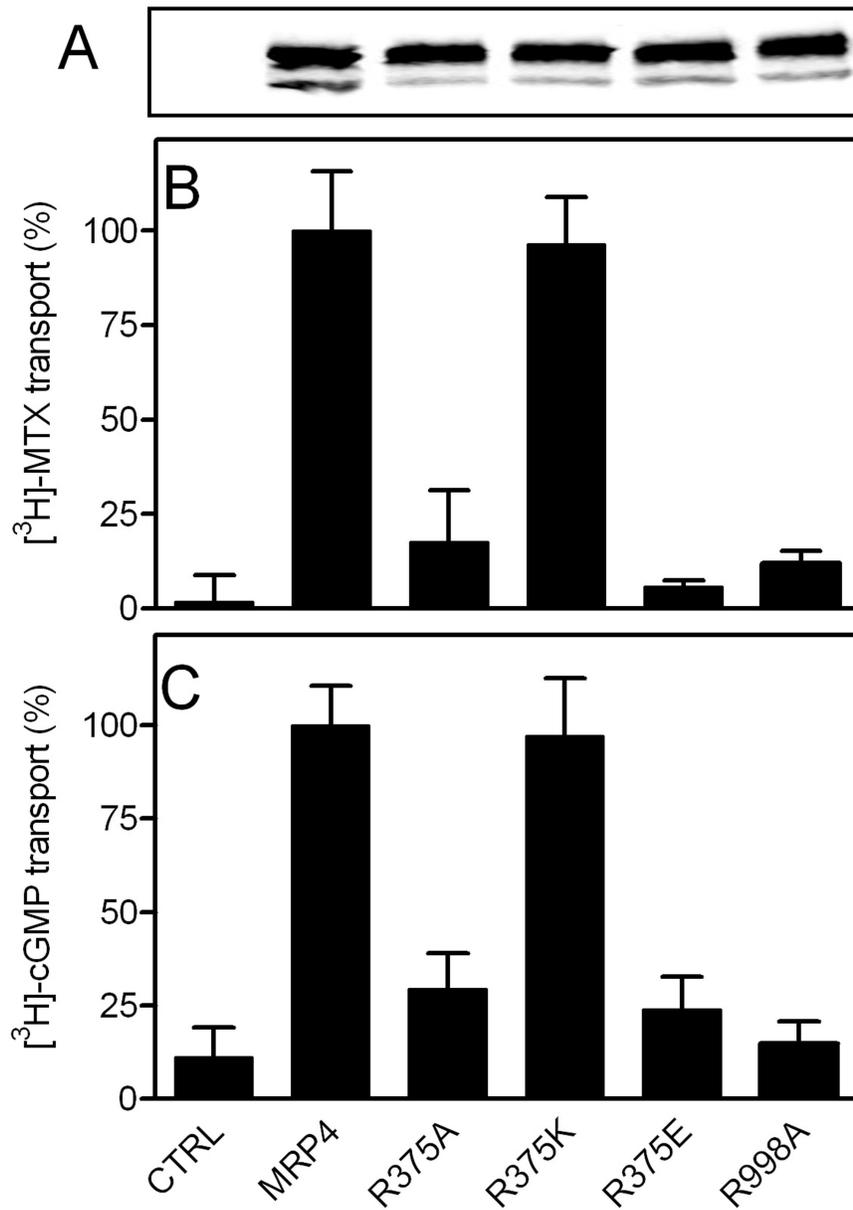
Human	LYGAVRLTVTL FF PSAI ER VSE E AIVSIRR	
Mouse	LYGAVRLTVTLFFPSAIERGSEAIVSIRR	
Rat	LYGAVRLTVTLFFPSAIERVSEAVSVRR	
Dog	LYGAVRLTVTLFFPSAIERVSESVSIQR	
Chicken	LYGAVRLTVTLFFPSAVERVSEAVVSIRR	
Cow	LYGAVRLTVTLFFPSAVEKVSEAFVSIRR	
Zebrafish	LYSAVRLTVTLFFPAAIEKVSESAISIRR	
Platypus	LYGAVRVTVTTLFFPAAVERVSETIISIRR	
Opossum	LYGAVRLTVTLFFPAAVEKMSETRVSIKR	
	** .***:*****:*:*: ** :*::*	
MRP1	LFNILRFPLNIL-PMVISSIVQASVSLKR	615
MRP2	LFNILRFPLSML-PMMISSMLQASVSTER	612
MRP3	LFNILRLPLNML-PQLISNLTQASVSLKR	601
MRP4	LYGAVRLTVTL FF PSAI ER VSE E AIVSIRR	385
MRP5	VFNSMTFALKVT-PFSVKSLSEASVAVDR	466
MRP6	VLNILNKAQAFL-PFSIHSLVQARVSFDR	600

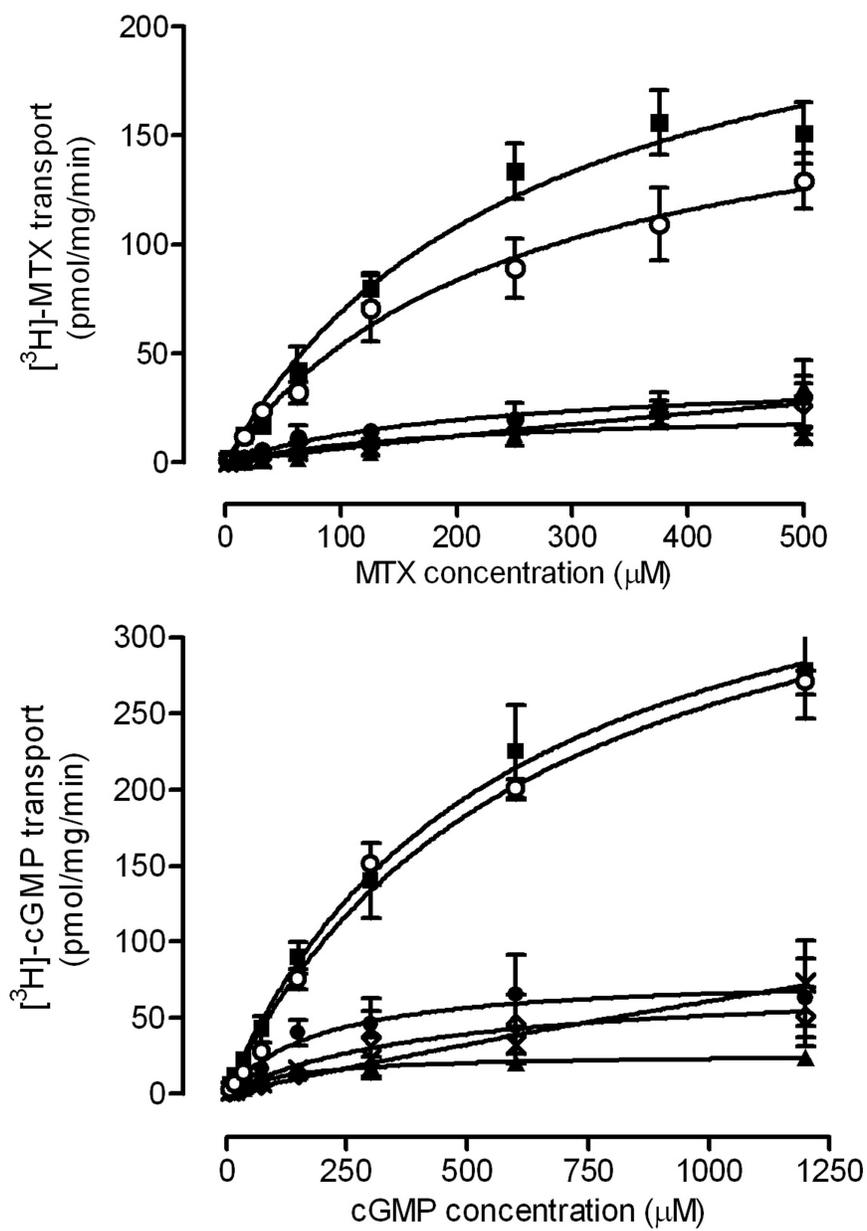












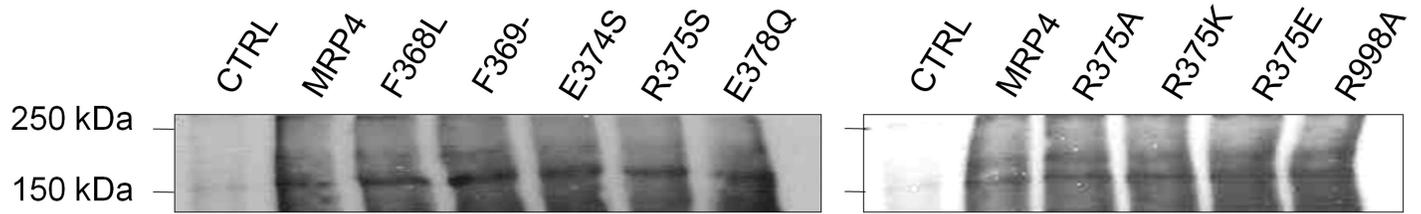


Figure 9

