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Title page

Interaction of Non-Steroidal Anti-Inflammatory Drugs with MRP2/ABCC2- and MRP4/ABCC4-Mediated Methotrexate Transport

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Running title page

Running title: NSAIDs inhibit MTX transport by MRP2 and MRP4

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Abbreviations:

ABC, ATP-binding-cassette; HEK293, Human embryonic kidney cells; MDCKII, Madin-Darby canine kidney cells; MRP/Mrp, multidrug resistance protein; MTX, methotrexate;

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NSAIDs, non-steroidal anti-inflammatory drugs; OAT/Oat, organic anion transporter; RFC/Rfc, reduced folate carrier.

Recommended section assignment: Metabolism, Transport, and Pharmacogenomics.

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Abstract

Methotrexate (MTX) has been used in combination with non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment of inflammatory diseases, as well as malignancies. Especially at high MTX dosages, severe adverse effects with this combination may occur usually resulting from an impaired renal elimination. It has been shown that the mechanism of this interaction cannot be fully attributed to inhibition of basolateral MTX uptake in renal proximal tubules. Here, we studied the effect of various NSAIDs on MTX transport in membrane vesicles isolated from cells over-expressing the proximal tubular apical efflux transporters human MRP2/ABCC2 and MRP4/ABCC4. MTX was transported by MRP2 and MRP4 with K_m values of 480 ± 90 and 220 ± 70 μM, respectively. The inhibitory potency of the NSAIDs was generally higher against MRP4- than MRP2-mediated MTX transport, with therapeutically relevant IC_{50} values, ranging from approximately 2 μM to 1.8 mM. Salicylate, piroxicam, ibuprofen, naproxen, sulindac, tolmetin, and etodolac inhibited MRP2- and MRP4-mediated MTX transport according to a one-site competition model. In some cases more complex interaction patterns were observed. Inhibition of MRP4 by diclofenac, and MRP2 by indomethacin and ketoprofen followed a two-site competition model. Phenylbutazone stimulated MRP2 and celecoxib MRP4 transport at low concentrations and inhibited both transporters at high concentration. Our data suggest that the inhibition by NSAIDs of renal MTX efflux via MRP2 and MRP4 is a potential new site and mechanism contributing to the over-all interaction between these drugs.

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Introduction

Methotrexate (MTX) has been successfully used for more than three decades in treating various malignant tumors and autoimmune disorders. Although MTX is generally well tolerated, unpredictable life-threatening toxicity still occurs at high dosages or concomitant use with other drugs, resulting in persistently high plasma concentrations. Of the drugs causing such interactions with MTX, non-steroidal anti-inflammatory drugs (NSAIDs) have been well known (Kremer and Hamilton, 1995). Numerous clinical case reports have documented that NSAIDs can cause a reduction in the MTX clearance, even at relatively low maintenance dosages used to treat rheumatoid arthritis. Ketoprofen, indomethacin, and diclofenac caused different levels of toxicities in patients receiving MTX, ending fatally in 3 out of 4 the patients receiving ketoprofen (Thyss et al., 1986), two of which had about twelve-fold higher MTX serum levels. Similar toxicities were found with salicylate (Stewart et al., 1991), ibuprofen (Cassano, 1989), and naproxen (Singh et al., 1986).

MTX is primarily excreted into urine in the unchanged form and renal handling involves tubular secretion and reabsorption, in addition to glomerular filtration. Renal tubular secretion of MTX has been thought to be a major site of interaction with other drugs (Takeda et al., 2002). The first step is active uptake from the blood across the basolateral membrane into the proximal tubular cells via the organic anion transporters 1 and 3 (OAT1 and OAT3) and the reduced folate carrier (RFC-1) (Nozaki et al., 2004). Extrusion from the cells across the luminal membrane into the urine is mediated by the ATP-dependent efflux pumps, multidrug resistance proteins 2 and 4 (MRP2 and MRP4) (Chen et al., 2002;Masuda et al., 1997;van Aubel et al., 2002). Furthermore, MTX is recognized by luminal OAT4 (Takeda et al., 2002), which reabsorbs anions from the primary urine back into the tubular cells (Ekaratanawong et al., 2004). The interaction of NSAIDs with MTX uptake at the basolateral membrane has been investigated recently, and the results showed that several NSAIDs

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inhibited MTX uptake via human OAT1 and OAT3 at clinically relevant concentrations (Takeda et al., 2002). Nevertheless, when studied in rat kidney slices, the interaction was not as potent as presumed, probably because Rfc-1, which is insensitive to NSAIDs, also contributes significantly to tubular MTX uptake (Nozaki et al., 2004). To date, the effect of NSAIDs on the luminal efflux of MTX via MRP2 and MRP4 has not yet been studied as potential site of this drug-drug interaction.

The expression of MRP2 (Schaub et al., 1997) and MRP4 (van Aubel et al., 2002) in the kidney is restricted to the luminal membrane of the proximal tubule cells. Mutations in the gene encoding MRP2 (*ABCC2*) causing Dubin-Johnson syndrome, a rare form of conjugated hyperbilirubinemia, can impair high dose MTX elimination leading to renal toxicity in vivo (Hulot et al., 2005). Previous studies showed that MRP4-mediated estradiol 17-beta-D-glucuronide transport was inhibited potently by several NSAIDs (Reid et al., 2003), suggesting that these drugs might be potential inhibitors of other MRP4 substrates. In addition, luminal efflux transporters may get exposed to relatively high inhibitor concentrations, as NSAIDs can be taken up actively into the proximal tubular cells via basolateral OATs (Apiwattanakul et al., 1999;Sekine et al., 1997). The objective of this study was to investigate the interaction potential of NSAIDs with the renal MTX efflux transporters MRP2 and MRP4. For this purpose we used membrane vesicles isolated from cells over-expressing human MRP2 and MRP4. Our findings show that various NSAIDs inhibit both MRP2- and MRP4-mediated MTX transport, in general with higher inhibitory potency against MRP4. Our study also shows different and sometimes complex inhibitory patterns of the various NSAIDs tested. This is the first study in which this putative drug-drug interaction is examined at the molecular level.

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Methods

Materials. [3',5',7'-^{3}H(n)]-MTX sodium salt (51.5 Ci mmol^{-1}) was purchased from Moravek Inc. (Brea, CA). The Bac-to-Bac and Gateway system were purchased from Invitrogen (Breda, the Netherlands). Creatine phosphate and creatine kinase were purchased from Roche diagnostics (Almere, the Netherlands). NC45 filters were obtained from Schleicher & Schuell (Den Bosch, the Netherlands). Protein concentrations were determined with an assay kit form Bio-Rad Laboratories (Veenendaal, the Netherlands). Celecoxib was purified from 200 mg Celebrex capsules, Pfizer (Capelle a/d Ijssel, the Netherlands) according to a previously described method (Tong et al., 2005).

Cell Lines and Culture Conditions. The MRP2-overexpressing MDCKII cell line was kindly provided by Dr. P. Borst, (Dutch Cancer Institute, Amsterdam, the Netherlands), and was cultured as described previously (Evers et al., 1998). The non-transfected MDCKII cell line was used as a control. HEK293 cells were grown in DMEM supplemented with 10% fetal calf serum at 37^{o}C under 5% CO_2-humidified air.

Generation of Human MRP4 Baculovirus. The Bac-to-Bac system, normally used for protein production in insect cells, was made suitable for protein expression in mammalian cells by cloning the vesicular stomatitis virus G protein cDNA behind the P10 promoter of the pFastBacDual vector. Next, the CMV promoter and Gateway destination elements (cassette that contains the chloramphenicol resistance gene and the ccdB gene flanked by attR1 and attR2 sites) were introduced in the pFastBacDual vector. The human MRP4 was cloned into the Gateway entry vector. The sequence of this MRP4 construct is equal to GenBank accession number NM_005845 except for the I18L polymorphism. This MRP4 cDNA was transferred to the newly constructed Bac-to-Bac vector with the gateway LR

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reaction. Baculoviruses were produced as described in the Bac-to-Bac manual. As a control, the enhanced yellow fluorescent protein (EYFP) was also cloned into the gateway entry vector (Invitrogen).

Transduction of HEK293 Cells. HEK293 cells were cultured in 182 cm^2 flasks till 70% confluence, after which the culture medium was removed and 1.5 ml virus and 3.5 ml medium were added. The cells were incubated for 30-60 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.

Isolation of Membrane Vesicles and Protein Analysis. Cells were harvested by centrifugation at 3,000x*g* 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 µM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 µM pepstatin, 1 µM E-64) and shaken at 4°C for 60 minutes. Lysed cells were centrifuged at 4°C at 100,000x*g* 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 100,000x*g* 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.

Immunoblotting. The membrane vesicle preparations were diluted in TS buffer. The indicated amount of protein was size fractionated on a 7.5% SDS-polyacrylamide gel and

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subsequently blotted on nitrocellulose membrane. Affinity-purified, polyclonal anti-human MRP2 (p Ab hM2-p1) and anti-human MRP4 (p Ab hM4-p4) antibodies (1:1000) were used to detect human MRP2 and MRP4, respectively (Smeets et al., 2004;van Aubel et al., 2002). Signals were visualized with chemiluminescence (ECL, Amersham Biosciences, Diegem, Belgie).

Vesicular Transport Assays. Uptake of [^{3}H]-MTX into membrane vesicles was performed as described (van Aubel et al., 1999). In brief, membrane vesicles were pre-warmed for 1 minute at 37°C and added to TS buffer, supplemented with an ATP-regeneration mixture (4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 mg/ml creatine kinase) in a final volume of 60 µl. The reaction mixture was incubated at 37°C, for 15 minutes and samples were taken from the mixture at indicated times, diluted in 900 µl ice-cold TS buffer. Diluted samples were filtered through 0.45-µm-pore NC filters that were pre-incubated with TS buffer, by a filtration device (Millipore, Bedford, MA), using a rapid filtration method. After adding 4 ml scintillation fluid and subsequent liquid scintillation counting, uptake of [^{3}H]-MTX 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. Measurements were corrected for the amount of ligand bound to the filters (usually <2% of total radioactivity). Each experiment was performed in triplicate. The sidedness of the membrane vesicles was not determined. ATP-dependent uptake can only occur in inside-out vesicles, and since inhibition by NSAIDs is expressed relatively as percentage of MTX uptake, the result is not affected by differences in expression levels and sidedness of the vesicles.

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Transport Inhibition Assays. To evaluate the inhibitory effects of NSAIDs on MTX uptake mediated by MRP2 and MRP4, the previously mentioned transport assay was performed using 0.5 μM [^{3}H]-MTX, in the absence or presence of various concentrations of NSAIDs. Hydrophilic NSAIDs, viz. salicylate, diclofenac, tolmetin, ibuprofen, and naproxen, were dissolved in H_2O. Hydrophobic NSAIDs, viz. sulindac, indomethacin, etodolac, ketoprofen, phenylbutazone, piroxicam, and celecoxib, were dissolved in dimethyl sulfoxide (DMSO) and diluted with incubation medium.

The final concentration of DMSO in the incubation medium was adjusted to less than 1% (Apiwattanakul et al., 1999). We tested the effect of 1% DMSO and observed a reduction of about 20% in transport. Because in all transport assays the same concentration of DMSO was used as a control, this reduction has not influenced the results. In all experiments with hydrophobic NSAIDs, control samples contained the same concentration of DMSO.

Kinetic Analysis. All data were expressed as means ± S.E. IC_{50} values of NSAIDs were obtained from curve-fitting of the resulting concentration-inhibition curves by nonlinear regression analysis using GraphPad Prism software version 4.03 (GraphPad Software Inc., San Diego, USA). Statistical differences were determined using a one-way ANOVA with Dunnett's post test. Differences were considered to be significant at $P < 0.05$.

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Results

Expression of MRP2 and MRP4 in Isolated Membrane Vesicles. Immunoblot analysis was performed on membrane vesicles prepared from MDCKII and HEK293 cells over-expressing human MRP2 and MRP4, respectively. Figure 1 demonstrates that both MRP2 and MRP4 were successfully expressed and were detected as 190 kd (Schaub et al., 1999;van Aubel et al., 2002) and 170 kd (Lee et al., 2000;van Aubel et al., 2002) bands, respectively, while control MDCKII and HEK293 vesicles did not show any protein expression at the corresponding heights.

Time-Dependent Uptake of MTX by MRP2 and MRP4. Membrane vesicles were incubated for 2.5, 5, 10, 15, 20, and 30 minutes at 37°C in uptake medium containing 0.5 µM [^{3}H]-MTX. A relatively low MTX concentration was chosen because the specific activity of the radiolabel was not very high and for an accurate determination of IC_{50} values a substrate concentration well below the K_m must be used. The relative contribution of MRP2 or MRP4 to overall uptake was assessed in parallel experiments performed on membrane vesicles isolated from control MDCKII and HEK293 cells, respectively. Both MRP2 and MRP4 mediated ATP-dependent [^{3}H]-MTX transport (Fig. 2). Transport of [^{3}H]-MTX increased with time and the uptake was linear over the first 20 min of the assay for both MRP2 and MRP4. Uptake at 20 min remained linear up to the highest [^{3}H]-MTX concentrations used in the experiments to assess the concentration-dependency of transport, viz. 2 mM for MRP2 and 1 mM for MRP4 vesicles (data not shown).

Concentration-Dependent Transport of MTX by MRP2 and MRP4. Membrane vesicles were incubated for 15 minutes at 37°C with [^{3}H]-labelled and unlabelled MTX to the final concentrations indicated (Fig. 3). ATP-dependent transport was calculated by subtracting the

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values obtained in the presence of AMP from those in the presence of ATP. MRP2- and MRP4- dependent uptakes were calculated by subtracting non-specific uptakes in control MDCKII and HEK293 vesicles, respectively. MRP2 and MRP4 efficiently transported MTX, and the concentration of MTX at half-maximum uptake rates (K_m) was relatively higher for MRP2 than for MRP4. The K_m and V_{max} values for MTX uptake in MRP2 and MRP4 vesicles were 480±90 and 220±70 μM, and 80±10 and 280±30 pmol.mg^{-1}.min^{-1}, respectively. It should be noticed that because of different expression systems the enrichment of the transporters may be different, it is therefore only meaningful to compare Km values.

Effect of NSAIDs on MRP2- and MRP4-mediated MTX transport. Membrane vesicles were incubated with 0.5 µM [^{3}H]-MTX at 37°C for 15 minutes, in the absence or presence of increasing concentrations of the various NSAIDs (Fig. 4 and 5). All NSAIDs studied inhibited MRP2- and MRP4-mediated MTX transport with different potencies and sometimes complex kinetic interactions. The calculated IC_{50} values are given in Table 1. Figure 4 shows that salicylate inhibited MTX transport with nearly the same IC_{50} values for MRP2 and MRP4, and with the lowest inhibitory potency of all the NSAIDs tested (Table 1). Piroxicam gave a similar inhibitory pattern, while the inhibitory potency of ibuprofen, naproxen, sulindac, and tolmetin was significantly higher against MRP4- than MRP2-mediated MTX transport.

Figure 5 shows that etodolac was the only NSAID that gave a steep inhibition curve of MRP2-mediated MTX transport, with a slope factor significantly higher than one. Furthermore, the inhibitory pattern of diclofenac on MRP4-mediated transport and of indomethacin and ketoprofen on MRP2-mediated MTX transport could not be simply described according to a one-site competition model, but was fitted significantly better using the two-site model. Phenylbutazone produced a bell-shaped curve against MRP2- but not

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MRP4-mediated transport, with significant stimulation up to 170±16 % at low and inhibition at higher concentrations. Celecoxib, the only selective cylcooxygenase II inhibitor tested, showed a mild but statistically significant stimulation of MRP4-mediated MTX transport at low concentrations. In contrast to what has been described by others (Zelcer et al., 2003), we could not find a stimulatory effect of indomethacin on MRP2-mediated MTX transport.

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Discussion

Severe and sometimes fatal side effects have been observed in patients after the co-administration of MTX and NSAIDs. To date the specific mechanisms contributing to this interaction have not been fully identified. It has been shown that inhibition of basolateral MTX uptake in renal proximal tubules via OAT1 and 3 is an important site of competition with NSAIDs, but not as powerful to account for the full interaction. In the present study, we show that a wide variety of NSAIDs inhibited MRP2- and MRP4-mediated MTX transport at concentrations to which the transporters may be exposed under therapeutic conditions, adding a new possible site and mechanism to the overall MTX-NSAIDs interaction.

Although all NSAIDs tested are weak organic acids, they are chemically diverse and possess a wide range of pharmacokinetic characteristics, which makes it difficult to study the relationship between structure and inhibitory activity.. In our study, sulindac had the highest and salicylate the lowest inhibitory potency for both MRP2 and MRP4. Etodolac was the only NSAID tested with an inhibitory curve with a slope factor significantly higher than unity, indicating positive cooperativity in inhibition. Most NSAIDs had a higher inhibitory potency against MRP4- than MRP2-mediated transport. The effect of diclofenac on MRP4 and of indomethacin and ketoprofen on MRP2 exhibited a two-site competition model. Furthermore, phenylbutazone and celecoxib showed a dual effect. At low concentrations phenylbutazone had a strong stimulatory effect on MTX transport via MRP2 but not MRP4, while celecoxib stimulated MRP4 transport modestly. At higher concentrations, phenylbutazone and celecoxib inhibited both transporters. This is compatible with the suggestion that both MRP2 (Zelcer et al., 2003) and MRP4 (van Aubel et al., 2005) have more than one binding site. This phenomenon was previously explained by the existence of two independent binding sites, one site that transports substrates and another site that can allosterically modulate the substrate transport site (Zelcer et al., 2003). Compounds that can

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stimulate as well as inhibit transport, probably bind at low concentrations preferentially to the modulatory site, whereas at high concentrations they can also compete for the substrate transport site. This type of allosteric interaction seems a common characteristic of the MRPs.

Several NSAIDs belonging to the selective cyclooxygenase II inhibitors were reported not to affect the clinical pharmacokinetics of MTX (Hartmann et al., 2004;Schwartz et al., 2001), including celecoxib (Karim et al., 1999). This may be due to the low immunosuppressive MTX dosages used in these studies. Our results show that celecoxib can be a potent inhibitor of MRP2- and MRP4-mediated renal MTX efflux.

The MTX concentration of 0.5 μM we used in the inhibition experiments is relatively low as compared to plasma concentrations after therapeutic dosages, which are highly variable and may range from 0.1 up to 20-50 μM. Because of tubular accumulation of MTX via active uptake it is likely that intracellular concentrations will exceed the plasma concentration. Apparently, MRP4 transports MTX with higher affinity than MRP2. Previous studies reported that K_m values of MTX were higher for MRP2 (2.5-3 mM) (Bakos et al., 2000) than for MRP4 (0.22-1.3 mM) (Chen et al., 2002;van Aubel et al., 2002), which is in line with our results. In addition, the protein expression of MRP4 is fivefold higher than MRP2 in human kidney cortex. (Smeets et al., 2004). Furthermore, by comparing the IC_{50} values of different NSAIDs we found that NSAIDs generally exhibited a higher inhibitory potency against MRP4. Given this information, we would expect that MRP4 plays a more important role than MRP2 in the inhibition of renal MTX efflux by NSAIDs. On the other hand, a recent study in Mrp2-/- mice showed 1.8-fold higher plasma concentrations of MTX as compared to wild type mice. Although Mrp4 expression was increased two-fold in the knockouts, apparently it was not able to compensate for the loss of Mrp2 in these mice (Vlaming et al., 2006). Furthermore, it was reported that in a patient with MRP2 protein dysfunction caused by a genetic variation in the *MRP2/ABCC2* gene, MTX excretion was

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impaired, leading to severe overdose manifestations as nephrotoxicity, amounting to renal failure (Hulot et al., 2005). The apparent contradiction between the expected higher effects of MRP4 participating in MTX elimination at the molecular needs further investigation. It is hard to define the relative influence of a certain NSAID on the urinary excretion of MTX, making it very difficult to recommend a specific NSAID with the lowest interaction potential in patients. In addition to the inhibitory potency of the NSAIDs, a number of other factors should be taken into consideration; the variation in NSAID dosing, which, in case of salicylate, may reach gram quantities per day producing serum concentration ranging from several hundred micromolars to several millimolars (Cerletti et al., 2003), the concentration of unchanged NSAIDs reached in proximal tubular cells by carrier-mediated uptake, and the possible effects of NSAID metabolites on MTX efflux. Because of extensive plasma protein binding ranging from 90-99%, the unbound concentrations of NSAIDs in plasma are low. The estimated unbound therapeutic concentration of the most potent inhibitors, sulindac and indomethacin, are about 1 μM and 8 μM, respectively, which is in the range of their IC_{50} values for MRP4 (Chen et al., 2006; Davies and Skjodt, 2000; Takeda et al., 2002). For the other NSAIDs tested, unbound plasma concentrations are considerably lower than their IC_{50} values, but it should be noticed that some of these compounds are also actively taken up in the proximal tubular cells by organic anion transporters, resulting in much higher concentrations to which MRP2 and MRP4 will be exposed (Apiwattanakul et al., 1999).

In summary, our study shows that MRP2- and MRP4-mediated efflux of MTX can be inhibited by various NSAIDs at therapeutically relevant concentrations. Since MRP2 and MRP4 are important efflux transporters for MTX in the kidney, they are potential new sites of MTX-NSAIDs interaction. The relative contribution of this mechanism to the overall inhibition of renal MTX excretion by NSAIDs and the individual roles of MRP2 and MRP4 herein, needs to be established in future in vivo studies.

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Footnotes

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Legends for Figures

Fig. 1. Immunoblot analysis of MRP2 and MRP4 expression in membrane vesicles used for transport studies. Membrane vesicles were prepared from control MDCKII and HEK293 as well as cells over-expressing human MRP2 and MRP4, respectively.

Fig. 2. Time-dependent uptake of [^{3}H]-MTX in MRP2 and MRP4 membrane vesicles. Vesicles were incubated for 2.5, 5, 10, 15, 20 and 30 minutes at 37°C in uptake medium containing 0.5 μM [^{3}H]-MTX. ATP-dependent [^{3}H]-MTX uptake was calculated by subtracting uptake in presence of AMP from uptake in presence of ATP in membrane vesicles from control (□) or MRP2-expressing (■) MDCKII cells, and control (○) or MRP4-expressing (●) HEK293 cells. Data present mean and S.E. of at least 3 different experiments, each performed in triplicate.

Fig. 3. Concentration-dependent uptake of [^{3}H]-MTX in MRP2 and MRP4 membrane vesicles. Vesicles were incubated for 15 minutes at 37°C with [^{3}H]-labeled and unlabeled MTX to the final concentrations indicated in the figure. ATP-dependent uptake is measured by subtracting uptake in the presence of AMP from that measured in the presence of ATP. ATP-dependent uptake of MTX by MRP2 (□) and MRP4 (●) was determined after subtraction of ATP-dependent uptake in the corresponding control vesicles from MDCKII and HEK293 cells, respectively. Data present mean and S.E. of at least 3 different experiments, each performed in triplicate.

Fig. 4. Effect of salicylate, piroxicam, ibuprofen, naproxen, sulindac, and tolmetin on MRP2- and MRP4-mediated [^{3}H]-MTX uptake in membrane vesicles. Vesicles were incubated with 0.5 μM [^{3}H]-MTX at 37°C for 15 minutes, in the absence or presence of increasing

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concentrations of the NSAIDs tested. Uptake was measured by subtracting the background (AMP) from the ATP-dependent uptake. Respective [^{3}H]-MTX transport rates for MRP2 (□) and MRP4 (●) were expressed as a percentage of control uptake against the log NSAID concentration. Data present mean and S.E. of 3 different experiments performed each in triplicate.

Fig. 5. Effect of etodolac, diclofenac, indomethacin, ketoprofen, celecoxib, and phenylbutazone on MRP2- and MRP4-mediated [^{3}H]-MTX uptake in membrane vesicles. Vesicles were incubated with 0.5 µM [^{3}H]-MTX at 37°C for 15 minutes, in the absence or presence of different concentrations of the NSAIDs tested. Uptake was measured by subtracting the background (AMP) from the ATP-dependent uptake. Respective [^{3}H]-MTX transport rates for MRP2 (□) and MRP4 (●) were expressed as a percentage of control uptake against the log NSAID concentration. Data present mean and S.E. of 3 different experiments performed each in triplicate.

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Tables

Table 1. IC_{50} values of various NSAIDs for MRP2- and MRP4-mediated MTX transport. [^{3}H]-MTX uptake was measured by subtracting the background (AMP) from the ATP-dependent uptake. Each value represents the mean ± S.E. of data obtained from three separate experiments, each performed in triplicate, calculated by non-linear regression analysis of the data from figure 4 and figure 5.

NSAID	MRP2 (µM)	MRP4 (µM)
Salicylate	1760 ± 30	1500 ± 20
Piroxicam	257 ± 2	216 ± 2
Ibuprofen	930 ± 20	26.3 ± 0.3
Naproxen	609 ± 7	42.3 ± 0.3
Sulindac	38 ± 1	2.11 ± 0.02
Tolmetin	494 ± 5	20.5 ± 0.3
Etodolac	480 ± 2[§]	120 ± 1
Diclofenac	97 ± 1	0.006 ± 0.001 (H)
		326 ± 6 (L)
Indomethacin	0.06 ± 0.01 (H)	6.1 ± 0.1
	46 ± 1 (L)	
Ketoprofen	1.4 ± 0.1 (H)	11.9 ± 0.1
	470 ± 20 (L)	
Celecoxib	100 ± 2	35 ± 1[#]
Phenylbutazone	605 ± 4[#]	130 ± 2

[§]Dose-inhibition curve with a slope factor > 1. High (H) and low (L) affinity IC_{50} determined according to a two-site competition model. [#]Stimulation of uptake at low concentrations.

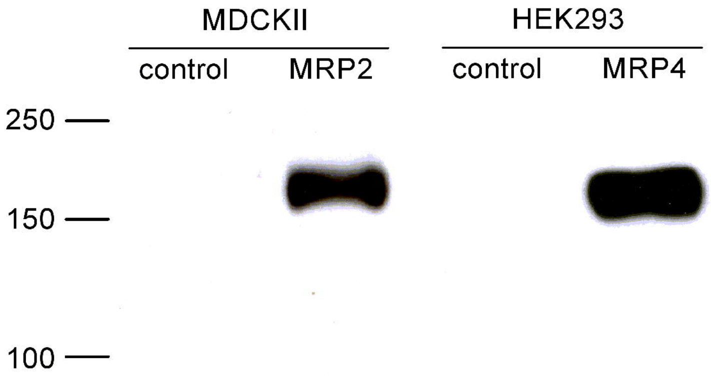


Figure 1

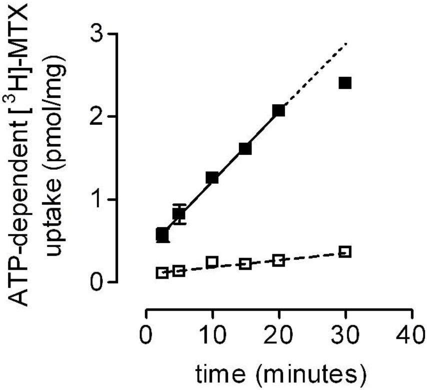
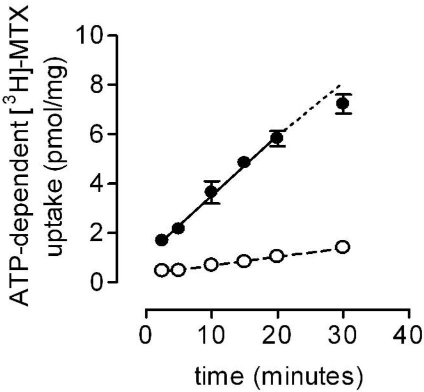

ATP-dependent [^{3}H]-MTX uptake (pmol/mg)
3
2
1
0
0
10
20
30
40
time (minutes)

ATP-dependent [^{3}H]-MTX uptake (pmol/mg)
10
8
6
4
2
0
0
10
20
30
40
time (minutes)

Figure 2

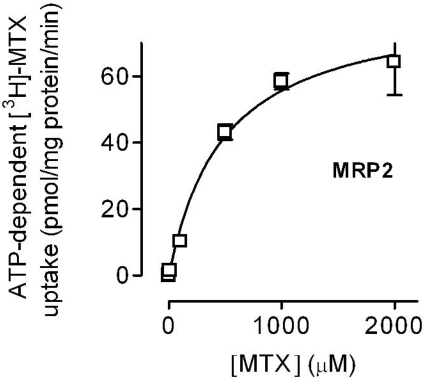
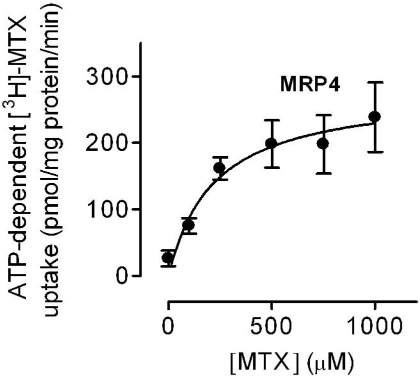

ATP-dependent [3H]-MTX uptake (pmol/mg protein/min)
60
40
20
0
MRP2
0
1000
2000
[MTX] (μM)

ATP-dependent [3H]-MTX uptake (pmol/mg protein/min)
300
200
100
0
MRP4
0
500
1000
[MTX] (μM)

Figure 3

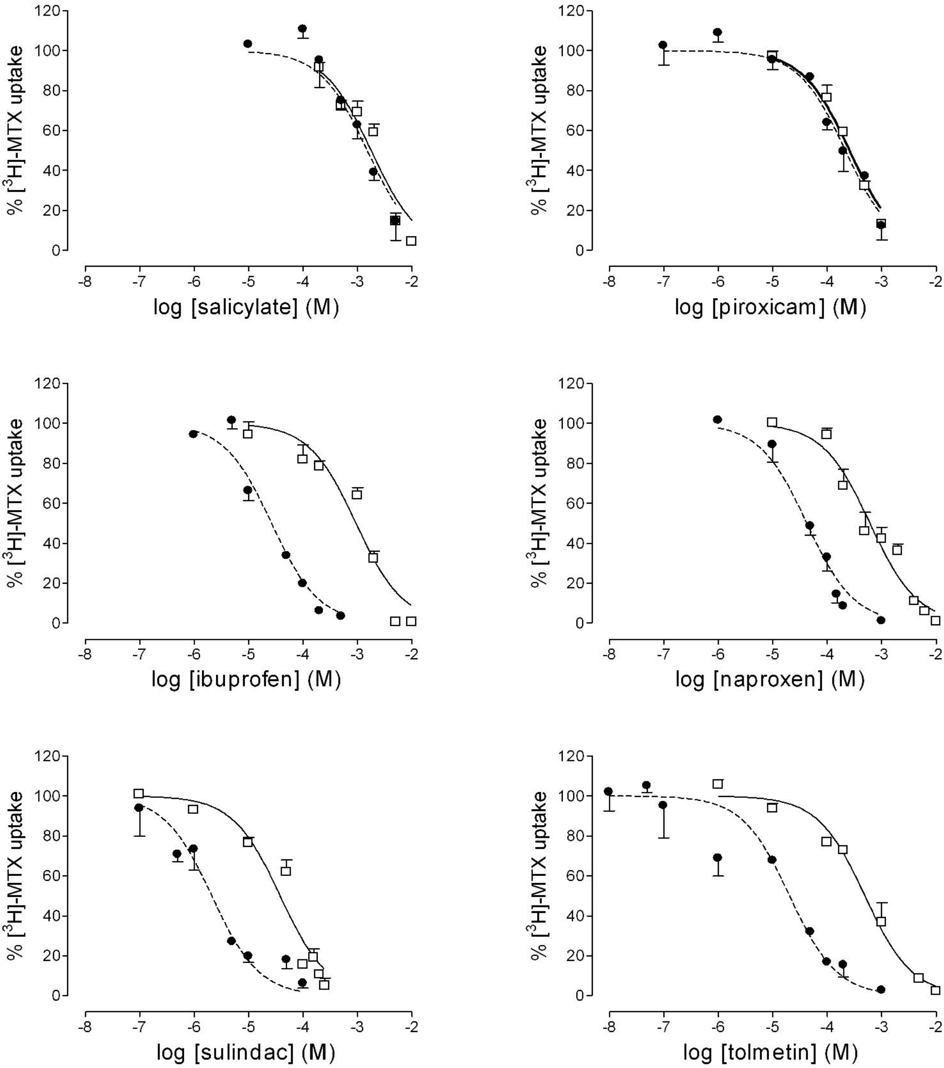

% [3H]-MTX uptake
log [salicylate] (M)
log [piroxicam] (M)
log [ibuprofen] (M)
log [naproxen] (M)
log [sulindac] (M)
log [tolmetin] (M)

Figure 4

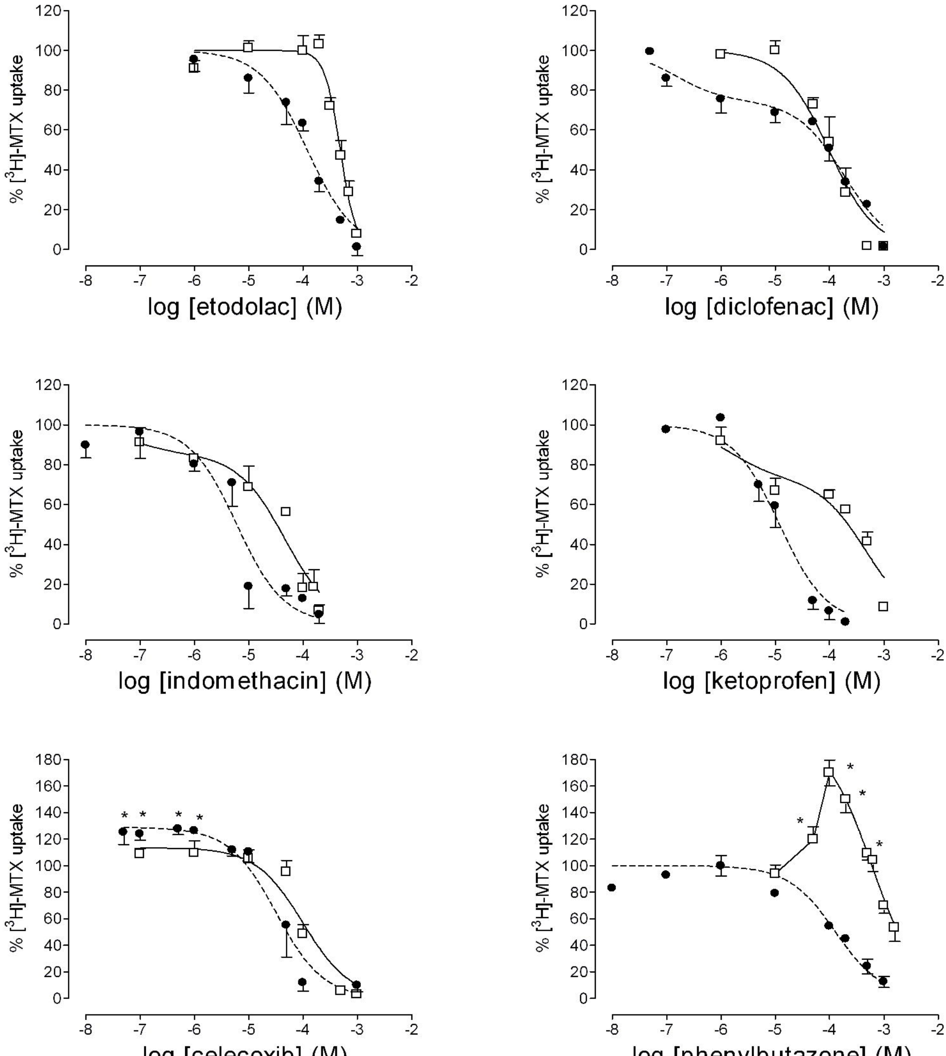

% [3H]-MTX uptake
log [etodolac] (M)
log [diclofenac] (M)
log [indomethacin] (M)
log [ketoprofen] (M)
log [celecoxib] (M)
log [phenylbutazone] (M)

Figure 5