Interaction of Nonsteroidal Anti-Inflammatory Drugs with Multidrug Resistance Protein (MRP) 2/ABCC2- and MRP4/ABCC4-Mediated Methotrexate Transport


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ABSTRACT

Methotrexate (MTX) has been used in combination with nonsteroidal 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 overexpressing the proximal tubular apical efflux transporters human multidrug resistance protein (MRP) 2/ABCC2 and MRP4/ABCC4. MTX was transported by MRP2 and MRP4 with \( K_m \) values of 480 ± 90 and 220 ± 70 \( \mu M \), 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 \( \mu M \) to 1.8 mM. Salicylate, piroxicam, ibuprofen, naproxen, sulindac, tometin, 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 overall interaction between these drugs.

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, nonsteroidal 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 three of four patients receiving ketoprofen (Thyss et al., 1986), two of which had approximately 12-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 (OATs) 1 and 3 and the reduced folate carrier-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 (MRPs) 2 and 4 (Masuda et al., 1997; Chen et al., 2002; van Aubel et al., 2002). Furthermore, MTX is recognized by luminal OAT4 (Takeda et al., 2002), which reabsorbs anions

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ABBREVIATIONS: MTX, methotrexate; NSAID, nonsteroidal anti-inflammatory drug; OAT/Oat, organic anion transporter; MRP/Mrp, multidrug resistance protein; MDCKII, Madin-Darby canine kidney; HEK, human embryonic kidney; DMSO, dimethyl sulfoxide; E-64, N-(trans-epoxy-succinyl)-l-leucine 4-guanidinobutyramide.
from the primary urine back into the tubular cells (Ekara-
tanawong 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 inhib-
ited 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 reduced folate car-
rier-1, which is insensitive to NSAIDs, also contributes signifi-
cantly to tubular MTX uptake (Nozaki et al., 2004). To date,
the effect of NSAIDs on the luminal efflux of MTX via MR
P2 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-β-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 trans-
porters may get exposed to relatively high inhibitor concen-
trations because NSAIDs can be taken up actively into the
proximal tubular cells via basolateral OATs (Sekine et al.,
1997; Apipattanakul et al., 1999). 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 some-
times complex inhibitory patterns of the various NSAIDs
studied. This is the first study in which this putative drug-
drug interaction is examined at the molecular level.

Materials and Methods

Materials. [3',5',7'-3H](n)MTX sodium salt (51.5 Ci/mmol) was
purchased from Moravek Inc. (Brea, CA). The Bac-to-Bac and Gate-
eway system were purchased from Invitrogen (Breda, The Nether-
lands). Creatine phosphate and creatine kinase were purchased from
Roche Diagnostics (Almere, The Netherlands). NC45 filters were
obtained from Schleicher and 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 de-
scribed previously (Evers et al., 1998). The nontransfected MDCKII
cell line was used as a control. HEK293 cells were grown in DMEM
supplemented with 10% fetal calf serum at 37°C under 5% CO2-
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 pro-
moter of the pFastBacDual vector. Next, the CMV promoter and
Gateway destination elements (cassette that contains the chloram-
phenicol 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 reaction. Baculoviruses were produced as described in
the Bac-to-Bac manual. As a control, the enhanced yellow fluorescent
protein was also cloned into the gateway entry vector (Invitrogen).

Transduction of HEK293 Cells. HEK293 cells were cultured in
182-cm² flasks until 70% confluence, after which the culture medium
was removed and 1.5 ml of virus and 3.5 ml of medium were added.
The cells were incubated for 30 to 60 min at 37°C, after which 20 ml
of medium was added. After 24 h 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 3000g for 30 min. The pellets
were resuspended in ice-cold homogenization buffer (0.5 mM sodium
phosphate, 0.1 mM EDTA, pH 7.4) supplemented with protease
inhibitors (100 μM phenylmethanesulfonyl fluoride, 5 μg/ml aprotinin,
5 μg/ml leupeptin, 1 μM pepstatin, and 1 μM E-64) and shaken at
4°C for 60 min. Lysed cells were centrifuged at 4°C at 100,000
g for 30 min, and the pellets were homogenized in ice-cold TS buffer
(10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) using a tight fitting
Dounce homogenizer for 30 strokes. After centrifugation at 500g at
4°C for 20 min, the supernatant was centrifuged at 4°C at 100,000g
for 60 min. The resulting pellet was resuspended in TS buffer and
passed through a 27-gauge needle 30 times. Protein concentration
was determined by a 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 di-
rilled in TS buffer. The indicated amount of protein was size frac-
tionated on a 7.5% SDS-polyacrylamide gel and subsequently blotted
on nitrocellulose membrane. Affinity-purified, polyclonal anti-hu-
mans MRP2 (pAb hM2-p1) and anti-human MRP4 (pAb hM4-p4)
were purchased from Santa Cruz (Santa Cruz, CA). Antibodies (1:1000) were used to detect human MRP2 and MRP4,
respectively (van Aubel et al., 2002; Smeets et al., 2004). Signals
were visualized with chemiluminescence (Amersham Biosciences,
Diegem, Belgium).

Vesicular Transport Assays. Uptake of [3H]MTX into mem-
brane vesicles was performed as described previously (van Aubel et
al., 1999). In brief, membrane vesicles were prewarmed for 1 min at
37°C and added to TS buffer, supplemented with an ATP-regenera-
tion mixture (4 mM ATP, 10 mM MgCl2, 10 mM creatine phosphate,
and 100 mg/ml creatine kinase) in a final volume of 60 μl. The
reaction mixture was incubated at 37°C for 15 min, and samples
were taken from the mixture at indicated times, diluted in 900 μl of
ice-cold TS buffer. Diluted samples were filtered through 0.45-μm-
pore NC filters that were preincubated with TS buffer by a filtration
device (Millipore, Bedford, MA) using a rapid filtration method. After
adding 4 ml of scintillation fluid and subsequent liquid scintillation
counting, uptake of [3H]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 determined. ATP-dependent uptake can only occur in inside-out
vesicles, and because 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.
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 \textmu M \[^3\text{H}\]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% (Apipattanakul et al., 1999). We tested the effect of 1% DMSO and observed a reduction of approximately 20% in transport. Because in all transport assays the same concentration of DMSO was used as a control, this reduction did not influence 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, CA). Statistical differences were determined using a one-way analysis of variance with Dunnett’s post test. Differences were considered to be significant at \( P < 0.05 \).

Results

Expression of MRP2 and MRP4 in Isolated Membrane Vesicles. Immunoblot analysis was performed on membrane vesicles prepared from MDCKII and HEK293 cells overexpressing human MRP2 and MRP4, respectively. Figure 1 demonstrates that both MRP2 and MRP4 were successfully expressed and were detected as 190-kDa (Schaub et al., 1999; van Aubel et al., 2002) and 170-kDa (Lee et al., 2000; van Aubel et al., 2002) bands, respectively, whereas 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 min at 37°C in uptake medium containing 0.5 \textmu M \[^3\text{H}\]MTX. A relatively low MTX concentration was chosen, whereas control MDCKII and HEK293 vesicles did not show any protein expression at the corresponding heights.

Effect of NSAIDs on MRP2- and MRP4-Mediated MTX Transport. Membrane vesicles were incubated with 0.5 \textmu M \[^3\text{H}\]MTX at 37°C for 15 min in the absence or presence of increasing concentrations of the various NSAIDs (Figs. 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, whereas 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 MRP4-mediated transport, with significant stimulation up to 170 ± 16% at low and inhibition at higher concentrations. Celecoxib, the only selective cyclooxygenase 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.
Discussion

Severe and sometimes fatal side effects have been observed in patients after the concomitant 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-NSAID 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, whereas 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.
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 (Schwartz et al., 2001; Hartmann et al., 2004), 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 with plasma concentrations after therapeutic dosages, which are highly variable and may range from 0.1 up to 20 to 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 (2.5–5 mM) (Bakos et al., 2000) than for MRP4 (0.22–1.3 mM) (Chen et al., 2002; van Oude Elferink et al., 1998) than for MRP4 (0.22–1.3 mM) (Chen et al., 2002; van Oude Elferink et al., 1998). 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 knockout mice showed 1.8-fold higher plasma concentrations of MTX as compared with wild-type mice. Although MRP4 expression was increased 2-fold in the knockouts, apparently it was not able to compensate for the loss of Mrp2 in these mice (Vlamling et al., 2006). Furthermore, it was reported that in a patient with MRP2 protein dysfunction caused by a genetic variation in the MRP2/ABC2 gene, MTX excretion was 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 level 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 the dose of NSAIDs in plasma are low. The estimated unbound therapeutic concentration of the most potent inhibitors, sulindac and indomethacin, are approximately 1 and 8 μM, respectively, which is in the range of their IC_{50} values for MRP4 (Davies and Skjodt, 2000; Takeda et al., 2002; Chen et al., 2006). 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. Because 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 need to be established in future in vivo studies.

References


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**NSAIDs Inhibit MTX Transport by MRP2 and MRP4**

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