ABSTRACT:

Diclofenac is an important analgesic and anti-inflammatory drug, widely used for treatment of postoperative pain, rheumatoid arthritis, and chronic pain associated with cancer. Consequently, diclofenac is often used in combination regimens and undesirable drug-drug interactions may occur. Because many drug-drug interactions may occur at the level of drugtransporting proteins, we studied interactions of diclofenac with apical ABC multidrug efflux transporters. Using Madin-Darby canine kidney (MDCK)-II cells transfected with human P-glycoprotein (P-gp; MDR1/ABCB1), multidrug resistance protein 2 (MRP2/ABCC2), and breast cancer resistance protein (BCRP/ABCG2) and murine Bcrp1, we found that diclofenac was efficiently transported by murine Bcrp1 and moderately by human BCRP but not by P-gp or MRP2. Furthermore, in Sf9-BCRP membrane vesicles diclofenac inhibited transport of methotrexate in a concentration-dependent manner. We next used MDCK-II-MRP2 cells to study interactions of diclofenac with MRP2-mediated drug transport. Diclofenac stimulated paclitaxel, docetaxel, and saquinavir transport at only 50 μM. We further found that the uricosuric drug benzbromarone stimulated MRP2 at an even lower concentration, having maximal stimulatory activity at only 2 μM. Diclofenac and benzbromarone stimulated MRP2-mediated transport of amphiphatic lipophilic drugs at 10- and 250-fold lower concentrations, respectively, than reported for other MRP2 stimulators. Because these concentrations are readily achieved in patients, adverse drug-drug interactions may occur, for example, during cancer therapy, in which drug concentrations are often critical and stimulation of elimination via MRP2 may result in suboptimal chemotherapeutic drug concentrations. Moreover, stimulation of MRP2 activity in tumors may lead to increased efflux of chemotherapeutic drugs and thereby drug resistance.

Transport of Diclofenac by Breast Cancer Resistance Protein (ABCG2) and Stimulation of Multidrug Resistance Protein 2 (ABCC2)-Mediated Drug Transport by Diclofenac and Benzbromarone

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Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) that exhibits potent analgesic and anti-inflammatory properties and is extensively used to treat postoperative pain, rheumatoid arthritis, osteoarthritis, and acute gouty arthritis (Davies and Anderson, 1997). Diclofenac is also widely used to treat pain associated with cancer, and treatment combinations of diclofenac with chemotherapeutic drugs are common. In addition, preclinical evidence has accummulated that NSAIDs, including diclofenac, have beneficial effects as adjuvant therapy in treatment of some types of cancer (Crokart et al., 2005; Johnsen et al., 2005). Due to this widespread co-use of drugs, interactions of NSAIDs with chemotherapeutic drugs can result in unexpected toxicities or failure of chemotherapy. For example, NSAIDs are known to restrict plasma clearance of methotrexate (MTX). When MTX is used at high-dose regimens for cancer treatment, interactions with NSAIDs can result in severe toxicity, with sometimes fatal outcome (Thyss et al., 1986). In a recent study, using human kidney slides, it was demonstrated that diclofenac and its acyl-glucuronide can inhibit the luminal urinary efflux of MTX via the ABC-binding cassette (ABC) multidrug transporters Mrp4 and Mrp2, respectively (Nozaki et al., 2007).

Because it is recognized more and more that many interactions of drugs occur at the level of drug-transporting proteins, we investigated whether diclofenac is a substrate of one of the apical ABC transporters, using MDCK-II cells transfected with human P-gp, MRP2, BCRP, or murine Bcrp1 cDNA. We further used the MDCK-II cells transduced with human and mouse MRP2/Mrp2 to examine whether diclofenac could modulate MRP2-mediated transport of taxane anticancer drugs. To date, several compounds, e.g., probenecid, sulfisopyrazone, and sulfanilamide, have been shown to stimulate the MRP2-mediated transport of lipophilic amphipathic drugs (Huisman et al.,...
2002; Zelcer et al., 2003). Stimulation of MRP2-mediated transport can be of interest, because we recently demonstrated in vivo that MRP2 is an important determinant for biliary excretion and thereby pharmacokinetics of the lipophilic amphipathic anticancer drug paclitaxel (Lagas et al., 2006). Stimulation of MRP2-mediated excretion of paclitaxel into the bile might thus seriously interfere with paclitaxel pharmacokinetics by enhancing its elimination. The same may apply to various other lipophilic amphipathic drugs transported by MRP2. Moreover, stimulation of MRP2 expressed in tumor cells may result in increased efflux of chemotherapeutics, thereby resulting in suboptimal drug concentrations and therapy failure.

Materials and Methods

Chemicals. [3H]Etoposide, [3H]paclitaxel, [3H]MTX, [3H]ulinin, and [14C]alinin were from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). [3H]Docetaxel was obtained from Sankyo (Tokyo, Japan). [3H]diclofenac was from Campro Scientific (Veenendaal, The Netherlands). [3C]Saquinavir originated from Roche Discovery Welwyn (Welwyn Garden City, UK). GlaxoSmithKline (Uxbridge, Middlesex, UK) kindly provided Elacridar (GFI20918). The BCRP/Bcrp1 inhibitor Ko143 was described previously (Allen et al., 2002). All other chemicals and reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

Transport across MDCK-II Monolayers. Polarized canine kidney MDCK-II cell lines were used in transport assays. MDCK-II cells transfected with human MDR1, MRP2, or BCRP or murine Mrp2 and Bcrp1 were described previously (Evers et al., 1998; Jonker et al., 2002; Zelcer et al., 2003). Transpentrabial transport assays using Transwell plates were performed as described, with minor modifications (Schinkel et al., 1995). Experiments with cells transfected with human MRP2 or mouse Mrp2 were done in the presence of 5 μM Elacridar, to inhibit any endogenous P-glycoprotein activity. Elacridar does not affect MRPL activity (Evers et al., 2000b). Experiments with MDCK-II-BCRP or MDCK-II-Bcrp1 cells were performed with or without 5 μM BCRP inhibitor Ko143. When applied, these inhibitors were present in both compartments during 2-h preincubation and during the transport experiment. After preincubation, experiments were started (t = 0) by replacing the medium in either the apical or basolateral compartment with fresh Opti-MEM (Invitrogen, Paisley, UK), either with or without 5 μM Elacridar or Ko143 and containing 5 μM drug of interest (diclofenac, docetaxel, saquinavir, or etoposide), traced with radiolabeled drug (0.09 μCi/well). When stimulation of MRPL was investigated, the stimulator was present in both compartments during preincubation as well as during the transport experiment. Cells were incubated at 37°C in 5% CO2, and 50-μl aliquots were taken at t = 1, 2, 3, and 4 h. The samples were diluted with 4 ml of scintillant fluid (Ultima-Gold; PerkinElmer Life and Analytical Sciences, Waltham, MA), and radioactivity was measured using a dual-channel scintillation counter. Transport was calculated as the fraction of drug found in the acceptor compartment relative to the total amount added to the donor compartment at the beginning of the experiment. Transport was given as mean percentage ± S.D. (n = 3). Membrane tightness was assessed using [13C]ulinin (0.09 μCi/well), which was added to the donor compartment. Leakage was not allowed to be >1% of the total added radioactivity per hour.

Vesicular Transport Assays. Inside-out membrane vesicles were prepared from S9 insect cells overproducing human BCRP, as described previously (Zelcer et al., 2003). Transport of [3H]MTX was studied at pH 7.4 and pH 5.5. The latter condition was included because the net transport of MTX by BCRP was reported to be higher at pH 5.5 (Breedveld et al., 2007). Vesicular uptake buffers for incubations with S9 vesicles consisted of 50 mM Tris and 250 mM sucrose, and the pH was adjusted with HCl to either 7.4 or 5.5. Both buffers for incubations with Sf9 vesicles consisted of 50 mM Tris and 250 mM sucrose, and the pH was adjusted with HCl to either 7.4 or 5.5. The latter condition was included because the net transport of MTX by BCRP was inhibited by diclofenac in a concentration-dependent manner under both pH conditions, as shown in Fig. 2, A and B.

Results

We used the polarized canine kidney cell line MDCK-II and its subclones transfected with human MDR1, MRP2, or BCRP or murine Mrp2 or Bcrp1 to study vectorial transport of 5 μM diclofenac. In murine Bcrp1-transduced MDCK-II cells, apically directed translocation was markedly increased and basolaterally directed translocation was markedly decreased (Fig. 1C). MDCK-II cells overexpressing human BCRP also displayed directional transport of diclofenac, albeit less pronounced than Bcrp1-transduced cells (Fig. 1, C and E). In the presence of 5 μM selective BCRP inhibitor Ko143 (Allen et al., 2002), net diclofenac transport in Bcrp1- and BCRP-transduced cells was effectively inhibited (Fig. 1, B, D, and F). In the MDCK-II cells transfected with human MDR1 or MRP2, no vectorial translocation of diclofenac was observed (data not shown). These results demonstrate efficient transport of diclofenac by murine Bcrp1, marked transport by human BCRP, and no transport by human MDR1 and MRP2. We also tested whether diclofenac could inhibit the transport of MTX by BCRP in vesicular uptake experiments performed at pH 7.4 and pH 5.5. Figure 2 shows that the net transport of MTX by BCRP was higher at pH 5.5 compared with pH 7.4, which is consistent with previous findings (Breedveld et al., 2007). The ATP-dependent transport of MTX by BCRP was inhibited by diclofenac in a concentration-dependent manner under both pH conditions, as shown in Fig. 2, A and B. The inhibitor concentrations at which the effect was 50% of the maximal inhibitory effect (IC50 values) were 78 μM (pH 7.4) and 71 μM (pH 5.5), respectively.

We next investigated whether diclofenac could modulate the transport of paclitaxel and docetaxel, using MDCK-II cells transfected with human MRP2. The MDCK-II-Neo cell-line was used as a control, because it contains only little endogenous canine Mrp2 (Evers et al., 1998). As shown previously (Huisman et al., 2005), doxetaxel and paclitaxel at 5 μM were efficiently transported by human MRP2 (Fig. 3, A, B, E, and F). This is evident from the increased relative transport ratios (r, defined under Materials and Methods) and the decreased intracellular taxane concentration in MDCK-II-MRP2 cells (Fig. 3). In the presence of 50 μM diclofenac, transport of docetaxel and paclitaxel by MRP2 was markedly stimulated (Fig. 3). For docetaxel, the transport ratio was increased 2.0-fold, and the intracellular concentrations were 1.6- and 2.3-fold decreased for apically and basolaterally applied doxetaxel, respectively (Fig. 3, B and D). For paclitaxel, the relative transport ratio was increased 2.6-fold, and the intracellular concentrations were 1.6- and 1.7-fold decreased for apically and basolaterally applied paclitaxel, respectively (Fig. 3, F and H). The stimulation of the relative transport ratio for paclitaxel was maximal for diclofenac concentrations ranging from 50 to 250 μM (Fig. 4A). Higher diclofenac concentrations (>500 μM) were toxic for the monolayers, as indicated by an increased paracellular inulin leakage. These results show that diclofenac can stimulate MRP2-mediated transport of paclitaxel and docetaxel at a concentration of only 50 μM, which is 10-fold lower than the optimal stimulatory concentrations we previously found in MDCK-II-MRP2 cells for the established MRP2 stimulators probencедин, sulfipyrazone, and sulfinatratin (Huisman et al., 2002, 2005; Zelcer et al., 2003).

Partly based on structural similarities with established MRP2 stimulators and partly based on reported MRP2 modulator activities, we tested four additional compounds for their ability to stimulate MRP2-mediated taxane transport. For the diuretic furosemide and the antidiabetic agents tolbutamide and glyburide, we found at best only weak
stimulatory activities (data not shown). However, the uricosuric drug benz bromarone (Fig. 4B) was found to stimulate MRP2 transport with a maximal stimulatory activity at only 2 μM (Figs. 4 and 5). Benz bromarone increased the transport ratio for docetaxel 3.3-fold and for paclitaxel 3.8-fold, and the intracellular concentrations of both taxanes were markedly decreased in the presence of benz bromarone (Fig. 5, B, D, F, and H). The MRP2-mediated transport of paclitaxel was stimulated in a concentration-dependent manner by benzbromarone. The relative transport ratio was maximal at 2 μM and gradually decreased to control levels at 10 μM benzbromarone (Fig. 4B).
Higher benzbromarone concentrations (>10 μM) were toxic for the monolayers, as indicated by increased paracellular inulin leakage. Because benzbromarone could stimulate taxane transport by MRP2 at a very low concentration, we tested its stimulatory potency for two other clinically relevant MRP2 substrates, the human immunodeficiency virus protease inhibitor saquinavir and the anticancer drug etoposide. In the presence of 2 μM benzbromarone, we found pronounced stimulation of net saquinavir transport (4.8-fold), and modest stimulation of net etoposide transport (1.4-fold; Fig. 5, I–P). These levels of stimulation of MRP2 are quantitatively similar to those seen before with 500 μM probenecid, sulfipyrazone, and sulfanitran (Huisman et al., 2002, 2005; Zelcer et al., 2003). These results thus demonstrate that benzbromarone is an effective MRP2 stimulator in MDCK-II-MRP2 cells for a wide range of drugs.

We next addressed the relevance of drug-drug interactions via stimulation of MRP2 in vivo. Because we recently demonstrated that hepatobiliary excretion of paclitaxel in the mouse is almost exclusively dependent on Mrp2 (Lagas et al., 2006), we tried to stimulate biliary paclitaxel excretion in mice by the coadministration of either diclofenac or benzbromarone. However, we were unable to show any in vivo stimulation (J. S. Lagas and A. H. Schinkel, unpublished data). This discrepancy might be the result of species differences between human MRP2 and murine Mrp2 in their modulatory responsiveness (Zimmermann et al., 2008). Therefore, we used the recently generated MDCK-II cells expressing murine Mrp2 cDNA (Zimmermann et al., 2008) and tested whether diclofenac or benzbromarone could modulate transport of paclitaxel. In contrast to stimulating transport activity of human MRP2 (Figs. 3 and 5), diclofenac and benzbromarone inhibited the net transport of paclitaxel in cells transfected with murine Mrp2 by 2.2- and 1.7-fold, respectively (Fig. 6B). As a result, intracellular accumulation of paclitaxel in MDCK-II-Mrp2 cells at 4 h was significantly increased in the presence of diclofenac or benzbromarone (Fig. 6C; \( P < 0.05 \)). In contrast, intracellular paclitaxel accumulation in human MRP2-transfected cells was significantly lower when diclofenac or benzbromarone were applied (Fig. 6C; \( P < 0.01 \)). Because benzbromarone showed most pronounced stimulation of human MRP2-mediated transport of saquinavir (Fig. 5L), we also tested the impact of diclofenac on saquinavir transport in both human and mouse MRP2/Mrp2-transfected cells. We observed a similar modulation pattern as for paclitaxel. Diclofenac increased the net transport of saquinavir by human MRP2 3.4-fold but decreased net saquinavir transport by murine Mrp2 2.1-fold (Fig. 7, A and B).
Furthermore, diclofenac markedly lowered the intracellular saquinavir concentrations in MDCK-II-MRP2 cells by 5.2-fold, whereas a 1.5-fold higher intracellular concentration was found in cells transfected with murine Mrp2. Taken together, these results show that profound species differences can occur in modulatory responsiveness of human MRP2 and murine Mrp2 for various compounds. The mouse therefore has limitations as a model to study drug-drug interactions that occur via stimulation of MRP2 by diclofenac and benzbromarone.

Discussion
In the present study, diclofenac was identified as an efficiently transported substrate for both murine and human BCRP. Diclofenac is often used to treat pain associated with cancer, and coadministration of diclofenac with anticancer drugs is common. Because BCRP is an important determinant in the pharmacokinetics of many anticancer agents, interactions of diclofenac with chemotherapeutics drugs at the level of BCRP may occur. In patients, diclofenac can limit plasma clearance of MTX, which can result in severe toxicity, especially when MTX is used in high-dose regimens for cancer treatment (Thyss et al., 1986). This interaction may be (partially) explained by the observation that diclofenac can inhibit luminal urinary efflux of MTX via Mrp4 in human kidney slices (Nozaki et al., 2007). In addition, MTX is a good substrate for BCRP (Volk et al., 2000), and, like MRP4, BCRP is expressed in the apical membrane of the proximal...
tubules of the human kidney (Huls et al., 2008). In vivo competition between diclofenac and MTX for transport by BCRP may therefore be a clinically relevant drug-drug interaction, because this may contribute to lower renal clearance of MTX. Using inside-out Sf9-BCRP plasma membrane vesicles, we show that diclofenac indeed can inhibit the BCRP-mediated transport of MTX in a concentration-dependent manner. However, we note that the IC50 values of 78 μM (pH 7.4) and 71 μM (pH 5.5) indicate that diclofenac is not a very potent BCRP inhibitor in vitro. Therefore, the clinical significance of drug-drug interactions through inhibition of BCRP by diclofenac needs to be studied in patients.

Using inside-out plasma membrane vesicles, extensive studies on the complex modulation (stimulation and/or inhibition) of MRP2/Mrp2-mediated transport of various anionic compounds have been performed (Bakos et al., 2000; Bodo et al., 2003; Zelcer et al., 2003; Chu et al., 2004; Gerk et al., 2004, 2007). Transport studies with estradiol-17β-glucuronide (E217βG) revealed that E217βG can stimulate its own transport by MRP2 (Bodo et al., 2003; Zelcer et al., 2003). This finding indicated that the MRP2 protein contains at least two binding sites that display a positive cooperative interaction, i.e., E217βG can bind to a substrate transport site and to a modulatory site that affects the transport rate allosterically by homotropic cooperative interaction (Zelcer et al., 2003; Borst et al., 2006). It is noteworthy that stimulation of its own transport, as was seen for E217βG, was recently also found for the bile salt tauroursodeoxycholate (Gerk et al., 2007). In addition, many examples have been reported of other substrates that can stimulate the MRP2-mediated transport of E217βG in a positive cooperative manner. Several of these compounds are themselves transported substrates of MRP2 and inhibit E217βG transport at higher concentrations via competition for the substrate transport site (Zelcer et al., 2003). Thus, at least several compounds can bind both the modulatory site and the substrate transport site.

Stimulated transport by MRP2 was also shown in intact MDCKII-cells overexpressing MRP2 (Evers et al., 2000a; Huisman et al., 2002; Zelcer et al., 2003). In this system, MRP2-mediated transport of the organic anion glutathione could be stimulated at relatively low concentrations by indomethacin and sulfinpyrazone (Evers et al., 2000a). Maximal stimulation of the MRP2-mediated transport of large lipophilic amphipathic drugs, however, was only observed at stimulator concentrations ≥500 μM (Huisman et al., 2002; Zelcer et al., 2003). We here report that diclofenac and benz bromarone can markedly stimulate MRP2-mediated transport of several lipophilic amphipathic compounds, including taxane anticaner drugs etoposide and saquinavir at 10- and 250-fold lower concentrations, respectively, than reported for other MRP2 stimulators (Huisman et al., 2002, 2005; Zelcer et al., 2003). We note that it is difficult to compare the detailed kinetic properties of MRP2 stimulators when monolayers of intact MDCK-II cells are used, because kinetic parameters (such as K_m and V_max) cannot be directly determined in this system, because the intracellular stimulator concentrations are unknown. In contrast, these parameters can be readily obtained from experiments with Sf9-MRP2 inside-out plasma membrane vesicles, because all applied transported substrates and stimulators have access to the transporters. However, transport of hydrophobic compounds, such as taxanes etoposide and saquinavir,
cannot be studied properly in inside-out plasma membrane vesicles, because these substrates easily diffuse back out and they also display extensive nonspecific binding to membranes, resulting in a low signal-to-noise ratio (Borst and Elferink, 2002). Detailed kinetic studies on stimulation of MRP2-mediated transport of amphipathic lipophilic compounds are therefore complicated. However, our observation that diclofenac is not transported by MRP2 might indicate that diclofenac binds primarily to the modulator site of the MRP2 protein.

To date, there are several MRP2 modulator compounds known that inhibit MRP2-mediated transport of some anionic substrates but stimulate the transport of lipophilic amphipathic compounds, e.g., probenecid inhibits transport of MTX by MRP2 but stimulates the MRP2-mediated transport of human immunodeficiency virus protease inhibitors and taxanes (Hooijberg et al., 1999; Huismann et al., 2002, 2005). This might also be true for diclofenac and benzbromarone. Several studies show that benzbromarone and diclofenac can inhibit MRP2-mediated transport of hydrophilic anionic compounds (Bakos et al., 2000; Evers et al., 2000a; Prime-Chapman et al., 2004; El-Sheikh et al., 2007; Nozaki et al., 2007). However, to our knowledge this is the first report showing that diclofenac and benzbromarone can stimulate MRP2-mediated transport of several clinically relevant lipophilic amphipathic drugs. Because the stimulator concentrations showing maximal stimulatory effect can be readily achieved in patients, confirmation of these results in vivo would indicate whether these drug-drug interactions are likely to be clinically relevant. Unfortunately, using mouse models we were unable to demonstrate stimulation with these compounds in vivo. We could attribute this to profound species differences in modulatory responsiveness for human MRP2 and murine Mrp2, which is in line with recent observations (Zimmermann et al., 2008). Thus, we conclude that the mouse has limitations as a model to study drug-drug interactions that occur via stimulation of human MRP2 by diclofenac and benzbromarone. However, because we observed strong stimulation of human MRP2 in vitro at very low modulator concentrations, drug-drug interactions via this mechanism may be relevant for the clinical situation in humans. Especially in cancer therapy, in which severe toxicity must be avoided and anticancer drugs are applied in a narrow therapeutic range, stimulation of MRP2 may result in suboptimal drug concentrations in the blood. Moreover, stimulation of MRP2 in tumors may result in increased drug efflux and thereby resistance against anticancer agents.
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References


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