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Stereoselective Inhibition of Methotrexate Excretion by Glucuronides of Nonsteroidal Anti-inflammatory Drugs via Multidrug Resistance Proteins 2 and 4

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ABSTRACT

Combined administration of methotrexate (MTX) and nonsteroidal anti-inflammatory drugs (NSAIDs) can result in a decreased systemic clearance of MTX. To date, inhibition of renal uptake via organic anion transporters and efflux via multidrug resistance–associated protein (MRPs) by NSAIDs has been recognized as possible sites of drug interaction between MTX and NSAIDs. Although most NSAIDs are glucuronidated in kidney tissue and excreted mainly as glucuronide conjugates, it is not fully known whether the glucuronides of NSAIDs (NSAIDs-Glu) inhibit MTX excretion via MRP2 and MRP4. The purpose of this study was to investigate the inhibitory effects of the glucuronides of several NSAIDs (diclofenac, *R*- and *S*-ibuprofen, *R*- and *S*-flurbiprofen, and *R*- and *S*-naproxen), as well as the parent NSAIDs on MTX uptake using human MRP2- and MRP4-expressing inside-out vesicles. Results confirm that all NSAIDs and NSAIDs-Glu examined exhibited stereoselective and concentration-dependent inhibitory effects on MTX uptake via MRP2 and MRP4. Notably, NSAIDs-Glu potently inhibited MTX uptake via MRP2 and MRP4 compared with the corresponding parent NSAIDs except for naproxen in MRP2 and *S*-flurbiprofen in MRP4. The present results support that the glucuronides of NSAIDs, as well as the parent NSAIDs, are involved in the inhibition of urinary excretion of MTX via MRP2 and MRP4.

Introduction

Methotrexate (MTX) is an analog of natural folate. MTX inhibits dihydrofolate reductase and is used widely for cancer chemotherapy (Frei et al., 1975; Jackson, 1984). Combined administration of MTX with other drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs) (Ellison and Servi, 1985; Maiche, 1986; Thyss et al., 1986; Ng et al., 1987; Tracy et al., 1992), penicillin antibiotics (Ronchera et al., 1993; Yamamoto et al., 1997; Titier et al., 2002), probenecid (Aherne et al., 1978), and ciprofloxacin (Dalle et al., 2002) can result in severe and life-threatening drug interactions. Of the drugs affecting the pharmacokinetics of MTX, NSAIDs have been well documented. Liegler et al. (1969) showed that renal clearance of MTX fell significantly to ≈60% of that seen for control samples when used in combination with salicylate. Several reports showed that NSAIDs, including indomethacin, ibuprofen, and naproxen, often induced an elevation of area under the plasma concentration curve of MTX (Dupuis et al., 1990; Tracy et al., 1992; Ekstrøm et al., 1997).

Elimination of MTX is almost entirely in an unchanged form in urine, which involves glomerular filtration and active tubular secretion (Shen and Azarnoff, 1978). Several mechanisms by which NSAIDs induce an increase in plasma concentrations of MTX have been postulated. It has been reported that NSAIDs decrease the glomerular filtration of MTX via reduced renal blood flow by inhibition of prostaglandin synthesis (Ahern et al., 1988; Tracy et al., 1992). Another mechanism is based on the involvement of transporters in drug interactions. MTX is taken up from blood across the basolateral membrane via organic anion transporters (OATs, SLC22A) 1 and 3 and reduced folate carrier-1 (Sekine et al., 1997; Hosoyamada et al., 1999; Cha et al., 2001; Nozaki et al., 2004), with subsequent excretion across the apical membrane via ATP-dependent efflux pumps, multidrug resistance proteins (MRPs, ABCC) 2 and 4 (Masuda et al., 1997; Chen et al., 2002; van Aubel et al., 2002) and breast cancer–resistant protein (BCRP) (Russel et al., 2002; Nozaki et al., 2007) into urine. Thus, the competition of renal tubular secretion between MTX and NSAIDs has been thought to be a major cause of the drug interaction (Frenia and Long, 1992; Maeda et al., 2008). With respect to the inhibitory effect of NSAIDs on MTX uptake at the basolateral membrane, many reports have shown that several NSAIDs inhibit the transport of MTX via OAT1 and OAT3 (Uwai et al., 2000; Khamdang et al.,2002; Takeda et al., 2002; Nozaki et al., 2004; Maeda et al., 2008). On the other hand, urinary excretion of MTX via MRPs is a determinant process for MTX elimination because a heterozygous

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ABBREVIATIONS: BCRP, breast cancer–resistance protein; CDCF, 5(6)-carboxy-2′,7′-dichlorofluorescein; $E_2$17β-Glu, estradiol 17β-D-glucuronide; HPLC, high-performance liquid chromatography; MRP, multidrug resistance-associated protein; MTX, methotrexate; NSAIDs, nonsteroidal anti-inflammatory drugs; NSAIDs-Glu, glucuronide of NSAIDs; OAT, organic anion transporter; UGT, UDP-glucuronosyl transferase.

mutation that results in the loss of function of MRP2 was observed in a patient who exhibited delayed elimination of MTX from the body (Hulot et al., 2005). Some reports have suggested 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 (El-Sheikh et al., 2007; Nozaki et al., 2007). The drug interaction between MTX and NSAIDs has been thought to involve not only the inhibition of basolateral OAT1 and 3 but also apical MRP2 and 4 by NSAIDs.

Meanwhile, most NSAIDs are mainly excreted into urine as their glucuronide conjugates (NSAIDs-Glu) (Davies and Anderson, 1997a,b) which are metabolized by human kidney microsomes, as well as human liver microsomes (Soars et al., 2002). Additionally, as NSAIDs are substrates of hOAT1 and hOAT3 (Apiwattanakul et al., 1999; Khamdang et al., 2002), the concentrations of NSAIDs and their glucuronides would be higher than those expected from their unbound concentrations in blood. Given that the renal apical efflux transporters are exposed to higher glucuronide levels, the effects of NSAIDs-Glu on the efflux transporters (MRP2 and MRP4) should be examined. Indeed, we have shown that diclofenac glucuronide inhibits the MTX transport mediated by MRP2 in a concentration-dependent manner (Nozaki et al., 2007).

In clinical situations, 2-arylpropionic acid NSAIDs, except naproxen, are commonly used in their racemic form. Physiologic characteristics such as metabolic profile (Knihinicki et al., 1990; Rudy et al., 1991), protein binding in serum (Lagrange et al., 2000; Nagao et al., 2003), plasma concentrations (Foster and Jamali, 1988; Geisslinger et al., 1993, 1994; Patel et al., 2003), as well as pharmacologic effects (Muller et al., 1990), differ among enantiomers. Accordingly, the effect of NSAIDs and NSAIDs-Glu on the renal excretion of MTX probably differs among enantiomers or diastereomers. There is a possibility that the *R*-enantiomers of NSAIDs, which are pharmacologically inactive or weak, may have an unwanted inhibitory effect on renal MTX excretion. It has been reported that NSAIDs show stereoselective inhibitory effects for OAT1 but not OAT3 (Honjo et al., 2011). To date, however, the involvement of NSAIDs-Glu in the mechanisms of the drug interaction of MTX and NSAIDs and their stereoselective differences in inhibitory effects have not been fully elucidated.

In this study, we examined the stereoselective inhibitory effects of diclofenac, *R*- and *S*-ibuprofen, *R*- and *S*-flurbiprofen, *R*- and *S*-naproxen, and their glucuronides on MTX transport using human MRP2- and MRP4-expressing inside-out vesicles. Our data show that inhibition by NSAIDs-Glu of MTX efflux via MRP2 and MRP4 is another potential site of drug interaction between MTX and NSAIDs. These findings also suggest the mechanisms underlying the drug interaction of MTX with NSAIDs involve complex drug-drug and metabolite-drug interactions for multiple transporters at basolateral and apical membranes of tubular cells.

Materials and Methods

Ethical Approval of the Study Protocol. The study protocol was approved by the Ethics Committee of Kinki University (Osaka, Japan). Animal studies were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the U.S. National Institutes of Health (Bethesda, MD).

Chemicals. Diclofenac was purchased from Sigma-Aldrich (Saint Louis, MO); naproxen enantiomers (*R*- and *S*-naproxen) and ibuprofen enantiomers (*R*- and *S*-ibuprofen) were purchased from Toronto Research Chemicals (Toronto, ON, Canada) and Enzo Life Science (Farmingdale, NY). Flurbiprofen enantiomers (*R*- and *S*-flurbiprofen) were obtained from Cayman Chemical Company (Ann Arbor, MI). Diclofenac glucuronide, ibuprofen glucuronide (mixture of diastereomers), flurbiprofen glucuronide (mixture of diastereomers), and MTX were purchased from Wako Pure Chemical Industries (Osaka, Japan). Human MRP2- and MRP4-expressing inside-out vesicles were purchased from Genomembrane (Kanagawa, Japan). [^{3}H]-MTX and [^{3}H]-estradiol 17β-D-glucuronide ($E_2$17β-Glu) were obtained from America Radiolabeled Chemicals (Saint Louis, MO); 5(6)-carboxy-2′,7′-dichlorofluorescein (CDCF) and $E_2$17β-Glu were purchased from Invitrogen (Carlsbad, CA) and Sigma-Aldrich, respectively. All other chemicals and solvents were of the highest purity available or of high-performance liquid chromatography (HPLC) grade.

Preparation of NSAIDs-Glu. β-1-*O*-glucuronides of NSAIDs were prepared biosynthetically in vitro from the respective parent drugs using rat liver microsomes according to published methods (Iwaki et al., 1995; Nozaki et al., 2007).

The identities of the glucuronides were confirmed by comparing the retention times of the glucuronides with those of authentic compounds using HPLC and cleavage to the respective parent drugs with β-1-glucuronidase and 1 M NaOH. The purity of the glucuronides obtained was determined by HPLC at an ultraviolet wavelength of 254 nm, with the remaining fraction consisting of polar impurities that did not yield the respective parent drugs. The purities of the glucuronides were almost homogeneous (diclofenac glucuronide, 99%; *S*-naproxen glucuronide, 100%; *R*-naproxen glucuronide, 98%; *S*-ibuprofen glucuronide, 100%; *R*-ibuprofen glucuronide, 94%; *S*-flurbiprofen glucuronide, 96%; *R*-flurbiprofen glucuronide, 100%). Obtained NSAIDs-Glu were stored at −80°C until use.

Determination of the Inhibitory Effects of NSAIDs and NSAIDs Glucuronide on Transport of MTX, CDCF, and $E_2$17β-Glu by MRPs. Uptake of [^{3}H]MTX into membrane vesicles was performed according to the previously described method (van Aubel et al., 1998; Nozaki et al., 2007), with some modifications. Briefly, the 25 μl-reaction buffer (10 mM Tris-HEPES, pH 7.4), 250 mM sucrose, and 10 mM $MgCl_2$) containing the ligands (50 μM MTX, including 1.0 μM [^{3}H]-MTX, 100 μCi/ml, 5.0 μM CDCF, or 5.0 μM $E_2$17β-Glu, including 2.5 μM [^{3}H]-$E_2$17β-Glu, 100 μCi/ml), 5 μM ATP or AMP, 2.5 μl of NSAIDs and NSAID glucuronide (final concentrations of 0.1–2000 μM), and ATP-regenerating system (10 mM creatine phosphate and 750 μg/ml creatine phosphokinase). An aliquot of transport medium (15 μl) was mixed rapidly with MRP2- and MRP4-expressing inside-out vesicle suspension (5 μg of protein/5 μl), incubated for 20 minutes for MRP2 (MTX) and 5 minutes for MRP2 (CDCF) and MRP4 at 37°C, and 200 μl of ice-cold stop solution (10 mM Tris-HEPES (pH 7.4), 50 mM sucrose, and 100 mM KNO_3) were added to reaction solutions. The reaction solutions were filtered through a Millex-HA filter (0.45 μm; Millipore, Darmstadt, Germany), and the filter was washed five times with ice-cold stop solution. A 5-ml scintillation medium Clearsol I (Wako Pure Chemical Industries) was added to the Millex-HA filter. The radioactivity was measured in a liquid scintillation counter (TRI-CARB; PerkinElmer, Waltham, MA). Fluorescence intensity was measured at 470 nm (excitation) and 529 nm (emission) (SH-9000; Corona Electric, Ibaragi, Japan). The ATP-dependent uptake was calculated by subtracting the uptake values obtained with AMP from that obtained with ATP.

The uptake rate was linear over the first 20 minutes of the incubation for MRP2 and MRP4. Uptake at 20 minutes for MRP2 and at 5 minutes for MRP4 remained linear up to the highest MTX concentration (200 μM) used in the preliminary experiments. The concentration of MTX used in this study is below the previously reported K_m values (480 ± 90 μM for MRP2 and 220 ± 70 μM for MRP4) (El-Sheikh et al., 2007).

Data Analyses. The IC_{50} values of NSAIDs and their glucuronides were obtained from curve-fitting of the resulting concentration-inhibition curves to the Hill equation by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Linear regression analysis was performed to assess the correlations between IC_{50} values for MTX transport via MRP2 and MRP4 and those for their typical substrates, CDCF (MRP2) or $E_2 17\beta$-Glu (MRP4).

Results

MRP2- and MRP4-expressing membrane vesicles were incubated with 50 μM MTX (1 μM [^{3}H]MTX) in the absence or presence of increasing concentrations of the NSAIDS and their glucuronides. Figures 1 and 2 show the inhibitory effects of NSAIDs and NSAIDs-Glu on MTX uptake via MRP2 and MRP4, respectively. Tables 1 and 2 summarize the IC_{50} values, their *R*/*S* and glucronide/aglycone ratios, and Hill slope values estimated from the data are shown in Figs. 1 and 2.

Inhibitory Effects of NSAIDs on MRP2- and MRP4-Mediated MTX Transport. All examined NSAIDs exhibited stereoselective and concentration-dependent inhibitory effects on MTX uptake via MRP2 and MRP4 with different potencies. For MRP2, relatively low IC_{50} values were observed for *S*-flurbiprofen and *S*-naproxen. In particular, *S*-naproxen exerted marked inhibitory effects on MTX uptake via MRP2. For MRP4, relatively low IC_{50} values were observed for *R*-flurbiprofen and *R*-naproxen. *S*-isomers of NSAIDs showed higher inhibitory effects on MTX uptake via MRP2 compared with *R*-isomers. Contrary to MRP2, the inhibitory effects of *R*-isomers of NSAIDs on MTX uptake via MRP4 were higher than those of *S*-isomers. Consequently, *R*/*S* ratios of IC_{50} values for MRP2 and MRP4 were above and below unity, respectively. In particular, among the 2-aryl propionic acid NSAIDs examined, remarkable stereoselectivity was observed in naproxen for MRP2 (*R*/*S* ratio = 71.7) and for MRP4 (R/S ratio = 0.16).

Inhibitory Effects of NSAIDs-Glu on MRP2- and MRP4-Mediated MTX Transport. To clarify the effects of NSAIDs-Glu on renal excretion of MTX via MRP2 and MRP4, we examined MTX uptake to MRP2- and MRP4-expressing inside-out vesicles under the presence of increasing NSAIDs-Glu. Stereoselective and concentration-dependent inhibitory effects of NSAIDs-Glu on MTX uptake via MRP2 and MRP4 were also observed, like their parent NSAIDs. For MRP2, relatively low IC_{50} values were observed for glucuronides of diclofenac and *R*- and *S*-flurbiprofen, and there was modest stereoselectivity (*R*/*S* ratios were around 2). For MRP4, relatively low IC_{50} values were observed for glucuronides of *R*-ibuprofen, *R*-flurbiprofen, *R*-naproxen, and the stereoselectivity was stronger and inverted: that is, R/S ratios were around 0.04. The glucuronides of *R*-enantiomers had more potent inhibition against MRP4-mediated MTX transport. On the contrary, the glucuronides of *S*-isomers had more potent inhibition against MRP2, although weak stereoselectivity of flurbiprofen and naproxen was observed.

In particular, *R*-naproxen glucuronide (which is not used clinically) showed marked differences in inhibitory effects on MTX transport between MRP2- and MRP4-expressing vesicles (MRP2/MRP4 ratio of IC_{50} was $\approx$470). Inhibitory effects of *R*-Ibuprofen glucuronide and *R*-naproxen also showed relatively higher selectivity for MRP4 compared with MRP2 (MRP2/MRP4 ratio of IC_{50} was $\approx$60). As seen in Glu/aglycone ratios in Tables 1 and 2, NSAIDs-Glu trended to more potently inhibit MTX uptake via MRP2 and MRP4 compared with the corresponding parent NSAIDs except for naproxen.

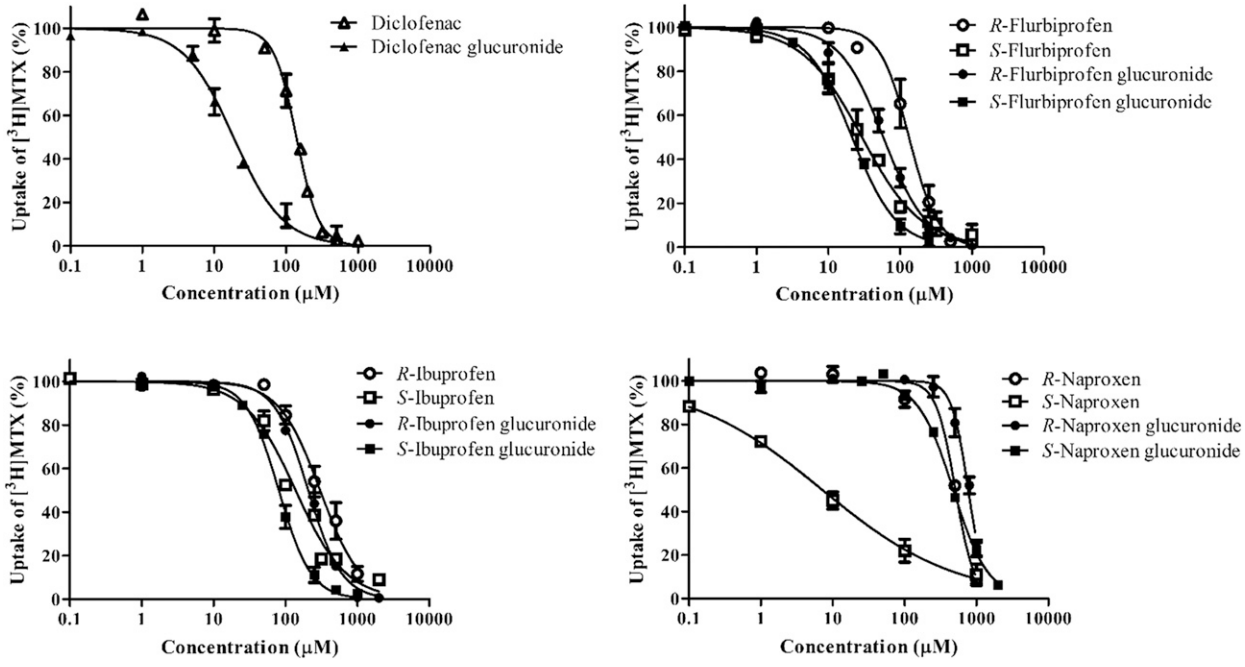

Fig. 1. MRP2-based vesicular transport of [^{3}H]-MTX in the presence of NSAIDs and NSAIDs-Glu. The uptake of MTX (50 μM) was measured with diclofenac, *R*- or *S*-ibuprofen, *R*- or *S*-flurbiprofen, *R*- or *S*-naproxen, and their glucuronides at concentrations between 0.1 and 2000 μM for 20 minutes at 37°C. Results are the mean ± S.E.M. (n = 3) with some exceptions.

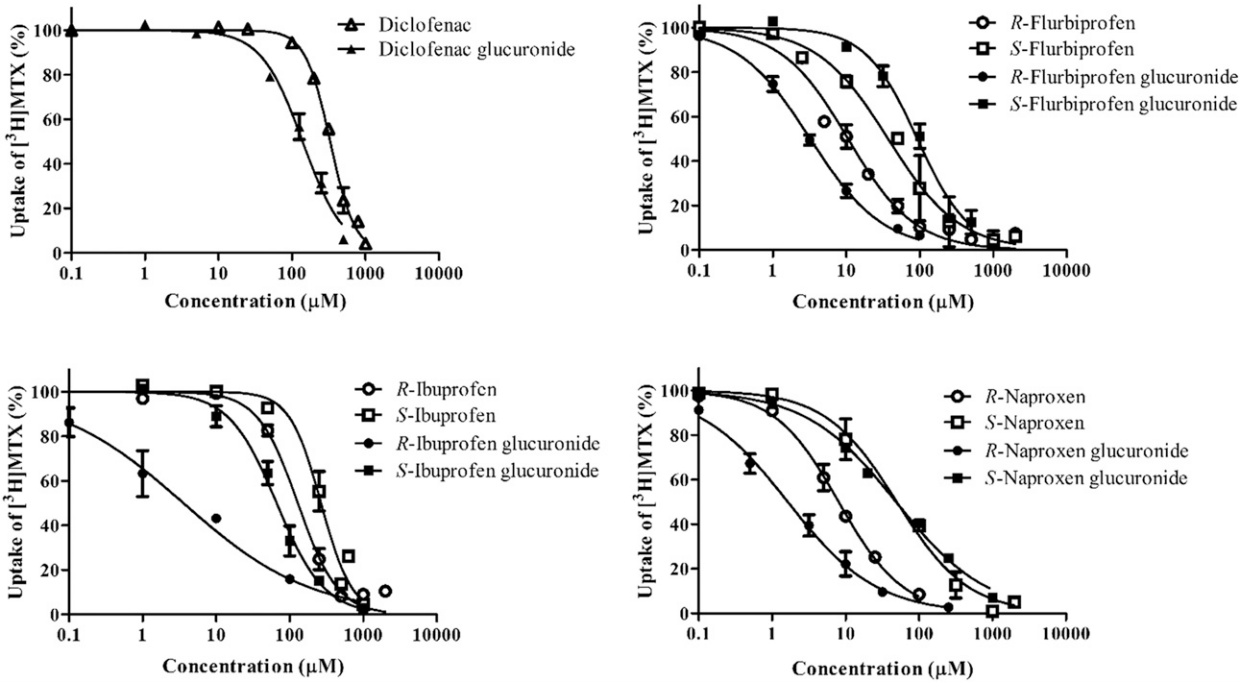

Fig. 2. MRP4-based vesicular transport of [^{3}H]-MTX in the presence of NSAIDs and NSAIDs-Glu. The uptake of MTX (50 μM) was measured with diclofenac, *R*- or *S*-ibuprofen, *R*- or *S*-flurbiprofen, *R*- or *S*-naproxen, and their glucuronides at concentrations between 0.1 and 2000 μM for 5 minutes. Results are the mean ± S.E.M. (n = 3) with some exceptions.

Inhibitory Effects of NSAIDs-Glu on MRP2-mediated CDCF and MRP4-Mediated $E_2$17β-Glu Transport. MRP2- and MRP4-expressing membrane vesicles were incubated with 5 μM CDCF and 50 μM $E_2$17β-Glu, which are typical substrates for MRP2 and MRP4, respectively, in the presence of increasing concentrations of the NSAIDS-Glu. From the obtained concentration-inhibitory profiles, IC_{50} of NSAIDs-Glu for CDCF and $E_2$17β-Glu were estimated. Figure 3 shows correlations between IC_{50} for MTX transport via MRP2 and MRP4 and the estimated IC_{50} for CDCF and $E_2$17β-Glu, respectively. Inhibitory effects of NSAIDs-Glu on MTX uptake via MRP2 and MRP4 correlated significantly with those on CDCF and $E_2$17β-Glu uptake, respectively; however, IC_{50} for MTX tended to be smaller than those for CDCF and $E_2$17β-Glu with some exceptions.

Discussion

Combined administration of MTX and NSAIDs to patients can result in severe (and sometimes fatal) side effects. NSAIDs can inhibit MTX uptake via OAT1 and OAT3 through the basolateral membrane (Takeda et al., 2002; Nozaki et al., 2004; Maeda et al., 2008) and MTX efflux via MRP2 and MRP4 (El-Sheikh et al., 2007; Nozaki et al., 2007) and BCRP (Nozaki et al., 2007) through the apical membrane. Human OAT1 and OAT3 actively transport NSAIDs from blood into tubular cells (Khamdang et al., 2002), which in turn are metabolized to glucuronide conjugates mainly by UDP-glucuronosyl transferase UGT2B7 (Jin et al., 1993; Sakaguchi et al., 2004). As a result, NSAIDs are excreted mainly into urine as their glucuronides (Davies and Anderson, 1997a; Aresta et al., 2006). Therefore, the inhibition of apical efflux of MTX by MRP2 and MRP4 and BCRP by NSAIDs-Glu, as well as their parent drugs, is possibly an important competitive site in drug interactions between MTX and NSAIDs. Nevertheless, to date, the effects of NSAIDs-Glu on MTX efflux via MRP2 and MRP4 as potential sites of MTX-NSAIDs interaction have not been fully examined.

In the present study, we evaluated the inhibitory effects of NSAIDs-Glu on MTX uptake via MRP2 and MRP4 by comparing them with those of their parent drugs by using membrane vesicles expressing hMRP2 and hMRP4. Our

TABLE 1
Inhibition parameters of NSAIDs and NSAIDs-Glu for uptake of MTX via MRP2

NSAID	IC_{50} (μM)		*R/S* Ratio		Glucuronide/Aglycone Ratio	Hill Slope	
	Aglycone	Glucuronide	Aglycone	Glucuronide		Aglycone	Glucuronide
Diclofenac	139 (128–151)[a]	18.6 (15.7–21.9)	—[b]	—	0.13	2.78	1.26
R-ibuprofen	303 (252–365)	208 (189–229)	2.19	2.57	0.69	1.52	1.87
S-ibuprofen	139 (113–171)	80.9 (74.2–88.2)			0.58	1.24	1.93
R-flurbiprofen	133 (112–158)	29.5 (23.9–36.3)	2.28	1.37	0.22	2.17	1.04
S-flurbiprofen	58.4 (50.4–67.6)	21.5 (19.4–23.8)			0.37	1.43	1.39
R-naproxen	510 (465–559)	771 (727–817)	71.7	1.62	1.51	3.28	3.75
S-naproxen	7.11 (5.21–9.70)	475 (449–504)			66.8	0.46	1.79

[a]The numbers in parentheses represent 95% confidence interval.
[b]Not available.

TABLE 2
Inhibition parameters of NSAIDs and NSAIDs-Glu for uptake of MTX via MRP4

NSAID	IC_{50} (μM)		*R/S* Ratio		Glucuronide/Aglycone Ratio	Hill Slope	
	Aglycone	Glucuronide	Aglycone	Glucuronide		Aglycone	Glucuronide
Diclofenac	332 (310–354)[a]	140 (123–159)	—[b]	—	0.42	2.57	1.55
R-ibuprofen	129 (108–154)	3.60 (2.04–6.33)	0.48	0.05	0.03	1.60	0.50
S-ibuprofen	267 (229–312)	66.6 (55.9–78.0)			0.25	2.06	1.34
R-flurbiprofen	10.6 (8.95–12.6)	3.24 (2.92–3.60)	0.29	0.04	0.31	0.96	0.88
S-flurbiprofen	37.2 (26.3–52.5)	93.0 (77.3–112)			2.50	0.90	1.23
R-naproxen	8.06 (7.19–9.04)	1.63 (1.32–2.03)	0.16	0.03	0.20	0.99	0.72
S-naproxen	49.8 (36.5–68.0)	48.7 (42.5–55.8)			0.98	0.90	0.70

[a]The numbers in parentheses represent 95% confidence interval.
[b]Not available.

studies showed that all the NSAIDs-Glu and NSAIDs examined inhibited MTX uptake via MRP2 and MRP4 in concentration-dependent manners with different potencies between enantiomers and between glucuronides and their parent drugs. Our results demonstrating that NSAIDs inhibit both hMRP2 and hMRP4 is consistent with previous findings using membrane vesicles isolated from cells overexpressing hMRP2 and hMRP4 (El-Sheikh et al., 2007) and using membrane vesicles prepared from HEK293 cells infected with hMRP2 and hMRP4 (Nozaki et al., 2007). Interestingly, most NSAIDs-Glu tested exerted stronger inhibitory effects on MTX uptake via MRP2 and MRP4 compared with corresponding NSAIDs except for naproxen in MRP2 (Fig. 1) and *S*-flurbiprofen in MRP4 (Fig. 2), suggesting that NSAIDs-Glu are probably involved in the decreased renal clearance of MTX and thus in the interaction between MTX and NSAIDs. The glucuronide conjugates are generally good substrates for MRP2 and MRP4. Although the reason glucuronides of NSAIDs tend to have more potent inhibitory effect on MRP2- and MRP4-mediated MTX transport is not clear, we speculate that MRPs have higher affinity for the glucuronides than do the parent NSAIDs.

The MTX concentration in inhibition experiments (50 μM) is comparable to plasma concentrations after administration of therapeutic doses (Widemann and Adamson, 2006). It should be noted, however, that intracellular concentrations probably could exceed the plasma concentration because of the active uptake of MTX accumulated in renal tubules. The IC_{50} values of diclofenac for MRP2- and MRP4-mediated MTX transport estimated with 50 μM of MTX in this study (139 μM for MRP2, 332 μM for MRP4) were consistent with values previously reported by El-Sheikh et al. (2008) (97 μM for MRP2, 326 μM for low-affinity MRP4). As they did not use enantiomers of NSAIDs, other IC_{50} values could not be directly compared. The inhibitory effects of diclofenac and naproxen glucuronides on MRP2- and MRP4-mediated transport of 0.1 μM of MTX were investigated using human embryonic kidney cell line HEK293 cells infected with MRP2 and MRP4, in which it was shown that 10 and 100 mM of diclofenac glucuronide significantly inhibited MRP2-mediated transport, whereas MRP4 was inhibited slightly or not at all inhibited by 100 μM of diclofenac and naproxen glucuronides (Nozaki et al., 2007). These results are in good agreement with our data using 50 μM MTX.

Plasma concentrations of NSAIDs range from several hundred micromolars to several millimolars (Cerletti et al., 2003). Concentrations of unbound NSAIDs in plasma are low because of extensive binding with plasma proteins (90%–99%). Although some NSAIDs, such as salicylate and indomethacin, were predicted to inhibit the uptake of MTX into tubular cells at clinically observed plasma concentrations (Nozaki et al.,

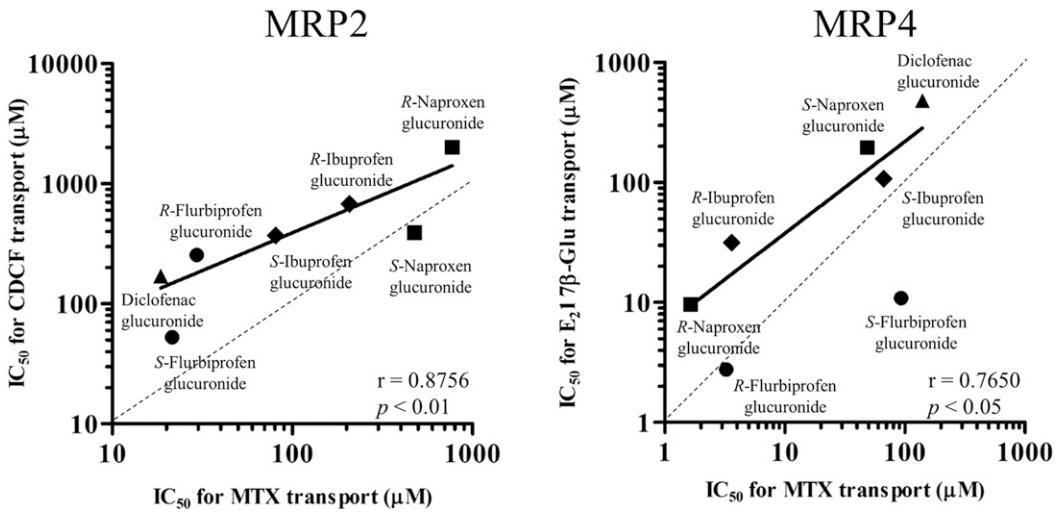

Fig. 3. Correlation between IC_{50} for [^{3}H]-MTX transport and IC_{50} for CDCF transport (MRP2) or IC_{50} for [^{3}H]-$E_2$17β-Glu transport. Solid line and dotted line show the linear regression curve and perfect correlation, respectively. The uptake of CDCF (5.0 μM) and $E_2$17β-Glu (5.0 μM) was measured with glucuronides of diclofenac, *R*- or *S*-ibuprofen, *R*- or *S*-flurbiprofen, and *R*- or *S*-naproxen at concentrations between 1 and 1000 μM for 5 minutes.

2007), the relative contribution of inhibition to the renal uptake of MTX may be small for drug interactions between MTX and other NSAIDs in clinical situations as a result of low unbound plasma concentrations. As described here, however, NSAIDs are possibly concentrated in the renal tubular cells by active transport. Moreover, the kidney has high levels of UGT2B7 (Ohno and Nakajin, 2009), which is the major isoform involved in the glucuronidation of NSAIDs, such as ibuprofen and ketoprofen (Sakaguchi et al., 2004). Indeed, the intrinsic clearance for glucuronidation by human kidney microsomes was 2.5-fold higher than that for human liver microsomes (Soars et al., 2002). Thus, much higher levels of glucuronides are expected to exist in the tubular cells. Some investigators have pointed out that not all the mechanisms underlying MTX-NSAID interactions can be explained merely by the inhibition of the uptake process involving OAT1 and OAT3 (Nozaki et al., 2004; Maeda et al., 2008). Given this information, the present study suggests that the inhibition of apical MRP2- and MRP4-mediated transport of MTX by glucuronides of NSAIDs may play an important role in the drug interaction, in addition to the parent drugs.

The ATP-dependent transport of MTX has been reported in MRP2-, MRP4-, and BCRP-expressing vesicles, in which the K_m values of MTX for MRP4 are much lower than those for MRP2 and BCRP (Nozaki et al., 2007). Similar results have been reported by several investigators (Bakos et al., 2000; Mitomo et al., 2003; Volk and Schneider, 2003). Furthermore, expression of MRP4 protein is 5-fold higher than that of MRP2 in human kidney cortices (Smeets et al., 2004). In addition to these findings, IC_{50} values of NSAIDs and NSAIDs-Glu for MRP4 tended to be smaller than those for MRP2 (Tables 1 and 2). Especially, all *R*-isomers of NSAIDs and their glucuronides tested have higher inhibitory potencies against MRP4-mediated MTX transport. Several investigators also showed that most NSAIDs have higher inhibitory potency against MRP4- than MRP2-mediated transport (Reid et al., 2003; El-Sheikh et al., 2007; Nozaki et al., 2007). Thus, it seems that MRP4 plays a more important role than MRP2 in the inhibition of apical MTX efflux by NSAIDs.

Several investigators have demonstrated stereoselective interactions between drugs and transporters (Ott and Giacomini, 1993; Gross and Somogyi, 1994; Wenzel et al., 1995; Hedman and Meijer, 1998; Pham et al., 2000; Tateishi et al., 2008). Regarding MTX-NSAID interactions, Karpf et al. (2003) reported that 50 μg/ml of *R*- and *S*-ketoprofen significantly reduced the clearance ratio of MTX using isolated perfused rat kidney, but the interaction was not enantioselective. Recently, Honjo et al. (2011) demonstrated the stereoselective inhibitory potencies of flurbiprofen, ibuprofen, and naproxen on hOAT1, but not for hOAT3. In this study, we also investigated the stereoselectivity for the inhibition of NSAIDs and NSAIDs-Glu on MRP2- and MRP4-mediated transport of MTX. Our study showed another intriguing finding that the *S*-enantiomers of NSAIDs and their glucuronides inhibited more strongly for MRP2 than for MRP4 (*R*/*S* ratio = ca. 2), whereas *R*-enantiomers and their glucuronides were much stronger for MRP4 (*R*/*S* ratio = 0.03–0.48). The precise mechanisms of stereoselective recognition of NSAIDs and NSAIDs-Glu for MRP2 and MRP4 remain unclear; however, differences in the accessibility of NSAIDs and NSAIDs-Glu to the binding sites of MRP2 and MRP4 seem to be involved in the stereoselective inhibition of MTX uptake via MRP2 and MRP4. Because we do not have any data about 2-aryl propionic NSAIDs other than ibuprofen, flurbiprofen, and naproxen, it is unclear whether this stereoselectivity is applicable to other 2-aryl propionic NSAIDs. Given that MRP4 is a key site of the drug interaction (Reid et al., 2003; El-Sheikh et al., 2007; Nozaki et al., 2007) and *R*-isomers of NSAIDs and their glucuronides can be potent inhibitors of MRP4, pharmacologically ineffective *R*-enantiomers including in marketing racemic NSAIDs may be undesirable and negative from the viewpoints of drug therapy and drug interaction of MTX.

Based on previous reports regarding possible mechanisms of drug interaction of MTX with NSAIDs and the present data, we propose a postulated mechanism underlying drug interactions (Fig. 4). Inhibition of OAT1 and OAT3 directly elevates the blood levels of MTX; on the other hand, inhibition of MRP2

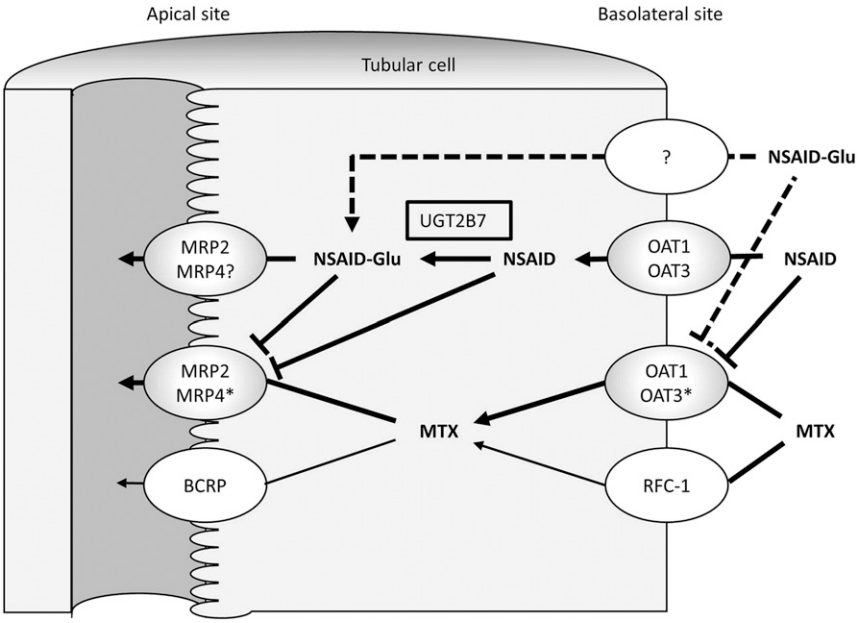

Fig. 4. Postulated mechanisms underlying drug interactions between MTX and NSAIDs. The mechanisms underlying the drug interaction of MTX with NSAIDs probably involve complex MTX-NSAIDs and MTX-glucuronide of NSAID interactions for multiple transporters expressed at basolateral and apical membranes. At least, inhibition of basolateral OATs by NSAIDs and of apical MRPs by NSAIDs and NSAIDs-Glu can be competitive sites. Inhibition of OATs by plasma NSAIDs-Glu may not be ignored.

and MRP4 increases the levels of MTX in tubular cells. Thus, in the case of coadministration of MTX with NSAIDs, both concentrations of MTX in blood and renal tubular cells will become elevated, resulting in a marked accumulation of MTX in kidney. To predict the magnitude of the pharmacokinetic interaction between MTX and NSAIDs, it is necessary to obtain not only the inhibition constant (K_i) values of inhibitors for basolateral OATs and apical MRPs by in vitro study but also the parent NSAIDs as well as their glucuronide levels in both clinical unbound levels and in tubular cells. It is difficult, however, to estimate or predict the levels of NSAIDs-Glu as well as NSAIDs in tubular cells. Thus, the contribution of the glucuronide conjugates to overall drug interaction between MTX and NSAIDs remains unclear.

In the inhibition experiment for MRP2-mediated transport, the reaction was performed for 20 minutes because the amounts of MTX accumulated in the vesicles were low. It is well known that acyl glucuronides are unstable in physiologic conditions and consequently undergo nonenzymatic hydrolysis or intramolecular rearrangement, which occurs by migration of the drug moiety from the 1-*O*-β position to the 2-, 3-, and 4-positions on the glucuronic acid ring (Smith et al., 1990; Benet et al., 1993, Iwaki et al., 1998; Bailey and Dickinson, 2003; Skonberg et al., 2008). We reported that *S*-naproxen acyl glucuronide was subjected predominantly to acyl migration resulting in a rapid appearance of the 2-*O*-acyl isomer and then gradual formation of other isomers at pH 7.4 and that hydrolysis of 1-*O*-glucuronide or its isomer to the parent drug was slow compared with acyl migration (Iwaki et al., 1998). A similar result was obtained for 1-*O*-glucuronide of *S*-naproxen in 25 mM potassium phosphate buffer (pH 7.4) using nuclear magnetic resonance analysis (the acyl migration rate constant of 1-*O*-glucuronode to 2-*O*-isomers was 0.18 hour^{-1}, and the hydrolysis rate constant was 0.025 hour^{-1}) (Mortensen et al., 2001). Since reaction mixture was incubated for 20 minutes in the MRP2-mediated transport experiment, part of the 1-*O*-β-glucuronides probably decomposed. Based on the published degradation rate constants or elimination half-lives of glucuronides at pH 7.4 (Iwaki et al., 1998; Walker et al., 2007; Sawamura et al., 2010), the remaining unchanged 1-*O*-glucuronides are calculated to be 68%–72% for diclofenac glucuronide, 81% for *R*-naproxen glucuronide, 89%–93% for *S*-naproxen glucuronide, and 92% for rac-ibuprofen glucuronide after 20-minute incubation at pH 7.4. We re-evaluated the stability of the glucuronides in the reaction buffer used in a MRP-mediated transport experiment. Less than 7% of the 1-*O*-glucuronides disappeared except for *R*-naproxen glucuronide and *R*-flurbiprofen glucuronide (both 14% loss); however, no detectable or negligible parent NSAIDs were found during 20-minute incubation from all 1-*O*-glucuronides tested. The IC_{50} values of the glucuronides for MRP2-mediated transport of MTX may be misestimated because their migration isomers probably react to MRPs with different potencies from 1-*O*-glucuronide. Whether the isomers have a stronger inhibitory effect on the MRP-mediated transport, the present study has shown that inhibition of MRP2 and MRP4 by NSAID glucuronide conjugates may contribute to drug interactions of MTX with NSAIDs.

Zelcer et al. (2003) demonstrated that two independent binding sites are present in MRPs: one site transports substrates, and another site can modulate the substrate transport site in an allosteric manner. Competitive inhibition and allosteric modulation of substrate transport via MRPs has been observed (Zelcer et al., 2003; El-Sheikh et al., 2007). At low concentrations (0.1–1 μM) of NSAIDs and NSAIDs-Glu, MTX uptake via MRP2- or MRP4-expressing inside-out vesicles was not promoted. Therefore, NSAIDs or NSAIDs-Glu did not undergo allosteric modulation of MTX excretion via MRP2 and MRP4.

We evaluated whether the inhibitory effects of NSAIDs-Glu on MTX excretion were correlated with uptake of the typical substrates of MRP2 and MRP4. Similar inhibitory effects of NSAIDs-Glu on CDCF (MRP2, $r = 0.876$) and $E_2$17β-Glu (MRP4, $r = 0.765$) were observed (Fig. 3). However, the IC_{50} values for MTX were smaller than those for the typical substrates in both MRP2- and MRP4-mediated transport, suggesting that MTX is susceptible to the inhibitory effects of NSAIDs-Glu. Consequently, we should consider that renal apical MTX transport may be much strongly affected by the glucuronides than that expected from the data using the typical substrates.

In conclusion, the present study shows that the glucuronide conjugates of NSAIDs as well as their parent drugs can inhibit MRP2- and MRP4-mediated MTX efflux, with a tendency of the glucuronides to have stronger potencies. These results suggest that the glucuronides of NSAIDs are likely to be involved in inhibition of the urinary excretion of MTX via MRP2 and MRP4 in addition to parent NSAIDs. Our study also shows the interesting stereoselective inhibitory effect of NSAIDs and their glucuronides in that the MRP2-mediated MTX efflux is potently inhibited by the *S*-NSAIDs and *S*-NSAID-Glu examined, whereas MRP4-mediated MTX efflux is potently inhibited by the *R*-isomers. These findings should contribute to better understanding of the renal mechanisms of drug-drug interactions and the nephrotoxicity caused by MTX and NSAIDs; however, the relative contribution of the glucuronides to overall inhibition of MTX excretion by NSAIDs in tubular cells remains one of the key issues to be clarified.

Authorship Contributions

Participated in research design: Iwaki.

Conducted experiments: Yamamoto, Egashira.

Performed data analysis: Kawase.

Wrote or contributed to the writing of the manuscript: Kawase, Iwaki.

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