BIOPHARMACEUTICS & DRUG DISPOSITION
Biopharm. Drug Dispos. **32**: 518–524 (2011)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/bdd.779

Stereoselective inhibitory effect of flurbiprofen, ibuprofen and naproxen on human organic anion transporters hOAT1 and hOAT3

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ABSTRACT: Nonsteroidal anti-inflammatory drugs (NSAIDs) delay the renal excretion of antifolate methotrexate by inhibiting human organic anion transporters hOAT1 (SLC22A6) and hOAT3 (SLC22A8). In this study, uptake experiments were performed using *Xenopus laevis* oocytes to assess stereoselectivity in the inhibitory characteristics of flurbiprofen, ibuprofen and naproxen against hOAT1 and hOAT3. Uptake of *p*-aminohippurate by hOAT1 was inhibited by each enantiomer of the three NSAIDs, and the inhibitory effect was superior in each (S)-enantiomer around 10 μM. The apparent 50% inhibitory concentrations were estimated to be 0.615 μM for (S)-flurbiprofen, 2.84 μM for (S)-ibuprofen and 1.93 μM for (S)-naproxen, and these values were significantly lower than those of the respective (R)-enantiomers [(R)-flurbiprofen: 2.35 μM, (R)-ibuprofen: 6.14 μM, (R)-naproxen: 5.26 μM]. Furthermore, the (S)-NSAIDs at 3 μM reduced methotrexate accumulation in hOAT1-expressing oocytes more strongly than the corresponding (R)-enantiomers. All enantiomers inhibited hOAT3-mediated transport of estrone sulfate and methotrexate, but there was no difference between both enantiomers of each NSAID in the inhibitory potencies. Eadie-Hofstee plot analysis showed that (S)-flurbiprofen and (R)-flurbiprofen inhibited hOAT1 and hOAT3 in a competitive manner. These findings represent the stereoselective inhibitory potencies of flurbiprofen, ibuprofen and naproxen on hOAT1, and the (S)-enantiomers are greater. In contrast, stereoselectivity was not recognized in their inhibitory effect on hOAT3. Copyright © 2011 John Wiley & Sons, Ltd.

Key words: organic anion transporter; transport; NSAID; inhibition; stereoselectivity

Introduction

Organic anion transporters, expressed in the renal proximal tubule, mediate tubular secretion of various drugs including antibiotics, antivirals, diuretics and antitumor agents into urine, and are involved in the regulation of their blood concentrations [1]. Accordingly, the simultaneous administration of their substrate and inhibitor introduces drug interaction. Especially, inhibition of human organic anion transporters hOAT1 (SLC22A6) and hOAT3 (SLC22A8) directly elevates the blood levels of their substrates, because these transporters are responsible for the basolateral uptake of anionic drugs in the proximal tubule [1,2].

Among the drug interactions that occur with the renal organic anion transporters, the interaction between antifolate methotrexate and nonsteroidal anti-inflammatory drugs (NSAIDs) is thought to be one of the most serious. This interaction may cause severe toxicity or death when high-dose methotrexate is used to treat malignancies [3,4]. Some laboratories reported that

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Received 23 August 2011
Revised 14 October 2011
Accepted 7 November 2011

methotrexate was transported by hOAT1 and hOAT3, and that NSAIDs, such as ibuprofen, indomethacin, ketoprofen, loxoprofen, phenylbutazone, piroxicam and salicylate, inhibited methotrexate uptake by hOAT1 and hOAT3 strongly, suggesting that hOAT1 and hOAT3 are involved, at least in part, in the interaction between methotrexate and NSAIDs [5–8].

Several NSAIDs such as flurbiprofen, ibuprofen and naproxen have an asymmetric carbon in their structures (Figure 1). The pharmacological activity of naproxen is known to reside mainly in the (S)-enantiomer, and naproxen is commercially available as the (S)-enantiomer. The others are being marketed as the racemates. Also in the pharmacokinetics of several NSAIDs, stereoselectivity was shown. For example, Knadler *et al*. reported the greater clearance of (R)-flurbiprofen in normal volunteers than the (S)-enantiomer [9], and Mano *et al*. showed that its glucuronidation was superior in (R)-flurbiprofen [10]. On the other hand, the binding constant of (R)-ibuprofen to the human serum albumin was 2.3-fold higher, compared with that of (S)-ibuprofen [11]. Although NSAIDs are known to interact stereoselectively with pharmacokinetic biomolecules, their stereoselective interaction with hOAT1 and hOAT3 has not been elucidated. Therefore, the present study was conducted to evaluate stereoselective inhibitory effects of flurbiprofen, ibuprofen and naproxen on hOAT1 and hOAT3, by performing uptake experiments using the *Xenopus laevis* oocyte expression system.

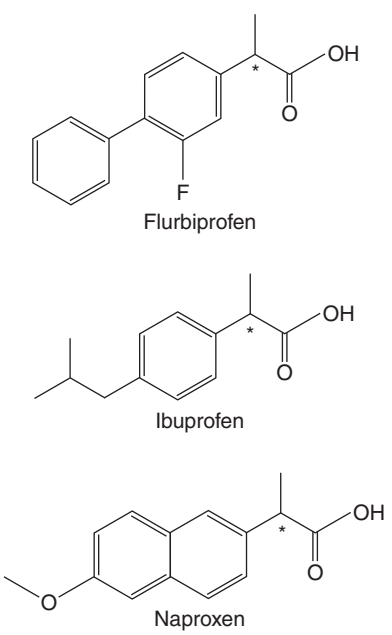

Figure 1. Chemical structures of flurbiprofen, ibuprofen and naproxen. An asymmetric carbon is indicated by an asterisk

Materials and Methods

Materials

[3′,5′,7-^{3}H(N)]Methotrexate, disodium salt (27.7 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). *p*-[Glycyl-2-^{3}H]aminohippurate (4.53 Ci/mmol) and [6,7-^{3}H(N)]estrone sulfate, ammonium salt (54.3 Ci/mmol) were obtained from PerkinElmer Life Science (Boston, MA, USA). (S)-Flurbiprofen, (R)-flurbiprofen and (S)-naproxen were from Cayman Chemical Company (Ann Arbor, MI, USA). (S)-Ibuprofen and (R)-ibuprofen were purchased from LKT Laboratories, Inc. (St Paul, MN, USA) and Enzo Life Sciences International, Inc. (Plymouth Meeting, PA, USA), respectively. (R)-Naproxen was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Unlabeled *p*-aminohippurate and estrone sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich (St Louis, MO, USA), respectively. All other chemicals used were of the highest purity available.

Uptake experiment using Xenopus laevis oocytes expressing hOAT1 or hOAT3

pBK-CMV plasmid vectors containing cDNA of hOAT1 or hOAT3 were a kind gift from Professor Ken-ichi Inui (Kyoto University Hospital, Kyoto, Japan). An uptake experiment using *Xenopus laevis* oocytes was performed as reported previously [12]. Briefly, capped RNA encoding hOAT1 or hOAT3 was transcribed from *Xba* I-linearized pBK-CMV containing cDNA of hOAT1 or hOAT3, respectively, with T3 RNA polymerase. After 50 nl water or cRNA (25 ng) was injected into defolliculated oocytes, the oocytes were maintained in modified Barth's medium (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$ and 5 mM HEPES; pH 7.4) containing 50 mg/l gentamicin at 18 °C. Two or three days after injection, the uptake reaction was

DOI: 10.1002/bdd

initiated by incubating the oocytes in 500 μl uptake buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES; pH 7.4) with each radiolabeled compound at room temperature in the absence or presence of an NSAID for 1 h. The uptake reaction was terminated by adding 2 ml ice-cold uptake buffer to each well, and the oocytes were washed three times with 2 ml ice-cold buffer. After washing, each oocyte was transferred to a scintillation counting vial, and solubilized in 150 μl of 10% sodium lauryl sulfate. Two milliliters of scintillation cocktail Clear-sol II (Nacalai Tesque, Kyoto, Japan) was added to each solubilized oocyte, and radioactivity was determined using a liquid scintillation counter.

Kinetic analysis

The apparent 50% inhibitory concentration (IC_{50}) of each enantiomer of NSAIDs for hOAT1 and hOAT3 was estimated by non-linear least squares regression analysis of the competition curve with a one-compartment model according to the following equation:

$$A = 100 \times IC_{50}/(IC_{50} + [I]) + B,$$

where A is the uptake amount of *p*-aminohippurate or estrone sulfate (% of control), $[I]$ is the concentration of NSAID, and B is the non-specific organic anion uptake (% of control).

The kinetic parameters of *p*-aminohippurate transport by hOAT1 and of estrone sulfate transport by hOAT3 were calculated using non-linear least squares regression analysis from the following Michaelis-Menten equation:

$$V = V_{max} \times [S]/(K_m + [S])$$

where V is the transport rate (pmol/oocyte/h), V_{max} is the maximum velocity by the saturable process (pmol/oocyte/h), $[S]$ is the concentration of *p*-aminohippurate or estrone sulfate (μM), K_m is the Michaelis-Menten constant (μM).

Statistical analysis

Data were analysed by an unpaired *t*-test or one-way analysis of variance followed by Dunnett's test, using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p < 0.05$.

Results

Stereoselectivity in concentration-dependent inhibitory effect of flurbiprofen, ibuprofen and naproxen on transport of p-aminohippurate by hOAT1 and of estrone sulfate by hOAT3

First, uptake amounts of *p*-aminohippurate were measured in *Xenopus* oocytes injected with hOAT1 cRNA in the absence or presence of each enantiomer of flurbiprofen, ibuprofen and naproxen at various concentrations, and their inhibitory effects were compared. As shown in Figure 2A, according to the increase of the concentration of both enantiomers of flurbiprofen, uptake of *p*-aminohippurate by hOAT1 was reduced. The uptake amounts of *p*-aminohippurate by hOAT1 with (S)-flurbiprofen at 0.01 μM, 0.1 μM, 100 μM and 1 mM were comparable to those with (R)-flurbiprofen at the respective concentrations. However, the greater inhibition by (S)-flurbiprofen was recognized at 1 μM and 10 μM. A similar tendency was exhibited with ibuprofen and naproxen (Figures 2B and 2C). The obtained IC_{50} value of (R)-flurbiprofen was about four times as high as that of (S)-flurbiprofen (0.615 ± 0.099 μM and 2.35 ± 0.36 μM, mean ± SEM from three independent experiments), and the difference was statistically significant ($p < 0.001$). The IC_{50} values of (R)-ibuprofen and (R)-naproxen were also significantly higher than those of the corresponding (S)-enantiomers (ibuprofen: 2.84 ± 0.22 μM and 6.14 ± 1.46 μM, mean ± SEM from four independent experiments, $p < 0.05$; naproxen: 1.93 ± 0.12 μM and 5.26 ± 1.04 μM, mean ± SEM from three independent experiments, $p < 0.05$). These findings indicate the stereoselectivity in the inhibitory potencies of flurbiprofen, ibuprofen and naproxen on *p*-aminohippurate transport by hOAT1, and that inhibition by the (S)-enantiomers is greater.

Figure 3 represents the stereoselectivity in inhibitory effect of the three NSAIDs on estrone sulfate transport by hOAT3. All enantiomers inhibited the uptake of estrone sulfate by hOAT3 concentration dependently, but there was no difference between the (S)-enantiomers and the

DOI: 10.1002/bdd

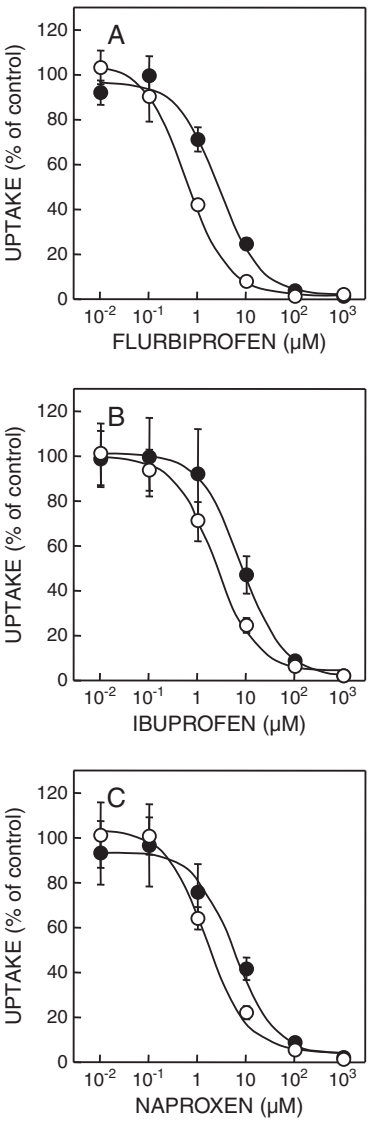

Figure 2. Stereoselective and concentration-dependent effect of flurbiprofen (A), ibuprofen (B) and naproxen (C) on *p*-aminohippurate uptake by hOAT1. Oocytes injected with hOAT1 cRNA were incubated with 221 nM [^{3}H]*p*-aminohippurate in the absence (control) or presence of (S)-enantiomer (open circle) or (R)-enantiomer (closed circle) of flurbiprofen, ibuprofen and naproxen at various concentrations for 1 h. The uptake amounts of [^{3}H]*p*-aminohippurate in each oocyte were determined and are represented as % of control. Each point represents the mean ± SEM of 7–10 oocytes

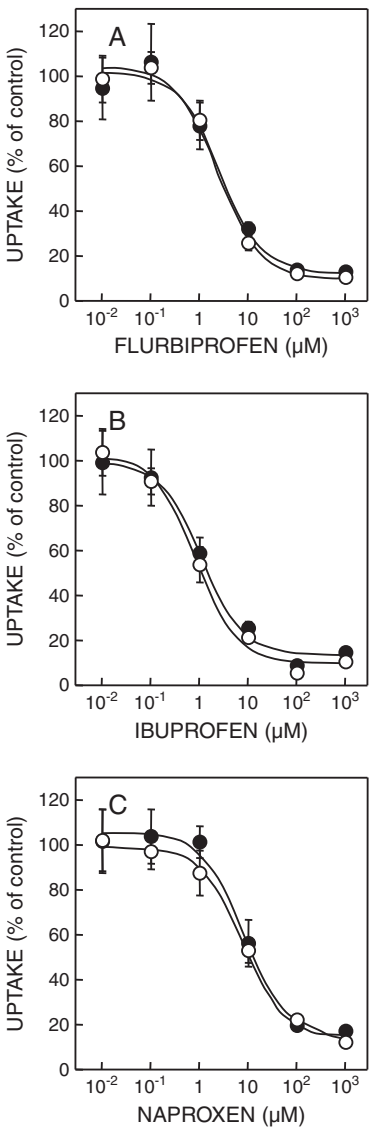

Figure 3. Stereoselective and concentration-dependent effect of flurbiprofen (A), ibuprofen (B) and naproxen (C) on estrone sulfate uptake by hOAT3. Oocytes injected with hOAT3 cRNA were incubated with 18.4 nM [^{3}H]estrone sulfate in the absence (control) or presence of (S)-enantiomer (open circle) or (R)-enantiomer (closed circle) of flurbiprofen, ibuprofen and naproxen at various concentrations for 1 h. The uptake amounts of [^{3}H]estrone sulfate in each oocyte were determined and represented as % of control. Each point represents the mean ± SEM of 7–10 oocytes

(R)-enantiomers of each NSAID in their inhibitory effect. The statistical difference was not recognized between each pair in the IC_{50} values (flurbiprofen: 1.80 ± 0.44 μM for the (S)-enantiomer and 2.13 ± 0.62 μM for the (R)-enantiomer; ibuprofen: 1.20 ± 0.39 μM for the (S)-enantiomer and 2.04 ± 0.48 μM for the (R)-enantiomer; naproxen: 6.79 ± 0.88 μM for the (S)-enantiomer and 8.09 ± 1.05 μM for the (R)-enantiomer, mean ± SEM from three independent experiments). This implies

DOI: 10.1002/bdd

that the inhibitory potencies of the (S)-enantiomers and the (R)-enantiomers of flurbiprofen, ibuprofen and naproxen on estrone sulfate transport by hOAT3 were comparable, respectively.

Stereoselectivity in inhibitory effect of flurbiprofen, ibuprofen and naproxen on methotrexate transport by hOAT1 and hOAT3

Next, the uptake amounts of methotrexate were quantified in oocytes expressing hOAT1 or hOAT3 with or without the NSAIDs at 3 μM. From Figures 2 and 3, this concentration was thought to be suitable to detect the stereoselective inhibitory potencies of the NSAIDs. As represented in Table 1, all enantiomers reduced methotrexate uptake by hOAT1, and a statistically stronger inhibition by each (S)-enantiomer was recognized. Table 2 shows their influence on hOAT3-mediated transport. Every enantiomer tested also inhibited methotrexate uptake by hOAT3, and there was no statistical difference between each pair in the inhibitory effect. These findings reveal that the (S)-enantiomers of flurbiprofen, ibuprofen and naproxen inhibited methotrexate transport by hOAT1 more strongly, and that their inhibitory potencies of the (S)-enantiomers and the (R)-enantiomers in each NSAID were matched by uptake of methotrexate by hOAT3.

Table 1. Stereoselective effect of flurbiprofen, ibuprofen and naproxen on methotrexate uptake by hOAT1

	Uptake (% of control)	
	(S)-Enantiomer	(R)-Enantiomer
Flurbiprofen	37.7 ± 1.3	57.9 ± 2.1[a]
Ibuprofen	50.9 ± 2.0	70.0 ± 2.4[a]
Naproxen	45.7 ± 1.8	64.1 ± 2.6[a]

Oocytes injected with hOAT1 cRNA were incubated with 36.1 nM [^{3}H] methotrexate for 1 h in the absence (control) or presence of each enantiomer of flurbiprofen, ibuprofen and naproxen at 3 μM. The uptake amounts of [^{3}H]methotrexate in each oocyte were determined and represented as % of control. The values represent the mean ± SEM of 28–30 oocytes from three independent experiments. [a]$p < 0.001$, significantly different from the value of (S)-enantiomer.

Table 2. Stereoselective effect of flurbiprofen, ibuprofen and naproxen on methotrexate uptake by hOAT3

	Uptake (% of control)	
	(S)-Enantiomer	(R)-Enantiomer
Flurbiprofen	55.7 ± 3.1	60.9 ± 3.5
Ibuprofen	56.2 ± 2.8	55.8 ± 2.7
Naproxen	74.0 ± 2.2	71.5 ± 3.0

Oocytes injected with hOAT3 cRNA were incubated with 36.1 nM [^{3}H] methotrexate for 1 h in the absence (control) or presence of each enantiomer of flurbiprofen, ibuprofen and naproxen at 3 μM. The uptake amounts of [^{3}H]methotrexate in each oocyte were determined and represented as % of control. The values represent the mean ± SEM of 26–30 oocytes from three independent experiments.

Stereoselectivity in inhibition manner of flurbiprofen for hOAT1 and hOAT3

Finally, to elucidate stereoselectivity in the inhibition manner of both enantiomers of flurbiprofen for hOAT1 and hOAT3, Eadie-Hofstee plot analysis was performed. The concentration-dependence of *p*-aminohippurate uptake by hOAT1 was observed, and (S)-flurbiprofen and (R)-flurbiprofen reduced the transport (Figure 4A). As represented in the inset of Figure 4A, the slope of the Eadie-Hofstee plots of hOAT1-mediated *p*-aminohippurate transport was affected by both enantiomers, but no change of its *y*-intercept was observed. Furthermore, the K_m value of *p*-aminohippurate transport by hOAT1 was significantly increased by (S)-flurbiprofen and (R)-flurbiprofen (control: 3.31 ± 0.50 μM; (S)-flurbiprofen: 13.7 ± 0.3 μM; (R)-flurbiprofen: 14.3 ± 3.9 μM, mean ± SEM from three independent experiments, $p < 0.05$), and no statistical alternation was recognized in the V_{max} values (control: 11.8 ± 3.1 pmol/oocyte/h; (S)-flurbiprofen: 13.0 ± 3.0 pmol/oocyte/h; (R)-flurbiprofen: 11.1 ± 1.8 pmol/oocyte/h). These findings imply that both enantiomers of flurbiprofen inhibited hOAT1 in a competitive manner.

On estrone sulfate uptake by hOAT3, similar findings were obtained. As shown in Figure 4B, (S)-flurbiprofen and (R)-flurbiprofen decreased the concentration-dependent transport of estrone sulfate by hOAT3, and influenced the K_m value (control: 2.76 ± 0.73 μM; (S)-flurbiprofen: 11.3 ± 1.5 μM; (R)-flurbiprofen: 13.5 ± 1.3 μM, mean ± SEM from three independent experiments, $p < 0.01$). In contrast, the V_{max} values did not change significantly (control: 12.3 ± 1.4 pmol/oocyte/h; (S)-flurbiprofen: 14.3 ± 1.9 pmol/oocyte/h; (R)-flurbiprofen: 14.3 ± 0.6 pmol/oocyte/h). These findings mean that (S)-flurbiprofen and (R)-flurbiprofen inhibited hOAT3 competitively. Accordingly, it is indicated that there is no difference between (S)-flurbiprofen and (R)-flurbiprofen in the inhibition manner for hOAT1 and hOAT3.

DOI: 10.1002/bdd

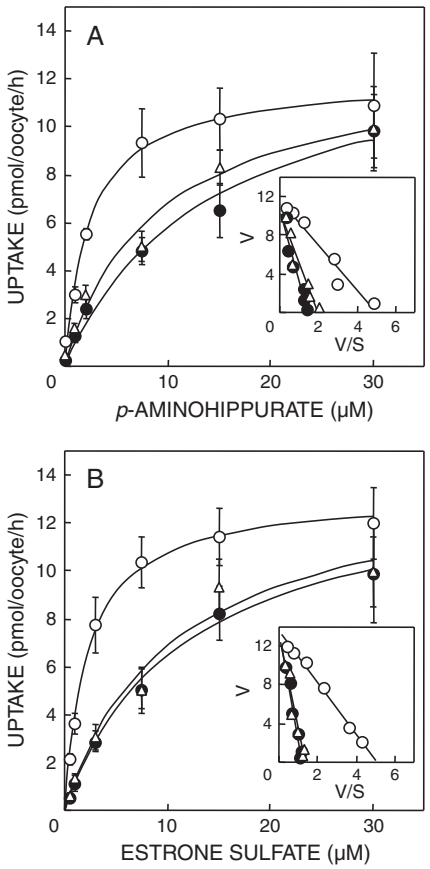

Figure 4. Stereoselective effect of flurbiprofen on concentration-dependent uptake of *p*-aminohippurate by hOAT1 (A) and of estrone sulfate by hOAT3 (B). (A) Oocytes injected with hOAT1 cRNA were incubated with [^{3}H]*p*-aminohippurate at various concentrations for 1 h in the absence (open circle) or presence of 1 μM (S)-flurbiprofen (closed circle) and 3 μM (R)-flurbiprofen (open triangle). hOAT1-mediated uptake of [^{3}H]*p*-aminohippurate was determined by subtracting its uptake amount in water-injected oocytes from that in oocytes injected with hOAT1 cRNA. (B) Oocytes injected with hOAT3 cRNA were incubated with [^{3}H]estrone sulfate at various concentrations for 1 h in the absence (open circle) or presence of 10 μM (S)-flurbiprofen (closed circle) and 10 μM (R)-flurbiprofen (open triangle). hOAT3-mediated uptake of [^{3}H]estrone sulfate was determined by subtracting its uptake amount in water-injected oocytes from that in oocytes injected with hOAT3 cRNA. Inset: Eadie–Hofstee plots of the data; *V*, uptake rate (pmol/oocyte/h); *S*, concentration of [^{3}H]*p*-aminohippurate or [^{3}H]estrone sulfate (μM). Each point represents the mean ± SEM of 7–10 oocytes

Discussion

In several NSAIDs, an asymmetric carbon exists in the structures, and stereoselectivity was reported in their pharmacokinetic profiles [9–11]. The present study assessed stereoselectivity in the inhibition of hOAT1 and hOAT3 by flurbiprofen, ibuprofen and naproxen. As represented in Figure 2, the uptake amounts of *p*-aminohippurate in hOAT1-expressing oocytes were lower with the (S)-NSAIDs around 10 μM, compared with those with the respective (R)-enantiomers. In addition, the calculated IC_{50} values of their (R)-enantiomers were statistically higher. In addition, 3 μM (S)-NSAIDs inhibited methotrexate uptake mediated by hOAT1 more strongly than the (R)-enantiomers (Table 1). These findings indicate a stereoselectivity in the inhibitory potencies of flurbiprofen, ibuprofen and naproxen on hOAT1, their (S)-enantiomers being superior.

Meanwhile, the transport of estrone sulfate or methotrexate by hOAT3 with the (S)-NSAIDs was comparable to that with the respective (R)-enantiomers (Figure 3 and Table 2). A significant difference was not recognized in the IC_{50} values of each pair of the enantiomers for hOAT3. These results mean no stereoselectivity in the inhibitory effects of flurbiprofen, ibuprofen and naproxen on hOAT3. Although the amino acid sequences of hOAT1 and hOAT3 show 51% identity [13], it is thought that the characteristics of drug transport by hOAT1 and hOAT3 are quite different. For instance, it was reported that hOAT1 and hOAT3 transported acyclic nucleotide antivirals adefovir, cidofovir and tenofovir, but that the transport activities of hOAT3 for the antivirals were negligible, in comparison with hOAT1 [14]. hOAT3 was shown to be a potent transporter of cephalosporin antibiotics, but not hOAT1 [15]. The present study suggests that stereoselectivity in the inhibition by NSAIDs is also not a common property for hOAT1 and hOAT3.

In this study, uptake experiments were performed using *Xenopus laevis* oocytes, to assess the stereoselectivity in the inhibition of hOAT1 and hOAT3 by NSAIDs. The obtained findings reveal that the inhibitory potencies of the (S)-enantiomers of flurbiprofen, ibuprofen and naproxen on hOAT1 were greater than those of the respective (R)-enantiomers. In contrast, they were comparable for hOAT3. To our knowledge, this is the first report showing the stereoselectivity in drug recognition of a renal organic anion transporter at molecular level. NSAIDs are one of the most prescribed medicines in the world, and they interact with various drugs. The obtained findings provide useful information for

DOI: 10.1002/bdd

the consideration for influence of NSAIDs on renal handling of anionic drugs.

Acknowledgements

We thank Professor Ken-ichi Inui (Kyoto University Hospital, Kyoto, Japan) for kindly providing pBK-CMV plasmid vectors containing hOAT1 or hOAT3.

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DOI: 10.1002/bdd