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Inhibitory Effect of Selective Cyclooxygenase-2 Inhibitor Lumiracoxib on Human Organic Anion Transporters hOAT1 and hOAT3

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Summary: Nonsteroidal anti-inflammatory drugs (NSAIDs) delay renal excretion of antifolate methotrexate by inhibiting human organic anion transporters hOAT1 (SLC22A6) and hOAT3 (SLC22A8). In this study, we performed uptake experiments using Xenopus laevis oocytes to assess the inhibitory effect of selective cyclooxygenase-2 inhibitors on hOAT1 and hOAT3. The uptake of methotrexate into oocytes was increased by the injection of hOAT1 and hOAT3 cRNA, and transport was strongly inhibited by lumiracoxib. The apparent 50% inhibitory concentrations of lumiracoxib were estimated to be 3.3 μM and 1.9 μM for uptake of p-aminohippurate by hOAT1 and of estrone sulfate by hOAT3, respectively. Eadie-Hofstee plot analysis showed that lumiracoxib inhibited hOAT1 and hOAT3 in a competitive manner. For other cyclooxygenase-2 inhibitors celecoxib, etoricoxib, rofecoxib and valdecoxib, slight to moderate inhibition of hOAT3 only was observed. These findings show that lumiracoxib has inhibitory potential toward hOAT1 and hOAT3, comparable to that of nonselective NSAIDs.

Keywords: hOAT1; hOAT3; selective cyclooxygenase-2 inhibitor; lumiracoxib; methotrexate; transport; drug interaction

Introduction

Organic anion transporters expressed in the renal proximal tubule regulate blood concentrations of various drugs by mediating tubular secretion of antibiotics, antivirals, diuretics and antitumor agents. Accordingly, renal organic anion transporters result in drug interaction when their substrate and inhibitor are co-administered to a patient. For instance, it is well known that probenecid delays renal excretion of cephalosporins, and it is accepted that renal organic anion transporters are involved in this drug interaction. Among the drug interactions involving renal organic anion transporters, the interaction between antifolate methotrexate and nonsteroidal anti-inflammatory drugs (NSAIDs) is thought to be one of the most serious, because this interaction may cause severe toxicity or death when high-dose methotrexate is used to treat malignancies.

In the mid-1990s, cDNA of renal organic anion transporters was isolated, and since then its function and expression have been characterized. Human organic anion transporters hOAT1 (SLC22A6) and hOAT3 (SLC22A8) are thought to be responsible for the renal tubular uptake of methotrexate from blood, because they have been shown to be expressed in the basolateral membrane of proximal epithelial cells and to transport methotrexate. In addition, several laboratories have reported that NSAIDs such as ibuprofen, indomethacin, ketoprofen, loxoprofen, phenylbutazone, piroxicam and salicylate have strong inhibitory effects on methotrexate uptake by hOAT1 and hOAT3, suggesting that hOAT1 and hOAT3 are involved, at least in part, in the interaction between methotrexate and NSAIDs, and this elucidation at the molecular level is useful for establishing optimal drug therapy.

Recently, selective cyclooxygenase-2 inhibitors have been developed, and these agents are increasingly prescribed instead of conventional NSAIDs. As shown in Figure 1, celecoxib and valdecoxib possess a sulfonamide group, and etoricoxib and rofecoxib have a methylsulfonyl moiety. Lumiracoxib is weakly acidic because of a carboxylic acid group. Although the influence of selective cyclooxygenase-2 inhibitors on hOAT1 and hOAT3 is of interest and its evaluation is important, to
our knowledge, no report on the subject has been published. In the present study, we assessed the inhibitory effects of selective cyclooxygenase-2 inhibitors on hOAT1 and hOAT3 by performing uptake experiments using the Xenopus laevis oocyte expression system.

Materials and Methods

Materials: [3H]Methotrexate (27.7 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). [3H]p-Aminohippurate (4.53 Ci/mmol) and [3H]estrone sulfate (54.3 Ci/mmol) were obtained from PerkinElmer Life Science (Boston, MA, USA). Lumiracoxib was from LKT Laboratories, Inc. (St. Paul, MN, USA). Celecoxib, etoricoxib, rofecoxib and valdecoxib were 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 hOAT1 or hOAT3 cDNA were a kind gift from Prof. Ken-ichi Inui (Kyoto University Hospital, Kyoto, Japan). An uptake experiment using Xenopus laevis oocytes was performed as previously reported. Briefly, capped RNA encoding hOAT1 or hOAT3 was transcribed from Xba I-linearized pBK-CMV containing 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(NO3)2, 0.4 mM CaCl2, 0.8 mM MgSO4, 2.4 mM NaHCO3 and 5 mM HEPES) containing 50 mg/L gentamicin at 18°C. Three days after injection, the uptake reaction was initiated by incubating the oocytes in 500 μL uptake buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2 and 5 mM HEPES; pH 7.4) with each radiolabeled compound at room temperature in the absence or presence of a selective cyclooxygenase-2 inhibitor for the indicated periods. Celecoxib, etoricoxib, rofecoxib and valdecoxib were dissolved in dimethyl sulfoxide, and the final concentration in uptake buffer was 0.1%. 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) were added to each solubilized oocyte, and radioactivity was determined using a liquid scintillation counter.

Kinetic analysis: The apparent 50% inhibitory concentration (IC50) of lumiracoxib 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 × IC50/[IC50 + [I]] + B, where A is the uptake amount of p-aminohippurate or estrone sulfate (% of control), [I] is the concentration of lumiracoxib, 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 = Vmax × [S]/(Km + [S]), where V is the transport rate (pmol/oocyte/hr), Vmax is the maximum velocity by the saturable process (pmol/oocyte/hr), [S] is the concentra-
tion of \( p \)-aminohippurate or estrone sulfate (\( \mu M \)) and \( K_m \) is the Michaelis-Menten constant (\( \mu M \)).

**Statistical analysis:** Data were analyzed by the unpaired \( t \) test or planned comparison using the Bonferroni test with GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at \( P < 0.05 \).

**Results**

Transport of methotrexate by hOAT1 and hOAT3 in the presence of lumiracoxib: To investigate whether the transport of methotrexate by hOAT1 and hOAT3 was affected by lumiracoxib, we measured the accumulation of methotrexate in oocytes exposed to the compound. The injection of hOAT1 and hOAT3 cRNA stimulated the uptake of methotrexate, and the uptake amounts increased in a time-dependent manner. In the presence of lumiracoxib at 100 \( \mu M \), the accumulation of methotrexate in oocytes expressing hOAT1 and hOAT3 was reduced to that of the control oocytes (Fig. 2). This finding means that lumiracoxib inhibited the transport of methotrexate by hOAT1 and hOAT3.

**Concentration dependence of inhibitory effects of lumiracoxib on hOAT1 and hOAT3:** To examine the influence of lumiracoxib on hOAT1 and hOAT3 in detail, the dose dependence of the inhibitory effect of lumiracoxib was investigated. As tracers for hOAT1 and hOAT3, their typical substrates, \( p \)-aminohippurate and estrone sulfate, respectively, were used instead of methotrexate. As shown in Figure 3, hOAT1 and hOAT3 were inhibited by lumiracoxib in a dose-dependent manner. The apparent IC\(_{50}\) values were calcu-
Fig. 4. Concentration-dependent uptake of p-aminohippurate by hOAT1 (A) and of estrone sulfate by hOAT3 (B) in the presence of lumiracoxib

(A) Oocytes injected with hOAT1 cRNA were incubated with [3H]p-aminohippurate at various concentrations for 1 hr in the absence (open circle) or presence (closed circle) of 20 μM lumiracoxib. hOAT1-mediated uptake of [3H]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 [3H]estrone sulfate at various concentrations for 1 hr in the absence (open circle) or presence (closed circle) of 5 μM lumiracoxib. hOAT3-mediated uptake of [3H]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/hr); S, concentration of [3H]p-aminohippurate or [3H]estrone sulfate (μM). Each point represents the mean±S.E.M. of 8 to 10 oocytes.

Inhibition manner of lumiracoxib toward hOAT1 and hOAT3: To elucidate the inhibition mode of lumiracoxib against hOAT1 and hOAT3, Eadie-Hofstee plot analysis was performed. As shown in Figure 4A, lumiracoxib affected the slope of the Eadie-Hofstee plots of hOAT1-mediated p-aminohippurate transport, but its y-intercept was not influenced. Furthermore, the K_m value of p-aminohippurate transport by hOAT1 was significantly increased by lumiracoxib from 3.6±0.2 to 13.3±0.5 μM (mean±S.E.M. from 3 separate experiments, P<0.0001). On the other hand, the V_max values were calculated to be 7.1±0.5 and 6.3±0.5 pmol/oocyte/hr in the absence or presence of lumiracoxib, respectively, and no statistical difference between these values was recognized (P=0.3701).

The results for hOAT3 showed a similar tendency to hOAT1 (Fig. 4B). The slope of the Eadie-Hofstee plot of estrone sulfate uptake by hOAT3 was influenced by lumiracoxib. The K_m values were estimated to be 4.7±1.2 and 12.0±1.9 μM (mean±S.E.M. from 3 separate experiments) in the absence or presence of lumiracoxib, respectively, and the difference was statistically significant (P=0.0315). On the other hand, no significant change of the V_max values was observed (without lumiracoxib, 6.2±0.7; with lumiracoxib, 6.4±0.5 pmol/oocyte/hr; P value = 0.8370). These findings imply that lumiracoxib inhibited hOAT1 and hOAT3 in a competitive manner.

Effect of celecoxib, etoricoxib, rofecoxib and valdecoxib on methotrexate transport by hOAT1 and hOAT3: Figure 5 shows the influence of celecoxib, etoricoxib, rofecoxib and valdecoxib on hOAT1 and hOAT3. No statistical change in hOAT1-mediated uptake of methotrexate by any cyclooxygenase-2 inhibitor was detected by planned comparison with the Bonferroni test. On the other hand, celecoxib slightly but significantly inhibited hOAT3 (P<0.05), and, moderate inhibition of hOAT3 was observed in the presence of etoricoxib, rofecoxib and valdecoxib (P<0.001).

Discussion

NSAIDs are some of the most commonly prescribed medicines, but they often induce drug interactions.9) In particular, severe toxicity has been observed by co-administration of NSAIDs and high-dose methotrexate, and one of the reasons is thought to be that NSAIDs prevent renal tubular uptake of methotrexate by hOAT1 and hOAT3.6,7) Accordingly, it is important to find or explore NSAIDs which do not affect drug metabolic enzymes and drug transporters. In this study, we examined the interaction of selective inhibitors of cyclooxygenase-2 with hOAT1 and hOAT3, and it was shown that lumiracoxib inhibited the uptake of methotrexate by these transporters. In addition, inhibition of hOAT3 by celecoxib, etoricoxib, rofecoxib and valdecoxib was observed.

To obtain more information on the inhibition of hOAT1 and hOAT3 by lumiracoxib, we examined the IC_{50} and inhibition mode of lumiracoxib. The IC_{50} was calculated to be 3.3 μM and 1.9 μM for hOAT1 and hOAT3, respectively. These values are comparable to...
IC\textsubscript{50} or K\textsubscript{i} values for the transporters of traditional NSAIDs, such as diclofenac, indomethacin, ibuprofen, ketoprofen, naproxen, loxoprofen and tolmetin.\textsuperscript{7,10} Eadie-Hofstee plot analysis showed that the inhibition manner of lumiracoxib toward hOAT1 and hOAT3 is competitive (Fig. 4). Takeda et al. and Maeda et al. reported that NSAIDs including indomethacin, mefenamic acid, sulindac and pranoprofen inhibited hOAT3 in a competitive manner.\textsuperscript{6,11} It seemed that there was no difference between lumiracoxib and nonselective NSAIDs in the inhibitory characteristics toward hOAT1 and hOAT3.

It was also of interest whether lumiracoxib inhibits hOAT1 and hOAT3 in the clinical setting. Compared with ibuprofen and naproxen, advantages of lumiracoxib have been shown in terms of gastrointestinal and cardiovascular safety\textsuperscript{12,13}; however, the countries where lumiracoxib is prescribed are limited due to its hepatotoxicity.\textsuperscript{14} Lumiracoxib shows pharmacological effects at a daily dosage of 100 mg to 400 mg,\textsuperscript{15} and Scott et al. reported that its concentration in plasma at the steady state ranged from 141 \(\mu\)g/L (0.5 \(\mu\)M) to 6635 \(\mu\)g/L (22.6 \(\mu\)M) when 400 mg lumiracoxib was orally administered once daily to patients with rheumatoid arthritis.\textsuperscript{16} From Figure 3, it can be seen that lumiracoxib in this concentration range inhibits hOAT1 and hOAT3 moderately; however, protein binding of lumiracoxib in plasma should be considered, and is estimated to be 98%.\textsuperscript{16} Using this percentage, the free level of lumiracoxib was calculated to be 9.6 nM to 450 nM in plasma. Taken together with the unbound plasma concentrations and the results shown in Figure 3, it is suggested that meaningful inhibition of hOAT1 and hOAT3 by lumiracoxib may not occur with a 400-mg daily dose. Hartmann et al. reported that no effect on methotrexate pharmacokinetics was observed with a 400 mg daily dose of lumiracoxib.\textsuperscript{17} They also measured the plasma concentrations of lumiracoxib, and reported that its mean maximum concentration was 7175 ng/mL (24.4 \(\mu\)M), and that its trough concentrations with median values ranged between 28.5 (97.0 nM) and 53.1 ng/mL (181 nM\textsuperscript{17}). The maximum unbound concentration of lumiracoxib was calculated to be 488 nM in these patients. The absence of interaction between methotrexate and lumiracoxib in the clinical trial by Hartmann et al. is considered to be reasonable. Lumiracoxib is mainly metabolized by cytochrome P450 (CYP) 2C9,\textsuperscript{15} and polymorphisms exist in its gene, affecting the metabolic activity. Using insect cell microsomes, Tang et al.\textsuperscript{18} showed that allelic variants reduced celecoxib hydroxylase activity. The possibility should not be excluded that the unbound level of lumiracoxib could reach the inhibitory range of hOAT1 and hOAT3 when the cyclooxygenase-2 inhibitor is administered at the usual dosage to patients with low CYP2C9 activity, followed by its mutation, hepatic failure and drug interaction. However, the frequencies of the allele with reduced CYP2C9 activity are reported to be very low, suggesting that the incidence of interaction between methotrexate and lumiracoxib in patients with CYP2C9 mutation would be rare, if it occurred at all.\textsuperscript{19}

Figure 5 shows that methotrexate uptake by hOAT1 was not inhibited by celecoxib, etoricoxib, rofecoxib or

**Fig. 5. Effect of various cyclooxygenase-2 inhibitors on methotrexate uptake by hOAT1 (A) and hOAT3 (B)**

(A) Water-injected oocytes were incubated with 36.1 nM \textsuperscript{3}H]methotrexate for 1 hr. Oocytes injected with hOAT1 cRNA were incubated with 36.1 nM \textsuperscript{3}H]methotrexate for 1 hr in the absence or presence of celecoxib, etoricoxib, rofecoxib and valdecoxib at the indicated concentrations with 0.1% dimethyl sulfoxide. (B) Water-injected oocytes were incubated with 36.1 nM \textsuperscript{3}H]methotrexate for 1 hr. Oocytes injected with hOAT3 cRNA were incubated with 36.1 nM \textsuperscript{3}H]methotrexate for 1 hr in the absence or presence of celecoxib, etoricoxib, rofecoxib and valdecoxib at the indicated concentrations with 0.1% dimethyl sulfoxide. The uptake amounts of \textsuperscript{3}H]methotrexate in each oocyte were determined. Each column represents the mean±S.E.M. of 9 to 10 oocytes. *\textit{P} value <0.05, significantly different from the values of hOAT3 cRNA-injected oocytes. ***\textit{P} value <0.001, significantly different from the values of hOAT3 cRNA-injected oocytes.
valdecoxib. The tested concentrations of the compounds were comparable with the IC50 of lumiracoxib for hOAT1, meaning that the inhibitory effects of the 4 cyclooxygenase-2 inhibitors on hOAT1 are weaker than that of lumiracoxib. On the other hand, inhibition of hOAT3 by celecoxib, etoricoxib, rofecoxib and valdecoxib was observed. Due to the very low solubility of celecoxib, etoricoxib, rofecoxib and valdecoxib in the uptake buffer used in the present study, unfortunately, we could not perform a more detailed examination of their inhibitory effects. However, these phenomena are interesting, because no difference in the inhibitory effect on hOAT1 and hOAT3 was recognized with anionic lumiracoxib.

In conclusion, this study shows that lumiracoxib exhibited an inhibitory effect on hOAT1 and hOAT3. The IC50 values and inhibition mode of lumiracoxib are similar to those of conventional NSAIDs. The clinical pharmacokinetics of lumiracoxib suggest that optimal administration would not induce interactions between the selective cyclooxygenase-2 inhibitor and the substrates of methotrexate. In addition, hOAT3 was inhibited by celecoxib, etoricoxib, rofecoxib and valdecoxib.

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References