Note

Inhibition of Methotrexate Uptake *via* Organic Anion Transporters OAT1 and OAT3 by Glucuronides of Nonsteroidal Anti-inflammatory Drugs

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Combination therapy of non-steroidal anti-inflammatory drugs (NSAIDs) and methotrexate (MTX) sometimes triggers adverse effects, such as liver injury, renal failure, gastrointestinal disorders, and myelosuppression, owing to the reduction of MTX clearance. Previous reports have suggested that NSAIDs inhibit renal MTX uptake *via* organic anion transporters (OATs) and reduced folate transporter (RFC)-1 and efflux *via* multidrug resistance-associated proteins (MRPs). Recently, our laboratory found inhibitory effects of NSAIDs-glucuronide (NSAIDs-Glu), a major metabolite of NSAIDs, on MRP-mediated MTX transport as a new site of interaction between MTX and NSAIDs. However, it remains unclear that whether NSAIDs-Glu inhibit renal uptake of MTX. Therefore, the present study aimed to evaluate inhibitory effects of several NSAIDs-Glu (diclofenac, *R*- and *S*-ibuprofen, *R*- and *S*-flurbiprofen, and *R*- and *S*-naproxen) on human OAT1 and OAT3-mediated MTX transport. In this study, [³H]MTX uptake was observed by using human OAT1 and OAT3-overexpressing HEK293 cells in the presence or absence of NSAIDs-Glu. All examined NSAIDs-Glu exhibited concentration-dependent inhibitory effects on MTX uptake *via* OAT1 and OAT3. Our results indicated that NSAIDs-Glu are more potent (5- to 15-fold) inhibitors of OAT3 than OAT1. Moreover, stereoselective inhibitory effects of NSAIDs-Glu on OATs-mediated MTX uptake were not observed, unlike on MRPs-mediated transport. These findings suggest that inhibition of OAT1 and OAT3-mediated renal uptake of MTX by plasma NSAIDs-Glu may be one of the competitive sites underlying complex drug interaction between MTX and NSAIDs.

Key words drug interaction; methotrexate; non-steroidal anti-inflammatory drug; glucuronide; organic anion transporter

Although methotrexate (MTX) is often co-administered with non-steroidal anti-inflammatory drugs (NSAIDs) to patients suffering from rheumatoid arthritis and neoplastic diseases, several NSAIDs, such as ketoprofen, indomethacin, naproxen, and diclofenac, reduce MTX clearance. Thus, combined administration of MTX and NSAIDs can result in elevated plasma MTX concentrations and severe adverse effects, such as liver injury, renal failure, gastrointestinal disorders, and myelosuppression.^{1–5)} In fact, the MTX prescribers' information states that combined use of MTX and NSAIDs may increase plasma MTX concentrations and adverse effects.

MTX is primarily excreted in urine *via* glomerular filtration and active tubular secretion in its unchanged form.⁶⁾ At a clinical plasma concentration of MTX (0.2–10 μ M), approximately 25% of renal clearance is excreted by glomerular filtration and the remaining 75% is excreted by active tubular secretion.⁷⁾ In the tubular secretory process, organic anion transporters (OAT) 1 and 3 and reduced folate carrier (RFC)-1 transport MTX from the blood across the basolateral membrane.^{8–11)} Multidrug resistance-related proteins (MRP) 2 and 4 and breast cancer-resistant protein (BCRP) transport MTX across the apical membrane into the urine where it is excreted.^{12–15)} Previous studies have shown that several mechanisms, including the inhibitory effect of NSAIDs on OATs and RFC-1-mediated MTX uptake at the basolateral membrane,^{16,17)} and MTX efflux *via* MRP2 and MRP4 at apical membrane in tubular cells,¹⁸⁾ are involved in the interaction between MTX and NSAIDs. However, it is also suggested that several

NSAIDs such as diclofenac, naproxen, and ketoprofen have no effect on the uptake and efflux of MTX at clinical doses because of low unbound plasma concentrations.¹⁵⁾

NSAIDs are primarily metabolized to glucuronide conjugates (NSAIDs-Glu) in the liver. For example, the percentage of glucuronide metabolites formed from diclofenac and ibuprofen in human liver microsomes was reported to be 89.4 and 53.3%, respectively.¹⁹⁾ The generated NSAIDs-Glu molecules are secreted into the blood along with bile.²⁰⁾ Separately, NSAIDs taken into tubular cells are also converted into glucuronide conjugates mainly by uridine diphosphate (UDP)-glucuronosyl transferase (UGT) 2B7.^{21,22)} Most NSAIDs-Glu molecules are excreted directly into urine.^{23,24)} Recently, our laboratory reported that NSAIDs-Glu, as well as parent NSAIDs, are involved in inhibition of urinary excretion of MTX *via* MRP2 and MRP4.²⁵⁾ Since the IC₅₀ of NSAIDs-Glu for MTX transport *via* MRPs was lower than that of parent NSAIDs, our results suggested that NSAIDs-Glu likely contribute to elevating the plasma concentration of MTX. However, despite the fact that some NSAIDs-Glu, such as glucuronides of diclofenac and ibuprofen, are detected in plasma after administration of NSAIDs,^{26,27)} inhibitory effect of NSAIDs-Glu on renal MTX uptake transporter such as OATs and RFC-1 is poorly understood.

In the present study, we evaluated the effects of several NSAIDs-Glu (glucuronides of diclofenac, *R*- and *S*-ibuprofen, *R*- and *S*-flurbiprofen, and *R*- and *S*-naproxen) on OAT1 and OAT3-mediated MTX transport. Our results indicate that

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NSAIDs-Glu inhibition of OAT1 and OAT3-mediated MTX uptake may be one of the mechanisms that contributes to the interaction between MTX and NSAIDs.

MATERIALS AND METHODS

Chemicals Diclofenac was purchased from Sigma-Aldrich (Saint Louis, MO, U.S.A.). Naproxen enantiomers (*R*- and *S*-naproxen) and ibuprofen enantiomers (*R*- and *S*-ibuprofen) were purchased from Toronto Research Chemicals (Toronto, Canada) and Enzo Life Science (Farmingdale, NY, U.S.A.). Flurbiprofen enantiomers (*R*- and *S*-flurbiprofen) were obtained from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Diclofenac glucuronide, ibuprofen glucuronide (mixture of diastereomers), flurbiprofen glucuronide (mixture of diastereomers), and MTX were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human OAT1 and OAT3-expressing HEK293 cells were purchased from Genomembrane (Kanagawa, Japan). [^3H]MTX was obtained from America Radiolabeled Chemicals (Saint Louis, MO, U.S.A.). All other chemicals and solvents were of the highest purity available, or of HPLC grade.

Preparation of NSAIDs-Glu Rat liver microsomes were prepared according to the method previously reported.^{28,29)} β -1-*O*-glucuronides of NSAIDs were prepared biosynthetically *in vitro* from respective NSAIDs using rat liver microsomes according to the published method.^{25,30)} The purity of the glucuronides obtained was determined by HPLC at a UV 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 homogenous (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-Glu on Transport of MTX via OAT1 and OAT3 Uptake experiments were performed using OAT1 and OAT3-over-expressing HEK293 cells according to the instructions provided by the supplier. Briefly, cells (4.0×10^5 cells/well) were plated in 24-well plates and cultured for 18–30 h. Then, an uptake experiment was initiated by adding transport buffer (250 mM NaCl, 9.6 mM KCl, 11.2 mM D-glucose, 2.4 mM CaCl_2 , 2.4 mM KH_2PO_4 , 2.4 mM MgSO_4 , 50 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid) containing [^3H]MTX (100 μM for OAT1 and 5 μM for OAT3) in the presence or absence of NSAIDs-Glu. Five minutes later, the cells were washed with ice-cold transport buffer and lysed in 1 M sodium hydroxide solution, which was then neutralized using hydrochloric acid. The radioactivity was measured using a liquid scintillation counter (TRI-CARB; PerkinElmer, Inc., Waltham, MA, U.S.A.).

The concentration of [^3H]MTX used in the uptake experiments was decided by reference to the previously reported K_m values ($724.0 \pm 74.9 \mu\text{M}$ for OAT1 and $21.2 \pm 5.7 \mu\text{M}$ for OAT3).^{31,32)} Moreover, since the uptake rate was linear over the first 5 min in preliminary experiments (data not shown), inhibitory effects of NSAIDs-Glu were evaluated at 5 min.

Kinetic Analysis The IC_{50} values of each NSAID-Glu for OAT1 and OAT3-mediated [^3H]MTX transport were estimated

by non-linear least squares regression analysis according to the following Hill equation: $A = 100 \times \text{IC}_{50} / (\text{IC}_{50} + [I])$, where A is the uptake amount of [^3H]MTX (% of control), and I is the concentration of NSAID-Glu, using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, U.S.A.).

RESULTS AND DISCUSSION

To date, although numerous studies have been conducted to investigate the inhibitory effect of parent NSAIDs on OATs, RFC-1, MRPs and BCRP-mediated MTX transport,^{10,14,15,17,28)} unbound plasma concentration of NSAIDs were too low to cause an interaction between MTX and NSAIDs at clinical doses.^{15,33,34)} Our recent study suggested that NSAIDs-Glu inhibit MRP2 and MRP4-mediated MTX efflux more strongly than parent NSAIDs.²⁵⁾ However, the inhibitory effect of plasma NSAIDs-Glu on renal uptake of MTX *via* OATs and RFC-1 remains unclear. In the present study, we evaluated the inhibitory potencies of NSAIDs-Glu on MTX uptake *via* OAT1 and OAT3.

As shown in Fig. 1, all examined NSAIDs-Glu exhibited concentration-dependent inhibitory effects on [^3H]MTX uptake *via* OAT1 and OAT3 with different potencies (Fig. 1). Although IC_{50} values are varied depending on experimental systems (*e.g.*, oocytes or expressing cells) in general, the IC_{50} values of NSAIDs-Glu for OAT1-mediated MTX transport were generally larger than those of parent NSAIDs reported by using OAT1-expressing oocytes.¹⁷⁾ On the other hand, the values for OAT3-mediated MTX transport were similar to those of parent NSAIDs (Table 1). Since previous studies using a kidney slice indicate that OAT3 and RFC-1 are likely to be major contributors to renal basolateral MTX transport and NSAIDs inhibit MTX transport *via* RFC-1,¹¹⁾ our results strongly suggested that coordinated inhibitory effect of NSAIDs and NSAIDs-Glu on OAT3-mediated MTX transport could involve in drug interaction between NSAIDs and MTX. However, the effect of NSAIDs-Glu on RFC-1-mediated MTX transport is still unclear. Since the effect of NSAID-Glu on RFC-1 is the only remaining mechanism of interaction between MTX and NSAIDs at tubular cells, future analysis is needed.

In the recent drug–drug interactions (DDI) guidance of Food and Drug Administration (FDA), unbound $C_{\text{max}} / \text{IC}_{50} \geq 0.1$ is considered positive as perpetrator of DDI. Although unbound plasma concentrations of NSAIDs-Glu have not been previously reported, total plasma concentrations of glucuronide conjugates of diclofenac ($1.8 \pm 0.5 \mu\text{M}$),²⁴⁾ ibuprofen ($3.6 \pm 2.0 \mu\text{M}$),²³⁾ naproxen (undetectable)³⁵⁾ and flurbiprofen (undetectable)³⁶⁾ have been determined after administration of clinical doses. Therefore, considering plasma concentration and inhibitory potencies, it is unlikely that NSAIDs-Glu other than diclofenac glucuronide inhibit OAT3-mediated MTX uptake at clinical doses. Among the NSAIDs-Glu tested in this study, diclofenac glucuronide had the most potent inhibitory effect against OAT3 ($\text{IC}_{50} = 3.2 \mu\text{M}$) (Table 1). Moreover, our previous studies suggested that the ratio of reversible binding to human serum albumin (HSA) of NSAIDs-Glu, such as naproxen glucuronide and fenoprofen glucuronide, tends to be weaker than that of their parent NSAIDs.^{37,38)} Assuming that diclofenac glucuronide has a similar tendency, it is unsurprising that inhibition of OAT3-mediated MTX uptake by diclof-

Fig. 1. OAT1 and OAT3-Mediated Transport of [^3H]MTX in the Presence of NSAIDs-Glu

The uptake of [^3H]MTX by OAT1- (left panels) and OAT3- (right panels) expressing HEK293 cells was measured in the presence of glucuronides of diclofenac (a, b), *R*- and *S*-ibuprofen (c, d), *R*- and *S*-flurbiprofen (e, f), and *R*- and *S*-naproxen (g, h) at various concentrations at 37°C. Results are the mean of duplicate experiments.

Table 1. Inhibition Parameters of NSAIDs-Glu for Uptake of MTX *via* OAT1 and OAT3

NSAIDs-Glu	IC ₅₀ (μM)		Ratio	
	OAT1	OAT3	R/S	OAT1/OAT3
Diclofenac-Glu	265 (183–384)	3.17 (0.35–28.3)	—	83.6
<i>R</i> -Ibuprofen-Glu	791 (464–1350)	60.1 (52.7–68.6)	OAT1: 0.82	13.2
<i>S</i> -Ibuprofen-Glu	960 (792–1160)	57.0 (26.6–122)	OAT3: 1.05	16.8
<i>R</i> -Flurbiprofen-Glu	198 (53–723)	19.4 (0.31–120)	OAT1: 1.14	10.2
<i>S</i> -Flurbiprofen-Glu	174 (108–281)	31.7 (0.66–151)	OAT3: 0.61	5.5
<i>R</i> -Naproxen-Glu	639 (457–894)	129 (60–277)	OAT1: 0.86	5.0
<i>S</i> -Naproxen-Glu	747 (565–987)	51.4 (10.6–250)	OAT3: 2.51	14.5

Numbers in parentheses represent 95% confidence interval.

Table 2. IC₅₀ Values of NSAIDs-Glu for MRP2- and MRP4-Mediated MTX Transport

NSAIDs-Glu	MRP2		MRP4	
	IC ₅₀ (μM)	R/S Ratio	IC ₅₀ (μM)	R/S Ratio
Diclofenac-Glu	18.6 (15.7–21.9)	—	140 (123–159)	—
<i>R</i> -Ibuprofen-Glu	208 (189–229)	2.57	3.6 (2.0–6.3)	0.05
<i>S</i> -Ibuprofen-Glu	80.9 (74.2–88.2)		66.6 (55.9–78.0)	
<i>R</i> -Flurbiprofen-Glu	29.5 (23.9–36.3)	1.37	3.2 (2.9–3.6)	0.04
<i>S</i> -Flurbiprofen-Glu	21.5 (19.4–23.8)		93.0 (77.3–112)	
<i>R</i> -Naproxen-Glu	771 (727–817)	1.62	1.63 (1.32–2.03)	0.03
<i>S</i> -Naproxen-Glu	475 (449–504)		48.7 (42.5–55.8)	

Numbers in parentheses represent 95% confidence interval.

enac glucuronide contributes to the DDI between diclofenac and MTX.

Inhibitory potencies of NSAIDs-Glu on OAT3-mediated MTX transport almost match with that on MTX transport *via* MRP2 and MRP4.²⁵⁾ The IC₅₀ values of NSAIDs-Glu for MRP2- and MRP4-mediated MTX transport were summarized in Table 2. Although stereoselectivity was observed in the inhibitory effect of NSAIDs-Glu on MRPs-mediated MTX transport (*R/S* ratio=0.03–2.57) (Table 2), it was not observed in the effect on OATs (*R/S* ratio=0.61–2.51) (Table 1). Although the combined administration of MTX and NSAIDs increases the risk of kidney injury, the different inhibitory potencies of NSAIDs-Glu against MTX transport *via* OATs, RFC-1 and MRPs may explain rates of kidney injury incidence. If NSAIDs and NSAIDs-Glu inhibit renal MTX uptake transporters (*e.g.*, OATs and RFC-1) more strongly than renal efflux transporter (*e.g.*, MRPs), then the incidence of kidney injury caused by MTX should decrease according to the rate of inhibition of MTX accumulation in renal tubular cells. Stereoselective inhibition of NSAIDs-Glu on MRPs may pos-

sibly relate to accumulation of MTX in tubular cells. Further investigation is needed to clarify the effect of NSAIDs-Glu on the accumulation of MTX in tubular cells by simultaneously evaluating both MTX uptake *via* OATs and RFC-1 and efflux *via* MRPs in a model such as a kidney slice.

In conclusion, the present study demonstrates for the first time that NSAIDs-Glu, as well as parent NSAIDs, can inhibit OAT1 and OAT3-mediated MTX transport. Stereoselectivity was not observed in the inhibitory effects on either OAT1 or OAT3. Our results suggest that inhibition of OAT3-mediated MTX uptake at the basolateral membrane of tubular cells by plasma NSAIDs-Glu may be the fourth competitive site underlying the complex drug interaction between MTX and NSAIDs, in addition to the inhibition of basolateral OATs by parent NSAIDs and of apical MRPs by NSAIDs and their glucuronides.

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Conflict of Interest The authors declare no conflict of interest.

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