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)-I 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 µM), 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 diphosphateglucuronosyl 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 (Farmingsdale, 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 Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [3H]MTX was obtained from America Radiolabeled Chemicals (Saint Louis, MO, U.S.A.).

Preparation of NSAIDs-Glu  Rat liver microsomes were prepared according to the method previously reported.28,29 β-1-D-glucuronides of NSAIDs were prepared biosynthetically in vitro from respective NSAIDs using rat liver microsomes according to the published method.28,29 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 (4.0×10^6 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 CaCl2, 2.4 mM KH2PO4, 2.4 mM MgSO4, 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 mM 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_{in} values (724±74.9 μM for OAT1 and 21.2±5.7 μM 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: 

\[ A = 100 \times \frac{[C]_{50}}{[C]_{50} + [J]} \]

where A is the uptake amount of [3H]MTX (% of control), and J 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, MRP1s, 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 MRPs 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_{max} 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±0.5 μM),29 ibuprofen (3.6±2.0 μM),29 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 (IC_{50}=3.2 μM) (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 diclo-
Fig. 1. OAT1 and OAT3-Mediated Transport of $[^3]$HMTX in the Presence of NSAIDs-Glu

The uptake of $[^3]$HMTX 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.
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. 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 MRP-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 possibly 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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**Table 1. Inhibition Parameters of NSAIDs-Glu for Uptake of MTX via OAT1 and OAT3**

<table>
<thead>
<tr>
<th>NSAIDs-Glu</th>
<th>IC₅₀ (µM)</th>
<th>Ratio</th>
<th>IC₅₀ (µM)</th>
<th>Ratio</th>
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<tbody>
<tr>
<td></td>
<td>OAT1</td>
<td>OAT3</td>
<td>OAT1/OAT3</td>
<td></td>
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<tr>
<td>Diclofenac-Glu</td>
<td>265 (183–384)</td>
<td>3.17 (0.35–28.3)</td>
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<td>83.6</td>
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<tr>
<td>R-Ibuprofen-Glu</td>
<td>791 (464–1350)</td>
<td>60.1 (52.7–68.6)</td>
<td>OAT1: 0.82</td>
<td>13.2</td>
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<tr>
<td>S-Ibuprofen-Glu</td>
<td>960 (792–1160)</td>
<td>57.0 (26.6–122)</td>
<td>OAT3: 1.05</td>
<td>16.8</td>
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<tr>
<td>R-Flurbiprofen-Glu</td>
<td>198 (53–723)</td>
<td>19.4 (0.31–120)</td>
<td>OAT1: 1.14</td>
<td>10.2</td>
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<tr>
<td>S-Flurbiprofen-Glu</td>
<td>174 (108–281)</td>
<td>31.7 (0.66–151)</td>
<td>OAT3: 0.61</td>
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<tr>
<td>R-Naproxen-Glu</td>
<td>639 (457–894)</td>
<td>129 (60–277)</td>
<td>OAT1: 0.86</td>
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<td>S-Naproxen-Glu</td>
<td>747 (565–987)</td>
<td>51.4 (10.6–250)</td>
<td>OAT3: 2.51</td>
<td>14.5</td>
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Numbers in parentheses represent 95% confidence interval.

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

<table>
<thead>
<tr>
<th>NSAIDs-Glu</th>
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<th>MRP4</th>
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<tbody>
<tr>
<td></td>
<td>IC₅₀ (µM)</td>
<td>R/S Ratio</td>
</tr>
<tr>
<td>Diclofenac-Glu</td>
<td>18.6 (15.7–21.9)</td>
<td>—</td>
</tr>
<tr>
<td>R-Ibuprofen-Glu</td>
<td>208 (189–229)</td>
<td>2.57</td>
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<tr>
<td>S-Ibuprofen-Glu</td>
<td>80.9 (74.2–88.2)</td>
<td>2.65</td>
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<tr>
<td>R-Flurbiprofen-Glu</td>
<td>29.5 (23.9–36.3)</td>
<td>1.37</td>
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<tr>
<td>S-Flurbiprofen-Glu</td>
<td>21.5 (19.4–23.8)</td>
<td>1.63</td>
</tr>
<tr>
<td>R-Naproxen-Glu</td>
<td>771 (727–817)</td>
<td>1.62</td>
</tr>
<tr>
<td>S-Naproxen-Glu</td>
<td>475 (449–504)</td>
<td>48.7</td>
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Numbers in parentheses represent 95% confidence interval.
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Conflict of Interest The authors declare no conflict of interest.

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