Long-Lasting Inhibitory Effect of Apple and Orange Juices, but Not Grapefruit Juice, on OATP2B1-Mediated Drug Absorption

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ABSTRACT

Enzyme-based grapefruit juice (GFJ)-drug interactions are mainly due to mechanism-based irreversible inhibition of metabolizing enzyme CYP3A4 by GFJ components, but the transporter organic anion transporting polypeptide (OATP)2B1 is also a putative site of interaction between drugs and fruit juices (FJ) in the absorption process. Here we aimed to investigate the effect of preincubation with FJ on OATP2B1-mediated transport of drugs in vitro. When OATP2B1-expressing Xenopus oocytes were preincubated with GFJ, orange juice (OJ), or apple juice (AJ), AJ induced a remarkable decrease in OATP2B1-mediated estrone-3-sulfate uptake in a concentration-dependent manner (IC50 = 1.5%). A similar but less potent effect was observed with OJ (IC50 = 21%), whereas GFJ had no effect. Similar results were obtained in preincubation studies using fexofenadine. Preincubation with OJ and AJ resulted in time-dependent inhibition of OATP2B1. Again, AJ had the more potent effect; its action lasted for at least 240 minutes, suggesting that AJ irreversibly inhibits OATP2B1-mediated drug uptake. Kinetic analysis revealed that coinubcation with AJ reduced OATP2B1-mediated estrone-3-sulfate uptake via competitive and noncompetitive mechanisms, respectively. Thus, OATP2B1 is functionally impaired through both competitive and long-lasting inhibition mechanisms by AJ and OJ, but not GFJ. Interestingly, although GFJ but not AJ is able to irreversibly inhibit CYP3A4, in the case of OATP2B1, AJ but not GFJ has a long-lasting inhibitory effect. Accordingly, complex FJ-drug interactions may occur in vivo, and their clinical significance should be examined.

Introduction

Fruit juice (FJ) interacts with a number of drugs in the intestinal absorption process, potentially resulting in altered pharmacokinetic behaviors of the drugs. The first FJ-drug interaction to be identified was an increase in the bioavailability of felodipine associated with grapefruit juice (GFJ) ingestion (Bailey et al., 1989). Subsequent work established that the mechanism of the elevation of drug bioavailability by GFJ is irreversible mechanism-based inhibition (MBI) of intestinal CYP3A4 by furanocoumarins present in the juice (Paine et al., 2006, 2008). Later, bergamottin and 6',7'-dihydroxybergamottin, two of the major furanocoumarins in GFJ, were identified as mechanism-based inhibitors, as well as reversible inhibitors of CYP3A4 (Schmiedlin-Ren et al., 1997; He et al., 1998; Guo et al., 2000). These furanocoumarins were not detected in orange juice (OJ) or apple juice (AJ), which is consistent with reports showing no effect of OJ on intestinal absorption of CYP3A4 substrates (Bailey et al., 1991; He et al., 1998; Fukuda et al., 2000; Paine et al., 2006, 2008).

On the other hand, recent investigations have demonstrated that GFJ also decreases the bioavailability of a variety of clinically used drugs (Dresser et al., 2002; Schwarz et al., 2005; Bailey et al., 2007; Shirasaka et al., 2009, 2010a). One of the putative mechanisms underlying this GFJ-drug interaction is thought to be a reduction in absorptive transport of drugs in small intestine owing to inhibition of organic anion transporting polypeptide (OATP) by GFJ and its components (Dresser et al., 2002). Fexofenadine was the first OATP substrate reported to interact with GFJ and has been the most extensively studied (Dresser et al., 2002). Subsequently, several OATP substrates whose bioavailabilities are reduced by GFJ ingestion have been reported (Reif et al., 2002; Litija et al., 2003; Schwarz et al., 2005; Tapaninen et al., 2010). We demonstrated that bioavailability of fexofenadine is decreased in individuals with the OATP2B1 c.1457C>T allele and is also decreased in the presence of AJ (Imanaga et al., 2011). These observations imply that AJ interacts with fexofenadine through inhibition of OATP2B1-mediated absorption, although a contribution of OATP1A2 to intestinal AJ-drug interactions cannot be ruled out (Kobayashi et al., 2003; Shirasaka et al., 2010a; Tamai, 2012). Involvement of OATP2B1 in FJ-drug interaction was also demonstrated by our recent report describing that coinubcation with GFJ, OJ, and AJ significantly inhibited OATP2B1-mediated uptake of estrone-3-sulfate with apparent IC50 values in the order of GFJ < OJ < AJ (Shirasaka et al., 2013).

In contrast to FJ-drug interactions involving CYP3A substrates, decreased bioavailability of OATP2B1 substrates was observed with not only GFJ, but also OJ and AJ (Dresser et al., 2002; Litija et al., 2004, 2005; Imanaga et al., 2011; Tapaninen et al., 2011; Jeon et al., 2013). Naringin, the main constituent flavonoid of GFJ, is thought to be a major inhibitor of OATP2B1-mediated drug transport in GFJ (Bailey et al., 2007; Shirasaka et al., 2009, 2010a, b, 2011a, b, 2013). However, because the concentration of naringin in OJ and AJ is much lower than...
that in GFJ, it is unlikely that naringin is a major contributor to OATP2B1-mediated drug interactions involving OJ and AJ (Ameer et al., 1996; Ho et al., 2000). We recently indicated that hesperidin is a major causal component in OJ through direct inhibition of intestinal OATP2B1, and the effect is likely to be clinically relevant (Shirasaka et al., 2013). However, the major inhibitor of OATP2B1 in AJ could not be determined. Although AJ was suggested to inhibit OATP2B1 due to the additive inhibitory effect of several flavonoids, this could not quantitatively account for the direct effect of AJ on OATP2B1 (Shirasaka et al., 2013). Therefore, we hypothesized that such AJ-drug interaction could be explained in terms of irreversible MBI or long-lasting inhibition of intestinal OATP2B1. The occurrence of MBI and/or long-lasting inhibition of OATP2B1 with FJ has not yet been evaluated. However, it was reported that preincubation enhanced the inhibitory effect of cyclosporine A on hepatic OATP1B1- and OATP1B3-mediated drug transport, suggesting a long-lasting inhibitory effect of cyclosporine A on OATP1B1 and OATP1B3 (Amundsen et al., 2010; Shitara et al., 2012).

In the present study, we aimed to clarify and differentiate the mechanisms of OATP2B1-mediated drug interactions with GFJ, OJ, and AJ, focusing on long-lasting inhibition of OATP2B1, by means of in vitro studies using OATP2B1-expressing Xenopus oocytes and a typical OATP2B1 substrate, estrone-3-sulfate. Our results indicate that OATP2B1 is functionally impaired via both competitive and long-lasting inhibition mechanisms by AJ and OJ, but not GFJ.

Materials and Methods

Chemicals and Reagents. [3H]Estrone-3-sulfate ammonium salt was purchased from PerkinElmer Life Sciences (Boston, MA). Grapefruit, orange, and apple juice were commercial products (Tropicana, Chicago, IL; 100% pure at normal strength) were purchased from a supermarket in Kanazawa city (Kanazawa, Japan). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Invitrogen (Carlsbad, CA), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), or Applied Biosystems (Foster City, CA).

Preparation of Xenopus laevis Oocytes. Xenopus oocytes expressing OATP2B1 were prepared as described previously (Nozawa et al., 2004; Shirasaka et al., 2010a, 2012). In brief, the construct pGEMHE containing OATP2B1 cDNA was used to synthesize cRNA in vitro. Manually defolliculated oocytes were injected with 50 nl of the cRNA solution (1 μg/μl) or water, and then incubated for 3 days at 18°C in modified Barth’s saline (MBS) (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, and 10 mM HEPES, pH 7.4) containing 50 μg/ml gentamicin.

Preincubation Experiments of Xenopus laevis Oocytes with Grapefruit, Orange, and Apple Juice. Oocytes expressing OATP2B1 were preincubated in uptake buffer (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4, 0.33 mM Ca(NO3)2, 0.41 mM CaCl2, and 10 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5) for 60 minutes at 25°C. Then, the oocytes were washed three times with MBS at 25°C, and uptake was initiated by replacing uptake buffer with [3H]estrone-3-sulfate solution in the uptake buffer (pH 6.5) in the absence of GFJ, OJ, and AJ. The oocytes were incubated for the designated time at 25°C. To avoid excess osmolality, 100% GFJ, OJ, and AJ were added to the uptake buffer to make the concentration of fruit juice the same as that in the preincubation condition. Uptake was measured in the absence of GFJ, OJ, and AJ for 15 minutes at 25°C. Uptake was terminated by washing the oocytes three times with ice-cold MBS.

LC/MS/MS Analysis. The concentrations of fexofenadine in all samples were quantified with a LC/MS/MS system consisting of a MDS-Sciex API 3200TM triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) coupled with a LC-20AD ultra-fast liquid chromatography system (Shimadzu Co., Kyoto, Japan). The LC/MS/MS analysis for fexofenadine was performed under the conditions described previously (Imanaga et al., 2011). The ultra-fast liquid chromatography gradient elution was performed using a mobile phase composed of 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.3 ml/min. The gradient profile was 5.0% B for 0–12.5 minutes, 5.0–95% B for 12.5–22.5 minutes, 95% B for 22.5–43.5 minutes, 95–5.0% B for 43.5–44.5 minutes, and 5.0% B for 44.5–5.5 minutes. The total run time was 5.5 minutes for each injection. The retention time of fexofenadine was 2.9 minutes. Mercury MS (C18, 10 × 4.0 mm, Luna 3 μm, Phenomenex, Torrance, CA) was used as the analytical column. The mass transitions (Q1/Q3) of m/z 502.3/466.3 was used for fexofenadine. Analyst software version 1.4 (AB Sciex, Foster City, CA) was used for data manipulation.

Data Analysis. Uptake (μl/oocyte) of estrone-3-sulfate was expressed as the cell-to-medium ratio by dividing the uptake amount by the initial concentration at 60 minutes.
of estrone-3-sulfate in the uptake medium. Uptake rate (μl/min/oocyte) was expressed as uptake of estrone-3-sulfate over a specified time. OATP2B1-mediated uptake rates were obtained after subtraction of the uptake by water-injected oocytes from that by OATP2B1 cRNA-injected oocytes.

Inhibition kinetic parameters were estimated by means of nonlinear least-squares analysis using the MULTI program or KaleidaGraph (Synergy Software, Reading, PA). The inhibitory effect on estrone-3-sulfate transport was expressed as percentage of control, and the inhibitor concentration giving half-maximum inhibition (IC50) was obtained by means of the following equation:

\[
\% \text{ of control} = \frac{100 \times \text{IC}_{50}}{\text{IC}_{50} + [I]}
\]

where [I] is inhibitor concentration (μM).

The affinity of estrone-3-sulfate for OATP2B1 (Km) and the maximal velocity of OATP2B1-mediated estrone-3-sulfate uptake (Vmax) were obtained by fitting the data to the following equation:

\[
V = \frac{V_{\text{max}(1)} \cdot C}{K_{\text{m}(1)} + C} + \frac{V_{\text{max}(2)} \cdot C}{K_{\text{m}(2)} + C}
\]

where V and C are uptake rate (pmol/min/oocyte) and initial concentration (μM), respectively. Suffixes 1 and 2 indicate high- and low-affinity sites, respectively.

Statistical Analysis. Data are given as the mean of values obtained in at least three experiments with the standard error (S.E.M.). Statistical analyses were performed with the unpaired Student’s t-test and a probability of less than 0.05 (P < 0.05) was considered to represent a statistically significant difference.

Results

Effect of Preincubation with Grapefruit, Orange and Apple Juices on OATP2B1-Mediated Uptake of Estrone-3-Sulfate. After preincubation with various concentrations of GFJ, OJ, and AJ for 60 minutes, uptake of estrone-3-sulfate (0.005 μM) by OATP2B1-expressing X. oocytes was evaluated in the absence of GFJ, OJ, and AJ (Fig. 1). Preincubation with OJ and AJ significantly inhibited OATP2B1-mediated estrone-3-sulfate uptake in a concentration-dependent manner, with IC50 values of 20.6 ± 1.7% and 1.46 ± 0.18%, respectively. However, OATP2B1-mediated uptake of estrone-3-sulfate was not affected by preincubation with 50% GFJ (Fig. 1).

In the following studies on preincubation effect, OJ and AJ were routinely used at the concentration close to IC50, namely 20% and 2%, respectively.

Time-dependence of Effect of Preincubation with Orange and Apple Juices on OATP2B1-Mediated Uptake of Estrone-3-Sulfate. After preincubation with 20% OJ or 2% AJ for 0–90 minutes, uptake of estrone-3-sulfate (0.005 μM) by OATP2B1-expressing X. oocytes was determined in the absence of OJ and AJ (Fig. 2). The extent of reduction in OATP2B1-mediated uptake of estrone-3-sulfate increased with increasing preincubation time (Fig. 2). Because the effect of preincubation with 20% OJ and 2% AJ on OATP2B1-mediated estrone-3-sulfate uptake was decreased beyond 60 minutes, the following experiments were performed using preincubation for 60 minutes.

Long-lasting Inhibitory Effect of Apple Juice on OATP2B1-Mediated Uptake of Estrone-3-Sulfate. After preincubation with 2% AJ for 60 minutes, OATP2B1-expressing X. oocytes were washed and further incubated for 0–240 minutes in MBS without AJ. Then, the uptake of estrone-3-sulfate (0.005 μM) was evaluated in the presence and absence of preincubation with AJ.

![Fig. 2. Time-dependence of effect of preincubation with orange or apple juice on estrone-3-sulfate uptake by OATP2B1-expressing X. oocytes. After preincubation with 20% OJ (A) or 2% AJ (B) for 0–90 minutes, OATP2B1-mediated uptake of estrone-3-sulfate (0.005 μM) was measured in the absence of OJ and AJ for 15 minutes at 25°C and pH 6.5. The effect of preincubation with 20% OJ (filled squares) and 2% AJ (filled circles) on OATP2B1-mediated estrone-3-sulfate uptake was evaluated by comparison with the uptake in the absence of OJ (open squares) and AJ (open circles). OATP2B1-mediated uptake of estrone-3-sulfate was determined by subtracting the uptake by water-injected oocytes from that by OATP2B1 cRNA-injected oocytes. *P < 0.05, significantly different from control (without AJ). Data are shown as the mean ± S.E.M. (n = 8–10).](image)

![Fig. 3. Long-lasting inhibitory effect of apple juice on estrone-3-sulfate uptake by OATP2B1-expressing X. oocytes. After preincubation with 2% AJ for 60 minutes, X. oocytes were incubated for 0–240 minutes in MBS without AJ, and the OATP2B1-mediated uptake of estrone-3-sulfate (0.005 μM) was measured in the absence of AJ for 15 minutes at 25°C and pH 6.5. The effect of preincubation with 2% AJ (filled circles) on OATP2B1-mediated estrone-3-sulfate uptake was evaluated by comparison with the uptake in the absence of preincubation with AJ (open circles). OATP2B1-mediated uptake of estrone-3-sulfate was determined by subtracting the uptake by water-injected oocytes from that by OATP2B1 cRNA-injected oocytes. *P < 0.05, significantly different from control (without AJ). Data are shown as the mean ± S.E.M. (n = 8–10).](image)
absence of AJ (Fig. 3). OATP2B1-mediated uptake of estrone-3-sulfate by oocytes preincubated with 2% AJ was significantly lower than that by oocytes preincubated without AJ over 240 minutes after the preincubation, indicating that AJ has an irreversible inhibitory effect on OATP2B1.

Kinetic Analysis of the Long-lasting Inhibitory Effect of Apple Juice on OATP2B1-Mediated Uptake of Estrone-3-Sulfate. The inhibitory mechanism of AJ on OATP2B1 was examined by analyzing the concentration dependence of OATP2B1-mediated uptake of estrone-3-sulfate by oocytes preincubated with 2% AJ for 60 minutes and comparing the results with those in the case of coincubation with 2% AJ (namely uptake in the presence of 2% AJ without preincubation) (Fig. 4). Under all conditions, the OATP2B1-mediated uptake of estrone-3-sulfate was saturable, and Eadie-Hofstee plot analysis showed biphasic kinetics. The results are summarized in Table 1.

Coincubation of oocytes with AJ significantly increased the $K_m$ value (0.550 ± 0.150 μM versus 1.32 ± 0.25 μM) for estrone-3-sulfate uptake mediated by the high-affinity site on OATP2B1, and a small but statistically significant change of $V_{max}$ value was observed (0.288 ± 0.067 pmol/min/oocyte versus 0.429 ± 0.007 pmol/min/oocyte), suggesting that the inhibition of OATP2B1 is competitive. Overall, the uptake efficiency, evaluated as $V_{max}/K_m$, was decreased from 0.524 to 0.325 μmol/min/oocyte. In contrast, no significant change was observed at the low-affinity site. In the case of preincubation, no significant change of the $K_m$ value (0.550 ± 0.150 μM versus 0.506 ± 0.109 μM) for estrone-3-sulfate uptake mediated by the high-affinity site on OATP2B1 was observed with AJ, whereas the $V_{max}$ value (0.288 ± 0.067 pmol/min/oocyte versus 0.132 ± 0.027 pmol/min/oocyte) was significantly decreased, suggesting noncompetitive inhibition (Table 1). Although the $K_m$ value at the low-affinity site was decreased by preincubation with 2% AJ, no apparent change in $V_{max}/K_m$ was observed, suggesting that the low-affinity site is essentially unaffected by preincubation with 2% AJ. These findings indicated that OATP2B1 is functionally impaired (in terms of estrone 3-sulfate uptake) by AJ via both competitive and non-competitive long-lasting inhibition mechanisms at the high-affinity site.

Combined Effect of Coincubation and Preincubation with Orange and Apple Juices on OATP2B1-Mediated Uptake of Estrone-3-Sulfate. The combined effect of preincubation and coinubcation with 20% OJ or 2% AJ on OATP2B1-mediated uptake of estrone-3-sulfate was examined using OATP2B1-expressing Xenopus oocytes (Fig. 5). As shown in Fig. 5A, OATP2B1-mediated uptake of estrone-3-sulfate was significantly decreased to 9.10 ± 1.08% and 56.3 ± 4.5% by coincubation alone and preincubation alone with 20% OJ, respectively, indicating that the inhibitory effect of coinubcation with OJ is more potent than that of preincubation with OJ. The uptake was also decreased by combined coinubcation and preincubation with 20% OJ, but the inhibition was similar to that in the case of preincubation alone (8.09 ± 0.56%). On the other hand, as shown in Fig. 5B, OATP2B1-mediated estrone-3-sulfate uptake was significantly decreased to 44.7 ± 2.2% and 25.6 ± 1.8% by coincubcation alone and preincubation alone with 2% AJ, respectively, indicating that the inhibitory effect of AJ on OATP2B1 was more potent in the case of preincubation, rather than coincubcation. Interestingly, OATP2B1-mediated estrone-3-sulfate uptake was further reduced by combined coinubcation and preincubation with 2% AJ (16.2 ± 0.6%) compared with coinubcation or preincubation alone, suggesting that inhibitory effect of coinubcation and preincubation with AJ on OATP2B1 (Fig. 5B).

Effect of Coincubation and Preincubation with Grapefruit, Orange, and Apple Juices on OATP2B1-Mediated Uptake of Fexofenadine. The effect of preincubation and coinubcation with 20% GFI, OJ, and AJ on OATP2B1-mediated uptake of fexofenadine (500 μM) was examined using OATP2B1-expressing Xenopus oocytes (Fig. 6). As shown in Fig. 6A, OATP2B1-mediated fexofenadine uptake was significantly inhibited by coinubcation with GFI, OJ, and AJ. On the other hand, after preincubation with OJ and AJ for 60 minutes, OATP2B1-mediated uptake of fexofenadine was significantly inhibited in the absence of OJ and AJ (Fig. 6B). However, OATP2B1-mediated fexofenadine uptake was not affected by preincubation with GFI.

### Table 1

<table>
<thead>
<tr>
<th>Condition of Administration</th>
<th>Affinity Sites</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/oocyte)</th>
<th>$V_{max}/K_m$ (μmol/min/oocyte)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without 2% AJ)</td>
<td>High</td>
<td>0.550 ± 0.150</td>
<td>0.288 ± 0.067</td>
<td>0.524</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>41.0 ± 10.3</td>
<td>3.30 ± 0.36</td>
<td>0.0805</td>
</tr>
<tr>
<td>Coincubation with 2% AJ</td>
<td>High</td>
<td>1.32 ± 0.25*</td>
<td>0.429 ± 0.007*</td>
<td>0.325</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>66.1 ± 13.9</td>
<td>2.99 ± 0.25</td>
<td>0.0452</td>
</tr>
<tr>
<td>Preincubation with 2% AJ</td>
<td>High</td>
<td>0.506 ± 0.109</td>
<td>0.132 ± 0.027*</td>
<td>0.261</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>27.8 ± 3.7</td>
<td>2.43 ± 1.89</td>
<td>0.0874</td>
</tr>
</tbody>
</table>

* $P < 0.05$, significantly different from control (without 2% AJ).
Discussion

In the present study, we aimed to clarify the mechanism of OATP2B1-mediated drug interactions with GFJ, OJ, and AJ, focusing on long-lasting inhibition of OATP2B1, by means of in vitro studies using OATP2B1-expressing Xenopus oocytes with a typical OATP2B1 substrate, estrone-3-sulfate.

Preincubation with AJ caused a remarkable decrease in OATP2B1-mediated estrone-3-sulfate uptake (IC50 = 1.5%), suggesting a long-lasting inhibitory effect of AJ on OATP2B1 (Fig. 1). A similar but smaller effect on OATP2B1 was observed with OJ (IC50 = 2%). Meanwhile, our previous study revealed that no significant effect of osmolality on OATP2B1-mediated estrone-3-sulfate uptake was observed up to 500 mOsM (Shirasaka et al., 2013). Because osmolality of 50% GFJ, OJ, and AJ was found to be 346, 351, and 471 mOsM, respectively, it is considered that reduction of OATP2B1-mediated estrone-3-sulfate transport is not due to change in osmolality conditions and/or its cytotoxic effect on oocytes by these juices. Accordingly, drug interactions with OJ and AJ during intestinal absorption described in recent reports are likely to be at least partly due to the long-lasting inhibition of OATP2B1. Preincubation with OJ and AJ resulted in a preincubation time-dependent inhibition of OATP2B1, which was more significant with 2% AJ than with 20% OJ, showing that AJ has a more potent long-lasting inhibitory effect on OATP2B1 than does OJ (Fig. 2, A and B). Interestingly, in contrast to the MBI of CYP3A4 with GFJ, GFJ did not show long-lasting inhibition of OATP2B1 (Fig. 1). This is consistent with a clinical report that no significant change in the area under the plasma concentration-time curve of fexofenadine was observed when GFJ was given 4 hours before fexofenadine administration (Glaeser et al., 2007). On the other hand, the reduction in plasma concentration of fexofenadine reportedly was the largest in the case of coadministration with AJ, followed by OJ and GFJ (Dresser et al., 2002). However, as shown in Fig. 6, effect of coincubation with GFJ, OJ, and AJ on OATP2B1-mediated fexofenadine uptake was almost comparable. Furthermore, we previously reported that coincubation with AJ had a weaker inhibitory effect on OATP2B1; the apparent IC50 values were in the order of GFJ, OJ, and AJ (Shirasaka et al., 2013). Because the effect of preincubation with AJ on OATP2B1-mediated fexofenadine uptake was most potent, these apparently inconsistent observations may be due to distinct effects of coincubation and preincubation (Fig. 6). Indeed, we found here that OATP2B1-mediated estrone-3-sulfate uptake was further reduced by the combination of coincubation and preincubation with fruit juices.
preincubation with AJ, compared with coincubation alone or preincubation alone (Fig. 5). Although there is no direct evidence that both effects of coincubation and preincubation with AJ are involved in the decreased absorption of fexofenadine after coadministration with AJ, our results shown in Figs. 5 and 6 strongly support the view that potent inhibitory effect of AJ on OATP2B1-mediated fexofenadine absorption in vivo. On the other hand, no combined effect was observed in the case of OJ, possibly because the long-lasting inhibition potency is weak. These findings suggested that differences in the effects of coincubation and preincubation among FJ on OATP2B1 might result in complex FJ-drug interactions in vivo.

Clinical investigation revealed that the recovery of CYP3A4 activity impaired by GFJ requires enzyme synthesis, and the half-life for this process is approximately 23 hours after GFJ ingestion (Greenblatt et al., 2003). Therefore, MBI of intestinal CYP3A4 with GFJ is clinically important, because up to 3 days may be necessary for the enzymatic activity to recover. On the other hand, the long-lasting inhibitory effect of AJ on OATP2B1-mediated drug absorption in humans has not yet been reported to have clinical consequences. We found here that the effect of preincubation with AJ on OATP2B1-mediated estrone-3-sulfate uptake lasted for at least 240 minutes, suggesting an irreversible inhibitory effect of AJ on OATP2B1 (Fig. 3). The effect of AJ preexposure on intestinal drug absorption in vivo should be examined in the future.

Eadie-Hofstee plot analysis showed biphasic kinetics of OATP2B1-mediated estrone-3-sulfate uptake in accordance with our previous report (Fig. 4) (Shirasaka et al., 2012). Coincubation with AJ significantly increased the $K_m$ value for estrone-3-sulfate uptake mediated by both high- and low-affinity sites on OATP2B1, whereas the change of $V_{max}$ value was minimal, suggesting competitive inhibition of OATP2B1 (Table 1). In contrast, in the case of the preincubation effect, change of the $V_{max}$ value at the high-affinity site appears to be more important than change of $K_m$ value, suggesting that the inhibition of the high-affinity site on OATP2B1 is noncompetitive. It was recently reported that protein kinase C (PKC) activation resulted in reduced OATP2B1 transport activity, with a decrease in $V_{max}$ of estrone-3-sulfate uptake, due to internalization of OATP2B1, followed by lysosomal degradation (Köck et al., 2010). Thus, the mechanism of the long-lasting inhibition of OATP2B1 by AJ and OJ may involve reduced membrane surface expression of OATP2B1, although it is unclear whether PKC is activated by components in AJ and OJ. Another possibility is a covalent binding of components in AJ and OJ with OATP2B1. In the MBI of CYP3A4, a reactive metabolite forms a covalent bond with CYP3A4 and irreversibly inactivates it. Similarly, components in AJ and OJ may covalently bond with OATP2B1, resulting in its inactivation. The precise mechanism of the long-lasting inhibition of OATP2B1 by AJ and OJ remains to be clarified.

Our kinetic analysis revealed that preincubation with AJ reduced transport activity of estrone-3-sulfate at the high-affinity but not low-affinity sites on OATP2B1. This implies that AJ-drug interactions may be observed only when a drug is accepted as a substrate by OATP2B1 at the binding site that is affected by preincubation with AJ. In other words, long-lasting inhibitory effect of AJ on OATP2B1-mediated drug transport may be substrate dependent due to inhibition of only the high-affinity site for estrone-3-sulfate on OATP2B1. This hypothesis is supported by previous report indicating that drug-drug and drug-beverage interactions occur only when two drugs share the same binding site on OATP2B1 (Shirasaka et al., 2012). Interestingly, in Fig. 6, preincubation with OJ and AJ but not GFJ significantly inhibited OATP2B1-mediated uptake of fexofenadine, consistent with preincubation study using estrone-3-sulfate. Assuming that multiple binding sites are involved in OATP2B1, it is possible to consider that fexofenadine (500 µM) and estrone-3-sulfate (0.005 µM) share the same binding site on OATP2B1, but the fact remains unclear. To examine whether the presence of multiple binding sites results in a substrate-dependent long-lasting inhibition of OATP2B1 by AJ, further studies would be needed.

In conclusion, OATP2B1 is functionally impaired through both competitive and long-lasting inhibition mechanisms by components of AJ and OJ, but not GFJ. In particular, AJ has a potent long-lasting inhibitory effect on OATP2B1 compared with the other juices. The different inhibition mechanisms of OATP2B1 by FJ may result in complex FJ-drug interactions in vivo, although the clinical significance of these interactions requires further study.

**Authorship Contributions**

**Participated in research design:** Shirasaka, Tamai.
**Conducted experiments:** Shichiri, Murata, Mori.
**Performed data analysis:** Shirasaka, Shichiri.
**Wrote or contributed to the writing of the manuscript:** Shirasaka, Nakanishi, Tamai.

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