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

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Running Title:

Long-Lasting Inhibition of OATP2B1 by Fruit Juices

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Abbreviations:

AJ, apple juice; DDI, drug-drug interactions; FJ, fruit juice; GFJ, grapefruit juice; MBI, mechanism-based inhibition; OATP, organic anion transporting polypeptide; OJ, orange juice.

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Abstract

Enzyme-based grapefruit juice (GFJ)-drug interactions are mainly due to mechanism-based irreversible inhibition of metabolizing enzyme cytochrome (CYP) 3A4 by GFJ components, but the transporter OATP2B1 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 (IC_{50} = 1.5%). A similar but less potent effect was observed with OJ (IC_{50} = 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 min, suggesting that AJ irreversibly inhibits OATP2B1-mediated drug uptake. Kinetic analysis revealed that coincubation and preincubation 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, while 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.

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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 cytochrome (CYP) 3A4 by furanocoumarins present in the juice (Paine et al., 2006; Paine et al., 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; Paine et al., 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; Shirasaka et al., 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; Bailey et al., 2007; Shirasaka et al., 2009; Shirasaka et al., 2010a). 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; Lilja 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 coincubation with GFJ, OJ and AJ significantly inhibited OATP2B1-mediated uptake of estrone-3-sulfate with apparent IC_{50} 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; Lilja 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, 2010b, 2011a, 2011b, 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,

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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.

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Materials and Methods

Chemicals and Reagents

[^{3}H]Estrone-3-sulfate ammonium salt was purchased from PerkinElmer Life Sciences (Boston, MA). Grapefruit, orange and apple juice were commercial products (Tropicana^{TM}; 100% pure at normal strength). All other chemicals were obtained from Sigma-Aldrich Co. (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 (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, and 10 mM HEPES, pH 7.4) containing 50 μg/mL gentamicin (MBS).

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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 $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, and 10 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.5) for 10 min at 25 ˚C. To exclude effect of pH condition caused by juice on OATP2B1 activity, all experiments were performed under condition of pH 6.5 even when each FJ was simultaneously applied. For the long-lasting inhibition studies, oocytes expressing OATP2B1 were preincubated in GFJ, OJ or AJ solution (uptake buffer, pH 6.5) for the designated time at 25 ˚C. To avoid excess osmolality, 100% GFJ, OJ and AJ solutions was firstly prepared using components of uptake buffer excluding NaCl (pH 6.5). Then, a series of GFJ, OJ and AJ solutions with different concentrations was prepared by diluting with uptake buffer including NaCl (pH 6.5). After preincubation, the oocytes were washed three times with MBS at 25 ˚C, and uptake was initiated by replacing uptake buffer with [^{3}H]estrone-3-sulfate or fexofenadine solutions in uptake buffer (pH 6.5) in the absence of GFJ, OJ or AJ. The oocytes were incubated for the designated time at 25 ˚C. Uptake was terminated by washing the oocytes three times with ice-cold MBS.

To examine the time dependence (irreversibility) of the inhibition of OATP2B1 by

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AJ, oocytes expressing OATP2B1 were preincubated in 2% AJ solution in uptake buffer excluding NaCl (pH 6.5) for 60 min at 25 ˚C. The oocytes were washed three times with MBS at 25 ˚C, and then incubated in uptake buffer (pH 6.5) in the absence of AJ for the designated time at 25 ˚C. Then, the uptake was initiated by replacing the outer medium with [^{3}H]estrone-3-sulfate solution in the uptake buffer (pH 6.5) in the absence of AJ for 15 min at 25 ˚C. Uptake was terminated by washing the oocytes three times with ice-cold MBS.

To determine the uptake of [^{3}H]estrone-3-sulfate, the oocytes were solubilized in 50 μL of 5% sodium dodecyl sulfate. One mL of scintillation fluid was added to the sample and after incubation for 6 to 12 hr at room temperature, the radioactivity was determined with a liquid scintillation counter (LSC 5100, Aloka, Tokyo, Japan). To determine the uptake of fexofenadine, the oocytes were solubilized in 400 μL of 70% methanol. After centrifugation at 15,000 rpm for 15 min, 350 μL of resultant supernatant was dried under a reduced pressure and then dissolved in mobile phase to analyze with a liquid chromatography-tandem mass spectrometry (LC/MS/MS) system.

Combined Coincubation and Preincubation Experiments of OATP2B1-expressing *Xenopus laevis* Oocytes with Orange and Apple Juice

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Oocytes expressing OATP2B1 were preincubated in uptake buffer for 10 min at 25 ˚C. All experiments were performed at pH 6.5 even when each FJ was simultaneously applied. For the combined studies of coincubation and preincubation with OJ and AJ, oocytes were preincubated in OJ or AJ solution in uptake buffer excluding NaCl (pH 6.5) for 60 min at 25 ˚C. Then, the oocytes were washed three times with MBS at 25 ˚C, and uptake was initiated by replacing uptake buffer with [^{3}H]estrone-3-sulfate solution in the uptake buffer (pH 6.5) in the presence of OJ and AJ. The oocytes were incubated for 15 min 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 (UFLC) system (Shimadzu Co., Kyoto, Japan). The LC/MS/MS analysis for fexofenadine was performed under the conditions described previously (Imanaga et al., 2011). The UFLC 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 0-1.25 min, 5.0-95% B for 1.25-2.25 min, 95% B for 2.25-4.35 min, 95-5.0% B for 4.35-4.5 min, and 5.0% B for 4.5-5.5 min. The total run time was 5.5 min for each injection. The retention time of fexofenadine was 2.9 min. Mercury MS (C18, 10 x 4.0 mm, Luna 3µm, 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 was used for data manipulation.

Data Analysis

Uptake (nL/oocyte) of estrone-3-sulfate was expressed as the cell-to-medium ratio by dividing the uptake amount by the initial concentration of estrone-3-sulfate in the uptake medium. Uptake rate (nL/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). The inhibitory effect on estrone-3-sulfate transport was expressed as percentage of control,

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and the inhibitor concentration giving half-maximum inhibition (IC_{50}) was obtained by means of the following equation:

$$\%\ \text{of control} = \frac{100 \times IC_{50}}{IC_{50} + [\text{I}]} \quad (1)$$

where [I] is inhibitor concentration (μM).

The affinity of estrone-3-sulfate for OATP2B1 (K_m) and the maximal velocity of OATP2B1-mediated estrone-3-sulfate uptake (V_{max}) were obtained by fitting the data to the following equation:

$$V = \frac{V_{max(1)} \cdot C}{K_{m(1)} + C} + \frac{V_{max(2)} \cdot C}{K_{m(2)} + C} \quad (2)$$

where V and C are uptake rate (fmol/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 (SEM). 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.

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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 min, uptake of estrone-3-sulfate (0.005 μM) by OATP2B1-expressing *Xenopus* 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 IC_{50} 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 IC_{50}, 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 min, uptake of estrone-3-sulfate (0.005 μM) by OATP2B1-expressing *Xenopus* oocytes was determined in the absence of OJ and AJ (Fig. 2). The extent of reduction in

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OATP2B1-mediated uptake of estrone-3-sulfate increased with increasing preincubation time (Fig. 2). Since the effect of preincubation with 20% OJ and 2% AJ on OATP2B1-mediated estrone-3-sulfate uptake was decreased beyond 60 min, the following experiments were performed using preincubation for 60 min.

Long-Lasting Inhibitory Effect of Apple Juice on OATP2B1-Mediated Uptake of Estrone-3-Sulfate

After preincubation with 2% AJ for 60 min, OATP2B1-expressing *Xenopus* oocytes were washed and further incubated for 0-240 min in MBS without AJ. Then, the uptake of estrone-3-sulfate (0.005 μM) was evaluated in the 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 min 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

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preincubated with 2% AJ for 60 min, 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 vs. 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 vs. 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 μL/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 vs. 0.506 ± 0.109 μM) for estrone-3-sulfate uptake mediated by the high-affinity site on OATP2B1 was observed with AJ, while the V_{max} value (0.288 ± 0.067 pmol/min/oocyte vs. 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 noncompetitive 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 coincubation 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 coincubation with OJ is more potent than that of preincubation with OJ. The uptake was also decreased by combined coincubation and preincubation with 20% OJ, but the inhibition was similar to that in the case of coincubation 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 coincubation 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 coincubation. Interestingly, OATP2B1-mediated estrone-3-sulfate uptake was further reduced by combined coincubation and preincubation with 2% AJ (16.2 ± 0.6 %) compared with coincubation or preincubation alone, suggesting an additive inhibitory effect of coincubation 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 coincubation with 20% GFJ, 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 coincubation with GFJ, OJ and AJ. On the other hand, after preincubation with OJ and AJ for 60 min, 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 GFJ.

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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 (IC_{50} = 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 (IC_{50} = 21%), whereas no effect was observed with GFJ. Meanwhile, our previous study revealed that no significant effect of osmolality on OATP2B1-mediated estrone-3-sulfate uptake was observed up to 500 mOsm/L (Shirasaka et al., 2013). Since osmolality of 50% GFJ, OJ and AJ was found to be 346, 351 and 471 mOsm/L, 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

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2% AJ than with 20% OJ, showing that AJ has a more potent long-lasting inhibitory effect on OATP2B1 than does OJ (Fig. 2A 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 IC_{50} values were in the order of GFJ < OJ < 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 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 min, suggesting an irreversible inhibitory effect of AJ on OATP2B1 (Fig. 3). The effect of AJ pre-exposure on intestinal drug absorption *in vivo* should be examined in the future.

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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, while 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 GFJ significantly inhibited OATP2B1-mediated uptake of fexofenadine, consisting 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*, though 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

References

Ameer B, Weintraub RA, Johnson JV, Yost RA, and Rouseff RL (1996) Flavanone absorption after naringin, hesperidin, and citrus administration. *Clin Pharmacol Ther* **60**:34-40.

Amundsen R, Christensen H, Zabihyan B, and Asberg A (2010) Cyclosporine A, but not tacrolimus, shows relevant inhibition of organic anion-transporting protein 1B1-mediated transport of atorvastatin. *Drug Metab Dispos* **38**:1499-1504.

Bailey DG, Spence JD, Edgar B, Bayliff CD, and Arnold JM (1989) Ethanol enhances the hemodynamic effects of felodipine. *Clin Invest Med* **12**:357-362.

Bailey DG, Spence JD, Munoz C, and Arnold JM (1991) Interaction of citrus juices with felodipine and nifedipine. *Lancet* **337**:268-269.

Bailey DG, Dresser GK, Leake BF, and Kim RB (2007) Naringin is a major and selective clinical inhibitor of organic anion-transporting polypeptide 1A2 (OATP1A2) in grapefruit juice. *Clin Pharmacol Ther* **81**:495-502.

Dresser GK, Bailey DG, Leake BF, Schwarz UI, Dawson PA, Freeman DJ, and Kim RB (2002) Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to decrease the oral availability of fexofenadine. *Clin Pharmacol Ther* **71**:11-20.

Fukuda K, Guo L, Ohashi N, Yoshikawa M, and Yamazoe Y (2000) Amounts and variation in grapefruit juice of the main components causing grapefruit-drug interaction. *J Chromatogr B Biomed Sci Appl* **741**:195-203.

Glaeser H, Bailey DG, Dresser GK, Gregor JC, Schwarz UI, McGrath JS, Jolicoeur E, Lee W, Leake BF, Tirona RG, and Kim RB (2007) Intestinal drug transporter expression and the impact of grapefruit juice in humans. *Clin Pharmacol Ther* **81**:362-370

Greenblatt DJ, von Moltke LL, Harmatz JS, Chen G, Weemhoff JL, Jen C, Kelley CJ, LeDuc BW, and Zinny MA (2003) Time course of recovery of cytochrome p450 3A function after single doses of grapefruit juice. *Clin Pharmacol Ther* **74**:121-129.

Guo LQ, Fukuda K, Ohta T, and Yamazoe Y (2000) Role of furanocoumarin derivatives on grapefruit juicemediated inhibition of human CYP3A activity. *Drug Metab Dispos* **28**:766–771.

He K, Iyer KR, Hayes RN, Sinz MW, Woolf TF, and Hollenberg PF (1998) Inactivation of cytochrome P450 3A4 by bergamottin, a component of grapefruit juice. *Chem Res Toxicol* **11**:252-259.

Ho PC, Saville DJ, Coville PF, and Wanwimolruk S (2000) Content of CYP3A4 inhibitors, naringin, naringenin and bergapten in grapefruit and grapefruit juice

products. *Pharm Acta Helv* **74**:379-385.

Imanaga J, Kotegawa T, Imai H, Tsutsumi K, Yoshizato T, Ohyama T, Shirasaka Y, Tamai I, Tateishi T, and Ohashi K (2011) The effects of the *SLCO2B1* c.1457C>T polymorphism and apple juice on the pharmacokinetics of fexofenadine and midazolam in humans. *Pharmacogenet Genomics* **21**:84-93.

Jeon H, Jang IJ, Lee S, Ohashi K, Kotegawa T, Ieiri I, Cho JY, Yoon SH, Shin SG, Yu KS, and Lim KS (2013) Apple juice greatly reduces systemic exposure to atenolol. *Br J Clin Pharmacol* **75**:172-179.

Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, and Tamai I (2003) Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J Pharmacol Exp Ther* **306**:703-708.

Köck K, Koenen A, Giese B, Fraunholz M, May K, Siegmund W, Hammer E, Völker U, Jedlitschky G, Kroemer HK, and Grube M (2010) Rapid modulation of the organic anion transporting polypeptide 2B1 (OATP2B1, SLCO2B1) function by protein kinase C-mediated internalization. *J Biol Chem* **285**:11336-11347.

Lilja JJ, Backman JT, Laitila J, Luurila H, and Neuvonen PJ (2003) Itraconazole increases but grapefruit juice greatly decreases plasma concentrations of celiprolol. *Clin Pharmacol Ther* **73**:192-198.

Lilja JJ, Juntti-Patinen L, and Neuvonen PJ (2004) Orange juice substantially reduces the bioavailability of the beta-adrenergic-blocking agent celiprolol. *Clin Pharmacol Ther* **75**:184-190.

Lilja JJ, Raaska K, and Neuvonen PJ (2005) Effects of orange juice on the pharmacokinetics of atenolol. *Eur J Clin Pharmacol* **61**:337-340.

Nozawa T, Imai K, Nezu J, Tsuji A, and Tamai I (2004) Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. *J Pharmacol Exp Ther* **308**:438-445.

Paine MF, Widmer WW, Hart HL, Pusek SN, Beavers KL, Criss AB, Brown SS, Thomas BF, and Watkins PB (2006) A furanocoumarin-free grapefruit juice establishes furanocoumarins as the mediators of the grapefruit juice-felodipine interaction. *Am J Clin Nutr* **83**:1097-1105.

Paine MF, Widmer WW, Pusek SN, Beavers KL, Criss AB, Snyder J, and Watkins PB (2008) Further characterization of a furanocoumarin-free grapefruit juice on drug disposition: studies with cyclosporine. *Am J Clin Nutr* **87**:863-871.

Reif S, Nicolson MC, Bisset D, Reid M, Kloft C, Jaehde U, and McLeod HL (2002) Effect of grapefruit juice intake on etoposide bioavailability. *Eur J Clin Pharmacol* **58**:491-494.

Schmiedlin-Ren P, Edwards DJ, Fitzsimmons ME, He K, Lown KS, Woster PM, Rahman A, Thummel KE, Fisher JM, Hollenberg PF, and Watkins PB (1997) Mechanisms of enhanced oral availability of CYP3A4 substrates by grapefruit constituents. Decreased enterocyte CYP3A4 concentration and mechanism-based inactivation by furanocoumarins. *Drug Metab Dispos* **25**:1228-1233.

Schwarz UI, Seemann D, Oertel R, Miehlke S, Kuhlisch E, Fromm MF, Kim RB, Bailey DG, and Kirch W (2005) Grapefruit juice ingestion significantly reduces talinolol bioavailability. *Clin Pharmacol Ther* **77**:291-301.

Shirasaka Y, Li Y, Shibue Y, Kuraoka E, Spahn-Langguth H, Kato Y, Langguth P, and Tamai I (2009) Concentration-dependent effect of naringin on intestinal absorption of beta(1)-adrenoceptor antagonist talinolol mediated by p-glycoprotein and organic anion transporting polypeptide (Oatp). *Pharm Res* **26**:560-567.

Shirasaka Y, Kuraoka E, Spahn-Langguth H, Nakanishi T, Langguth P, and Tamai I (2010a) Species difference in the effect of grapefruit juice on intestinal absorption of talinolol between human and rat. *J Pharmacol Exp Ther* **332**:181-189.

Shirasaka Y, Suzuki K, Nakanishi T, and Tamai I (2010b) Intestinal absorption of HMG-CoA reductase inhibitor pravastatin mediated by organic anion transporting polypeptide. *Pharm Res* **27**:2141-2149.

Shirasaka Y, Suzuki K, Shichiri M, Nakanishi T, and Tamai I (2011a) Intestinal absorption of HMG-CoA reductase inhibitor pitavastatin mediated by organic anion transporting polypeptide and P-glycoprotein/multidrug resistance 1. *Drug Metab Pharmacokinet* **26**:171-179.

Shirasaka Y, Suzuki K, Nakanishi T, and Tamai I (2011b) Differential effect of grapefruit juice on intestinal absorption of statins due to inhibition of organic anion transporting polypeptide and/or P-glycoprotein. *J Pharm Sci* **100**:3843-3853.

Shirasaka Y, Mori T, Shichiri M, Nakanishi T, and Tamai I (2012) Functional pleiotropy of organic anion transporting polypeptide OATP2B1 due to multiple binding sites. *Drug Metab Pharmacokinet* **27**:360-364.

Shirasaka Y, Shichiri M, Mori T, Nakanishi T, and Tamai I (2013) Major active components in grapefruit, orange and apple juices responsible for OATP2B1-mediated drug interactions. *J Pharm Sci* **102**:280-288.

Shitara Y, Takeuchi K, Nagamatsu Y, Wada S, Sugiyama Y, Horie T (2012) Long-lasting inhibitory effects of cyclosporin A, but not tacrolimus, on OATP1B1- and OATP1B3-mediated uptake. *Drug Metab Pharmacokinet* **27**:368-378.

Tamai I (2012) Oral drug delivery utilizing intestinal OATP transporters. *Adv Drug Deliv Rev* **64**:508-514.

Tapaninen T, Neuvonen PJ, and Niemi M (2010) Grapefruit juice greatly reduces the plasma concentrations of the OATP2B1 and CYP3A4 substrate aliskiren. *Clin Pharmacol Ther* **88**:339-342.

Tapaninen T, Neuvonen PJ, and Niemi M (2011) Orange and apple juice greatly reduce the plasma concentrations of the OATP2B1 substrate aliskiren. *Br J Clin Pharmacol* **71**:718-726.

Footnotes

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Figure Legends

Figure 1. Effect of preincubation with grapefruit, orange or apple juice on estrone-3-sulfate uptake by OATP2B1-expressing *Xenopus* oocytes. After preincubation with various concentrations of GFJ (filled triangles), OJ (filled squares) or AJ (filled circles) for 60 min, OATP2B1-mediated uptake of estrone-3-sulfate (0.005 μM) was measured in the absence of GFJ, OJ and AJ for 15 min at 25 ˚C and pH 6.5. OATP2B1-mediated uptake of estrone-3-sulfate was determined by subtracting the uptake by water-injected oocytes from that by OATP2B1 cRNA-injected oocytes. Data are shown as the mean ± SEM (n = 8-10).

Figure 2. Time-dependence of effect of preincubation with orange or apple juice on estrone-3-sulfate uptake by OATP2B1-expressing *Xenopus* oocytes. After preincubation with 20% OJ (A) or 2% AJ (B) for 0-90 min, OATP2B1-mediated uptake of estrone-3-sulfate (0.005 μM) was measured in the absence of OJ and AJ for 15 min 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 uptake without OJ and AJ. Data are shown as the mean ± SEM (n = 8-10).

Figure 3. Long-lasting inhibitory effect of apple juice on estrone-3-sulfate uptake by OATP2B1-expressing *Xenopus* oocytes. After preincubation with 2% AJ for 60 min, *Xenopus* oocytes were incubated for 0-240 min in MBS without AJ, and the OATP2B1-mediated uptake of estrone-3-sulfate (0.005 μM) was evaluated in the absence of AJ for 15 min 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 ± SEM (n = 8-10).

Figure 4. Eadie-Hofstee plots of estrone-3-sulfate uptake by OATP2B1-expressing *Xenopus* oocytes. For coincubation analysis,

OATP2B1-mediated uptake of estrone-3-sulfate was measured at various concentrations in the presence of 2% AJ for 15 min at 25 ˚C and pH 6.5 (filled squares). For preincubation analysis, the oocytes were preincubated with 2% AJ for 60 min, and then OATP2B1-mediated uptake of estrone-3-sulfate was measured at various concentrations in the absence of AJ for 15 min (filled triangle). The effect of coincubation and preincubation with AJ on the concentration dependence of OATP2B1-mediated estrone-3-sulfate uptake was evaluated by comparison with the uptake in the absence of 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. Data are presented as means ± SEM (n = 8-10).

Figure 5. Combined effect of coincubation and preincubation with orange or apple juice on estrone-3-sulfate uptake by OATP2B1-expressing *Xenopus* oocytes. For coincubation study, OATP2B1-mediated uptake of estrone-3-sulfate (0.005 μM) was measured in the presence of 20 % OJ (A) or 2% AJ (B) for 15 min at 25 ˚C and pH 6.5. For preincubation study, after preincubation with 20% OJ or 2% AJ for 60 min, OATP2B1-mediated uptake of estrone-3-sulfate was measured in the absence of OJ and AJ for 15 min. For the combined effect study, after preincubation with 20% OJ and

2% AJ for 60 min, OATP2B1-mediated uptake of estrone-3-sulfate (0.005 μM) was measured in the presence of 20% OJ and 2% AJ for 15 min. 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 (nonincubation). Data are shown as the mean ± SEM (n = 8-10).

Figure 6. Effect of coincubation and preincubation with grapefruit, orange or apple juice on fexofenadine uptake by OATP2B1-expressing *Xenopus* oocytes.

For coincubation study (A), OATP2B1-mediated uptake of fexofenadine (500 μM) was measured in the presence of 20 % GFJ, OJ or AJ for 120 min at 25 ˚C and pH 6.5. For preincubation study (B), after preincubation with 20% GFJ, OJ or AJ for 60 min, OATP2B1-mediated uptake of fexofenadine (500 μM) was measured in the absence of GFJ, OJ and AJ for 120 min at 25 ˚C and pH 6.5. OATP2B1-mediated uptake of fexofenadine was determined by subtracting the uptake by water-injected oocytes from that by OATP2B1 cRNA-injected oocytes. Data are shown as the mean ± SEM (n = 8-10).

Table 1. Kinetic Parameters (K_m, V_{max}) of estrone-3-sulfate uptake by OATP2B1-expressing *Xenopus* oocytes.

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

AJ, apple juice. Kinetic parameters were obtained from Fig. 4. 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 2% AJ). Data are shown as means ± SEM (n = 4).

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Figure 1.

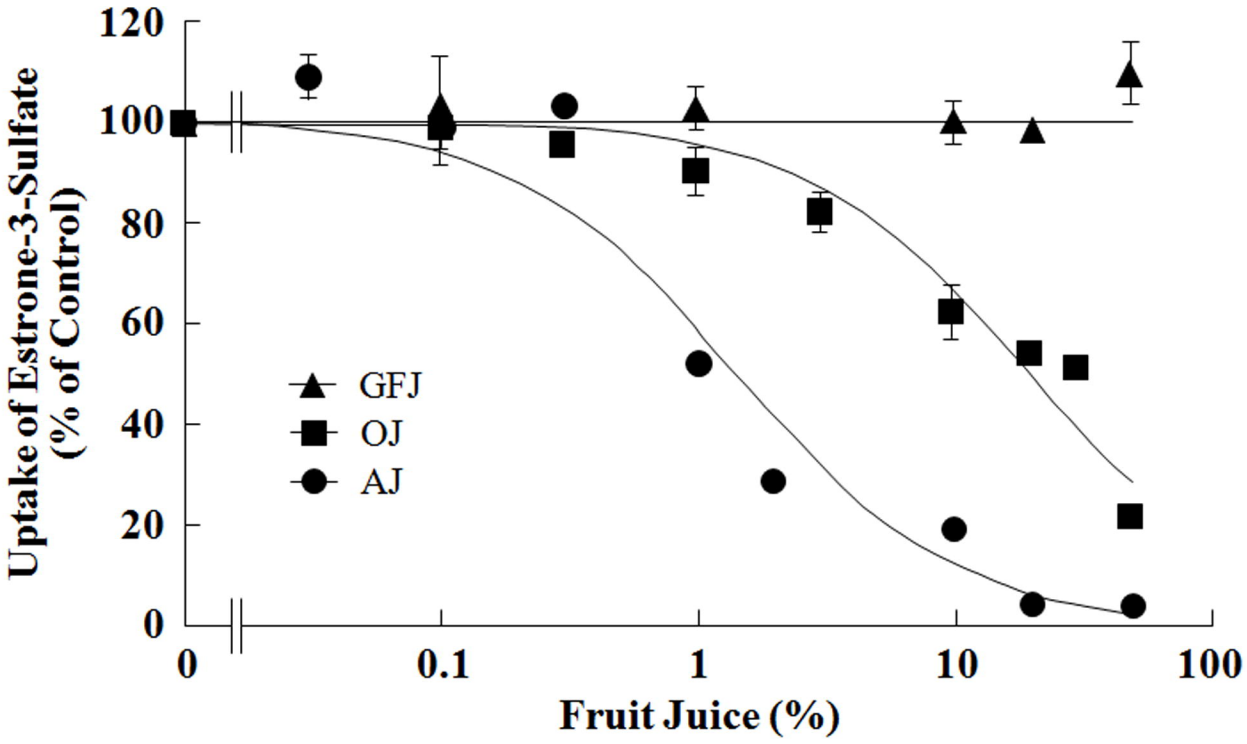

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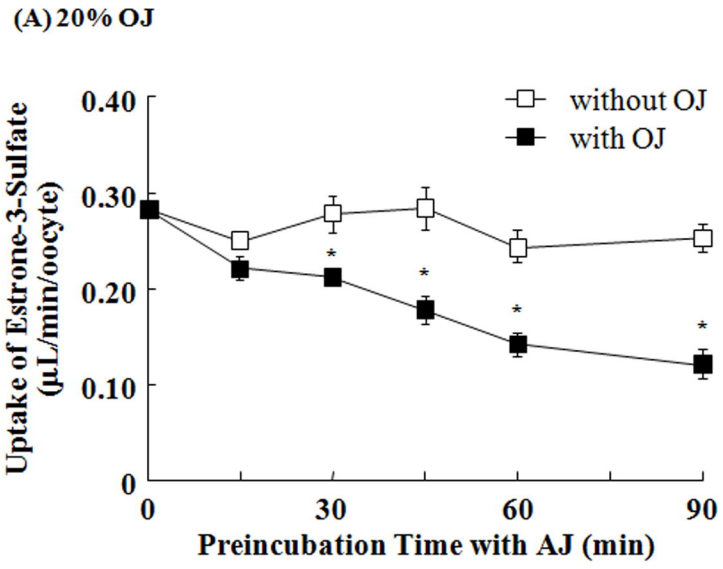
Figure 2.



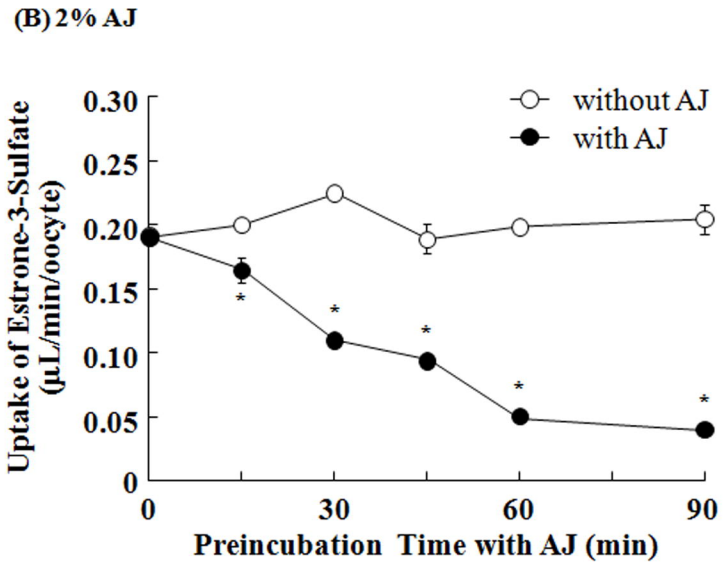


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Figure 3.

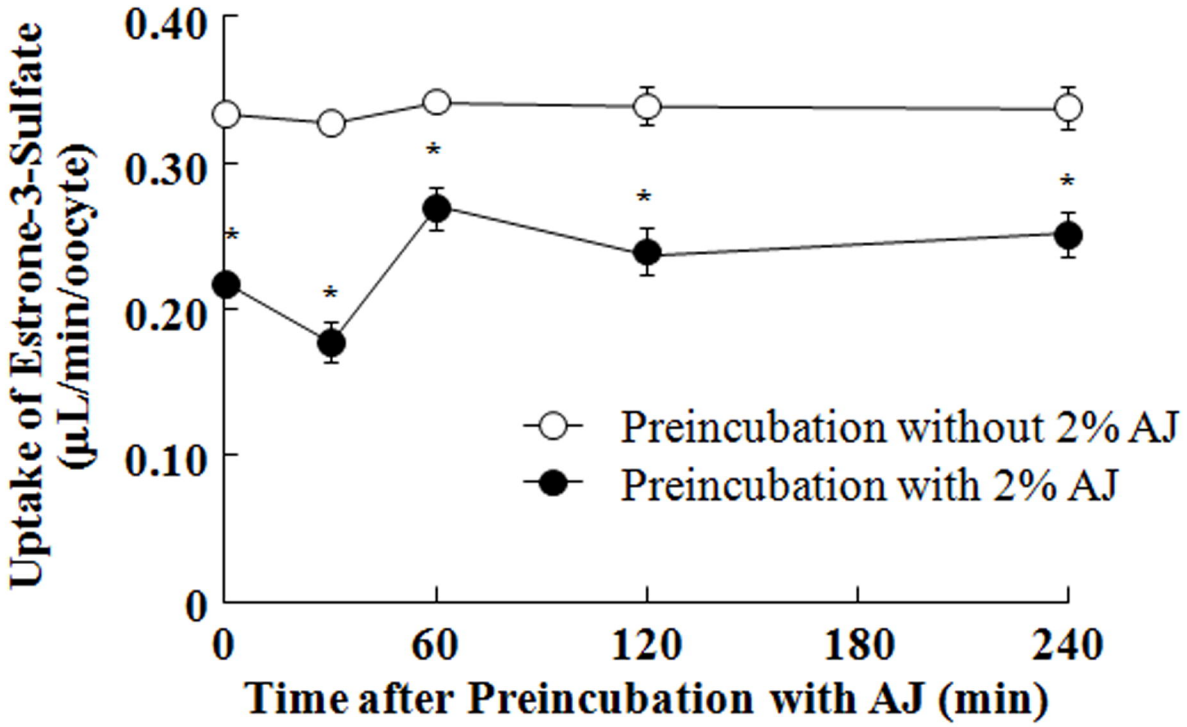

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Figure 4.

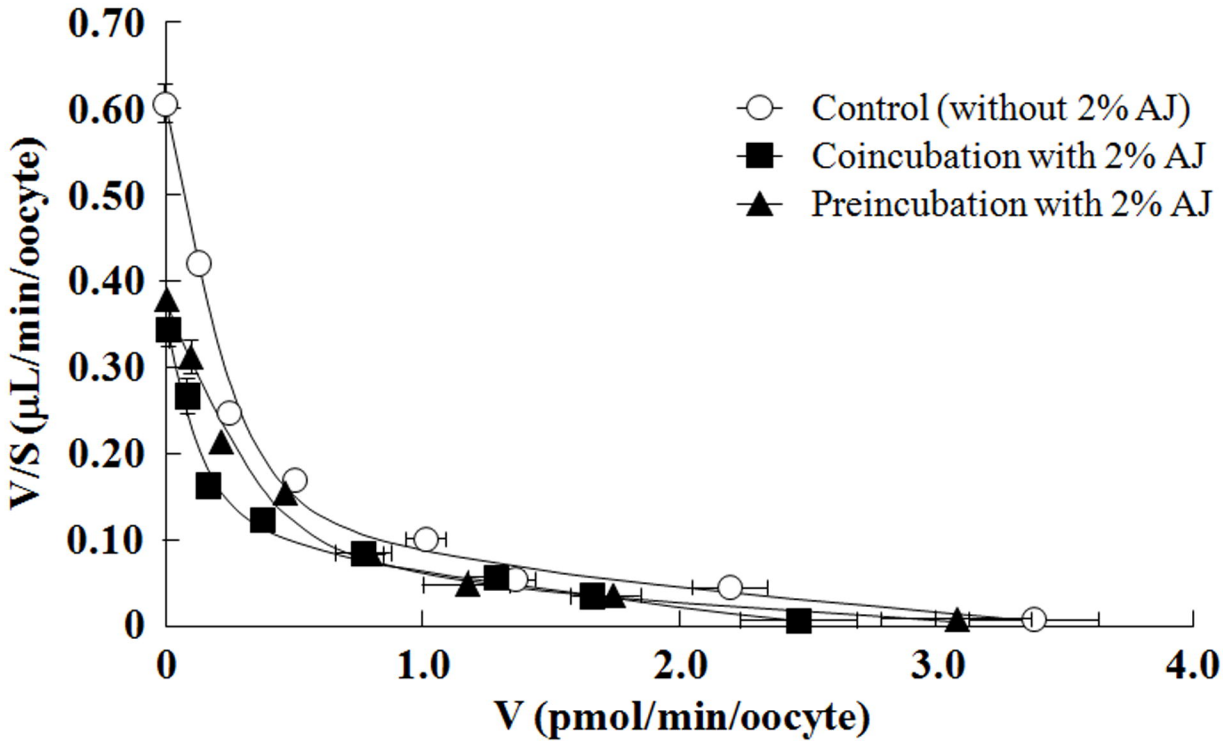

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Figure 5.

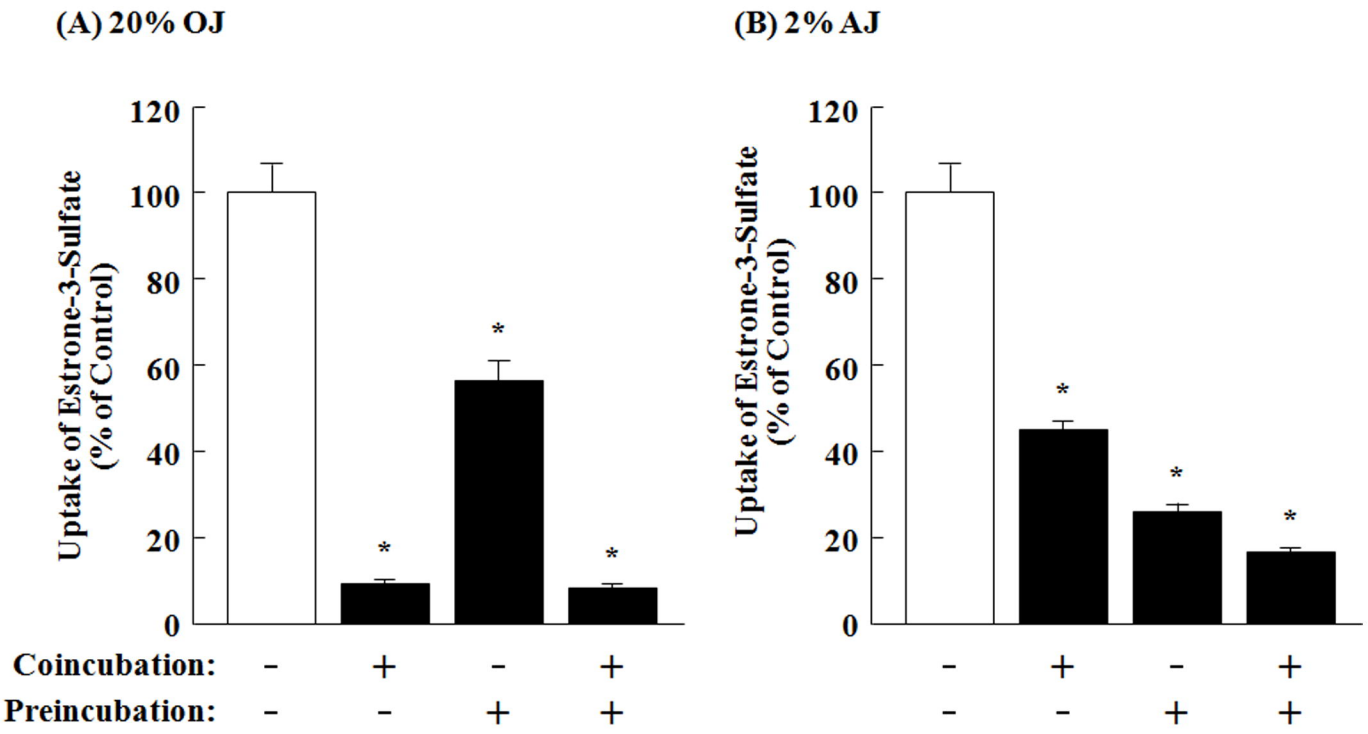

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Figure 6.