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RESEARCH PAPER

Substrate- and Dose-Dependent Drug Interactions with Grapefruit Juice Caused by Multiple Binding Sites on OATP2B1

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ABSTRACT

Purpose OATP2B1-mediated grapefruit juice (GFJ)-drug interactions are substrate-dependent; for example, GFJ ingestion significantly reduces bioavailability of fexofenadine, but not pravastatin. In the present study, we aimed to establish whether this observation can be explained by the presence of multiple binding sites (MBS) on OATP2B1.

Methods OATP2B1-mediated drug uptake was evaluated using a *Xenopus* oocyte expression system. Drug concentration was quantified by LC/MS/MS analysis.

Results OATP2B1-mediated uptake of pravastatin and fexofenadine exhibited biphasic saturation kinetics, indicating the presence of MBS on OATP2B1. GFJ strongly inhibited pravastatin uptake mediated by the high-affinity site on OATP2B1, while no significant inhibition of the low-affinity site was observed. In contrast, high-affinity transport of fexofenadine was only modestly inhibited by GFJ, while significant inhibition of the low-affinity site was observed. Contribution analysis indicated that both drugs are transported via the low-affinity site on OATP2B1 at therapeutically relevant concentrations. These findings indicate that only fexofenadine is expected to interact with GFJ on OATP2B1 at therapeutic concentrations, in accordance with the clinical observations.

Conclusion Substrate- and dose-dependent GFJ-drug interactions mediated by OATP2B1 might be explained in terms of the presence of MBS: interaction occurs only when drug and GFJ components share the same binding site on OATP2B1.

KEY WORDS Drug interaction · Grapefruit juice · Intestinal absorption · Multiple binding sites · OATP2B1

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ABBREVIATIONS

AJ	Apple juice
GFJ	Grapefruit juice
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
MBS	Multiple binding sites
OATP	Organic anion transporting polypeptide
OJ	Orange juice

INTRODUCTION

Organic anion transporting polypeptides (OATP), which belong to the family of membrane solute carrier (SLC) transporters, are expressed in a variety of tissues, including small intestine, liver, brain, and placenta, and are involved in the cellular uptake of a wide range of endogenous compounds and many therapeutic drugs (1). In the intestine, OATP1A2 and OATP2B1 contribute to the absorption of clinically used drugs (2–9). In particular, OATP2B1, which is relatively highly expressed in the apical membrane of enterocytes, is likely to play a significant role in intestinal absorption of various drugs, including fexofenadine, talinolol, celiprolol, pravastatin, pitavastatin, rosuvastatin, sulfasalazine and motelukast (3,10–16). We have demonstrated that intestinal absorption of fexofenadine is reduced in individuals with the OATP2B1 c.1457C>T allele, suggesting a contribution of OATP2B1 to the intestinal absorption of fexofenadine (12).

In addition, OATP2B1 is believed to be a site of drug interactions with grapefruit juice (GFJ). Inhibition of intestinal OATP2B1 by constituents of GFJ may lead to decreased systemic availability of its substrates, with consequent potential for reduced drug efficacy. As a result of extensive work on GFJ-drug interactions mediated by OATP2B1, it has been established that the bioavailability of a number of OATP

substrates (e.g., fexofenadine, celiprolol, talinolol, and aliskiren) is reduced by GFJ ingestion (17–20). A reduction in the bioavailability of OATP2B1 substrates (e.g., fexofenadine, talinolol, celiprolol, atenolol, ciprofloxacin and aliskiren) has also been observed after orange juice (OJ) and/or apple juice (AJ) ingestion (12,17,21–25). We recently revealed that naringin and hesperidin are the major active components responsible for OATP2B1-mediated drug interactions in GFJ and OJ, respectively (26). In addition, AJ was considered to inhibit OATP2B1 due to the combined effect of multiple flavonoids in AJ and/or a long-lasting inhibitory effect of AJ on OATP2B1 (26,27). Therefore, it is considered that OATP2B1 substrates have the potential to interact with GFJ, OJ and AJ in the intestinal absorption process.

On the other hand, OATP2B1-mediated drug interactions with these fruit juices are reportedly substrate-dependent. For example, GFJ ingestion clinically reduces the intestinal absorption of fexofenadine, talinolol, and celiprolol, but not digoxin, pravastatin or glibenclamide, although they are all OATP2B1 substrate drugs (28). In addition, OATP2B1-mediated transport of pitavastatin, estrone-3-sulfate and glibenclamide, but not talinolol or pravastatin, was inhibited by naringin (7,15,29). We hypothesized that such substrate-dependent GFJ-drug interaction could be explained by the presence of multiple binding sites (MBS) on OATP2B1. In a recent study, we demonstrated the presence of two binding sites on OATP2B1 for estrone-3-sulfate transport, with different affinities and pH-sensitivity (30). Naringin significantly inhibited estrone-3-sulfate uptake mediated by the high-affinity site, but not that mediated by the low-affinity site on OATP2B1, indicating that naringin could be specific inhibitor of the high-affinity site (30). Therefore, GFJ-drug interactions may be observed only when a drug has affinity for the binding site that is inhibited by GFJ and/or naringin.

In the present study, in order to verify the MBS hypothesis, we assessed GFJ-drug interaction mediated by OATP2B1, using OATP2B1-expressing *Xenopus* oocytes with two model drugs: fexofenadine, which clinically interacts with GFJ, and pravastatin, which does not. Our results indicated that both drugs are mainly transported via the low-affinity site on OATP2B1 at therapeutic concentrations. However, GFJ strongly inhibited pravastatin uptake mediated by the high-affinity site but not the low-affinity site, while the opposite was the case for fexofenadine. Thus, only fexofenadine is likely to interact with GFJ on OATP2B1 at therapeutic concentrations. Our findings support the idea that substrate- and dose-dependency in OATP2B1-mediated GFJ-drug interactions can be at least partly explained by the presence of MBS on OATP2B1 with different affinities for substrate drugs.

MATERIALS AND METHODS

Materials

Fexofenadine hydrochloride was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Pravastatin sodium was kindly provided by Kobayashi Kako Co., Ltd. (Fukui, Japan). Naringin was purchased from Chromadex (Irvine, CA, USA). Grapefruit, orange and apple juices (Tropicana™; 100% pure at normal strength) were purchased from a supermarket in Kanazawa city (Kanazawa, Japan). Gentamicin was purchased from Sigma-Aldrich Co. (St. Louis, MO). All other compounds and general reagents were obtained from Sigma-Aldrich Co., Gibco Laboratories (Lenexa, KS), Invitrogen (Carlsbad, CA), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Nacalai Tesque, Inc. (Kyoto, Japan) or Applied Biosystems (Foster City, CA).

Uptake Experiments in *Xenopus laevis* Oocytes

Preparation of oocytes, *in vitro* synthesis of OATP2B1 cRNA, and uptake experiments were conducted as described previously (4,10). In brief, the construct pGEMHE containing OATP2B1 cDNA was used to synthesize cRNA. For standard experiments, defolliculated oocytes were injected with 50 nL of the cRNA solution (0.5 μg/μL) or water, and then incubated for 3 days at 18°C in modified Barth's solution (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.

For the uptake studies, oocytes expressing OATP2B1 were transferred to a 24-well culture plate and pre-incubated 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 initiate uptake, the uptake buffer was completely replaced with fexofenadine or pravastatin solution (uptake buffer, pH 6.5) and the oocytes were incubated for the designated time at 25°C. The uptake was terminated by washing the oocytes three times with ice-cold modified Barth's solution.

For the inhibition studies, oocytes expressing OATP2B1 were transferred to a 24-well culture plate and pre-incubated in uptake buffer for 10 min at 25°C. To initiate uptake, the uptake buffer was completely replaced with fexofenadine or pravastatin solution in the absence or presence of GFJ, OJ, AJ or naringin (pH 6.5) and the oocytes were incubated for the designated time at 25°C. The GFJ, OJ and AJ solution was composed of uptake buffer excluding NaCl (pH 6.5). The uptake was terminated by washing the oocytes three times with ice-cold modified Barth's solution.

To determine uptake of fexofenadine and pravastatin, the oocytes were disrupted by sonication in 70% methanol

solution. The concentrations of fexofenadine and pravastatin in all samples were quantified with a liquid chromatography-tandem mass spectrometry (LC/MS/MS) system.

Data Analysis

Uptake (nL/oocyte) was calculated as the cell-to-medium ratio by dividing the uptake amount by the initial concentration of the substrate drug in the uptake medium. Uptake rate (nL/min/oocyte) was expressed as uptake of the substrate drug 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.

Kinetic parameters were estimated by means of nonlinear least-squares analysis using the MULTI program (31). The affinity of the substrate drug 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 (Eq. (1)):

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

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.

The inhibitory effect of GFJ, OJ, AJ or naringin on the substrate drug uptake was expressed as percent of control, and the inhibitor concentration giving half-maximum inhibition (IC_{50}) was obtained from the following equation (Eq. (2)):

$$\%\,\text{of control} = \frac{100 \times IC_{50}}{IC_{50} + [I]} \quad (2)$$

where [I] is inhibitor concentration (μM).

LC/MS/MS Analysis

The concentrations of fexofenadine and pravastatin 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-20 AD ultra-fast liquid chromatography (UFLC) system (Shimadzu Co., Kyoto, Japan). LC/MS/MS analysis for fexofenadine and pravastatin was performed under the conditions described previously (10,12,15). Mercury MS (C18, 10× 4.0 mm, Luna 3 μm, Phenomenex, Torrance, CA) was used as the analytical column for both fexofenadine and pravastatin. The gradient elution mobile phase consisted of 0.1% formic acid and acetonitrile for fexofenadine and ammonium acetate (12 mM; pH 4.5) and methanol for pravastatin. The mass transitions (Q1/Q3) of m/z 502.3/466.3 and 442.2/209.0 were used for fexofenadine and pravastatin, respectively. Analyst software version 1.4 was used for data manipulation.

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 be statistically significant.

RESULTS

Uptake of Fexofenadine and Pravastatin by *Xenopus* Oocytes Expressing OATP2B1

To determine whether fexofenadine and pravastatin are transported by OATP2B1, uptake of fexofenadine (1 μM) and pravastatin (10 μM) was measured using *Xenopus* oocytes expressing OATP2B1 (Fig. 1). The uptake of fexofenadine and pravastatin by OATP2B1 cRNA-injected oocytes was

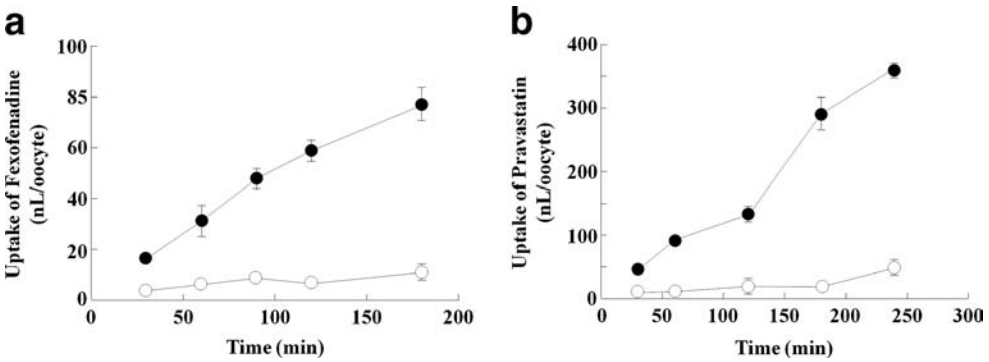

Fig. 1 Time courses of fexofenadine and pravastatin uptake by *Xenopus* oocytes expressing OATP2B1. (**a**) Uptake of fexofenadine (1 μM) by water-injected oocytes (*open circles*) or OATP2B1 cRNA-injected oocytes (*filled circles*) was measured at 25°C and pH 6.5. (**b**) Uptake of pravastatin (10 μM) by water-injected oocytes (*open circles*) or OATP2B1 cRNA-injected oocytes (*filled circles*) was measured at 25°C and pH 6.5. Data are shown as the mean ± SEM ($n=8–10$).

significantly higher than that by water-injected oocytes, suggesting that both fexofenadine and pravastatin are substrates of OATP2B1. The OATP2B1-mediated uptake of fexofenadine and pravastatin increased linearly up to at least 180 and 240 min, respectively. Therefore, in the subsequent studies, uptake was routinely measured at 120 and 180 min for fexofenadine and pravastatin, respectively, to evaluate the uptake rates.

Kinetic Analysis of OATP2B1-Mediated Uptake of Fexofenadine and Pravastatin

To kinetically analyze OATP2B1-mediated transport of fexofenadine and pravastatin, we examined the concentration-dependence of the uptake of fexofenadine and pravastatin using oocytes expressing OATP2B1. The OATP2B1-mediated uptake of both fexofenadine and pravastatin was saturable, and Eadie-Hofstee plot analysis showed multiphasic kinetics (Figs. 2 and 3). When the concentration dependence of OATP2B1-mediated fexofenadine uptake shown in Fig. 2a and b was kinetically analyzed with multiple affinity models, the data were best fitted to the two-affinity model based on the basis of the Akaike Information Criterion (−18.7, -36.5 and −32.2 for the one, two and three affinity models, respectively) (32). The kinetic parameters at the high- and low-affinity sites for OATP2B1-mediated fexofenadine uptake were evaluated to be 0.140±0.062 μM and 885±624 μM (K_m values) and 0.183±0.060 fmol/min/oocyte and 288±162 fmol/min/oocyte (V_{max} values), respectively (Table I). Based on these kinetic parameters, the contributions of the high- and low-affinity sites to total fexofenadine transport were estimated in the fexofenadine concentration range of 0.01 through 1000 μM (Fig. 2c).

Further, when the concentration dependence of OATP2B1-mediated pravastatin uptake shown in Fig. 3a and b was kinetically analyzed with multiple affinity models, the data were again best fitted to the two-affinity model on the basis of the Akaike Information Criterion (−6.64, -35.9 and −31.8 for the one, two and three affinity models, respectively) (32). The kinetic parameters at the high- and low-affinity sites for OATP2B1-mediated pravastatin uptake were evaluated as 6.25±1.43 μM and 225±34 μM (K_m values) and 10.5±2.3 fmol/min/oocyte and 142±9 fmol/min/oocyte (V_{max} values), respectively (Table I). Based on these kinetic parameters, the contributions of the high- and low-affinity sites to total pravastatin transport were estimated in the pravastatin concentration range of 0.01 through 1000 μM (Fig. 3c).

Inhibitory Effects of Grapefruit Juice and Naringin on OATP2B1-Mediated Uptake of Fexofenadine and Pravastatin

The inhibitory effects of GFJ (20%) and naringin (1000 μM) on OATP2B1-mediated uptake of fexofenadine and

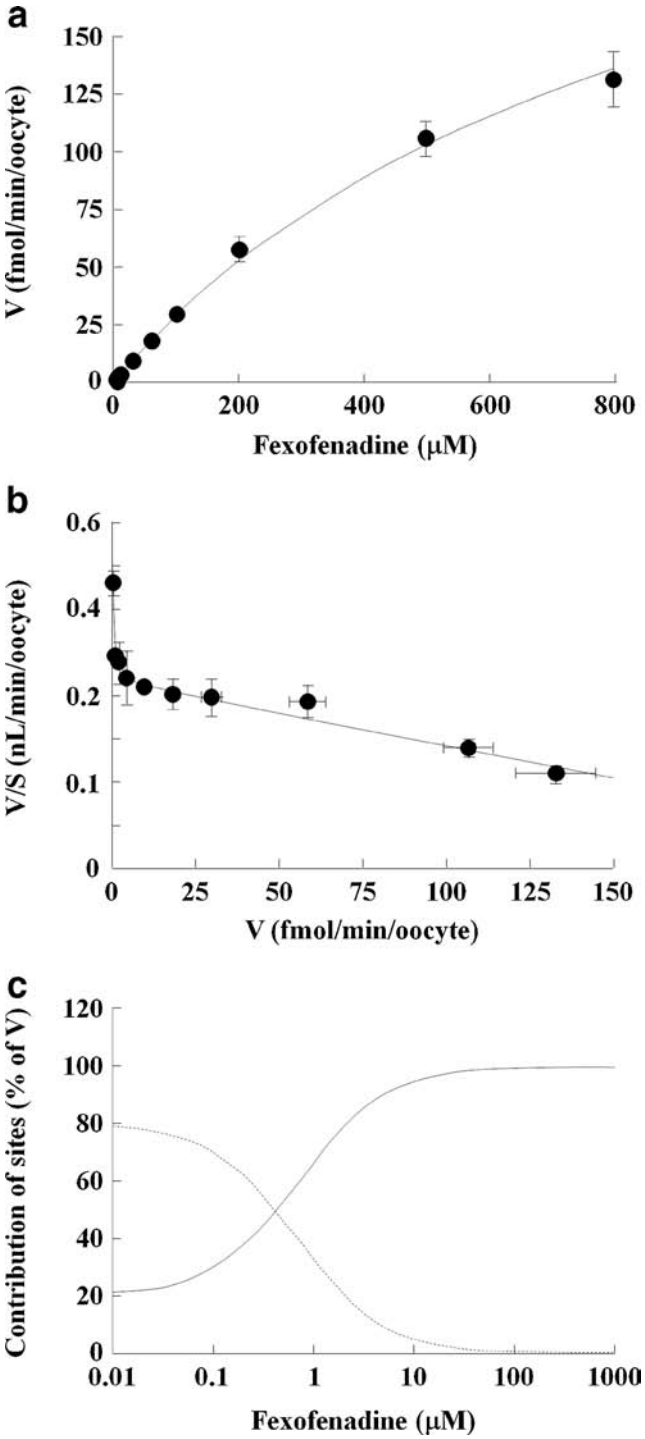

Fig. 2 Concentration dependence of OATP2B1-mediated transport of fexofenadine. (**a**) Michaelis-Menten and (**b**) Eadie-Hofstee plots of the concentration dependence of fexofenadine uptake by *Xenopus* oocytes expressing OATP2B1. Uptake of fexofenadine by water-injected or OATP2B1 cRNA-injected oocytes was measured for 120 min at 25°C and pH 6.5, and OATP2B1-mediated uptake 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). (**c**) Contributions of high-affinity (*dashed line*) and low-affinity sites (*solid line*) to OATP2B1-mediated transport of fexofenadine were simulated using the kinetic parameters. The contributions of the high- and low-affinity sites to OATP2B1-mediated transport of fexofenadine are presented as percent of total velocity ($V = V_{high} + V_{low}$).

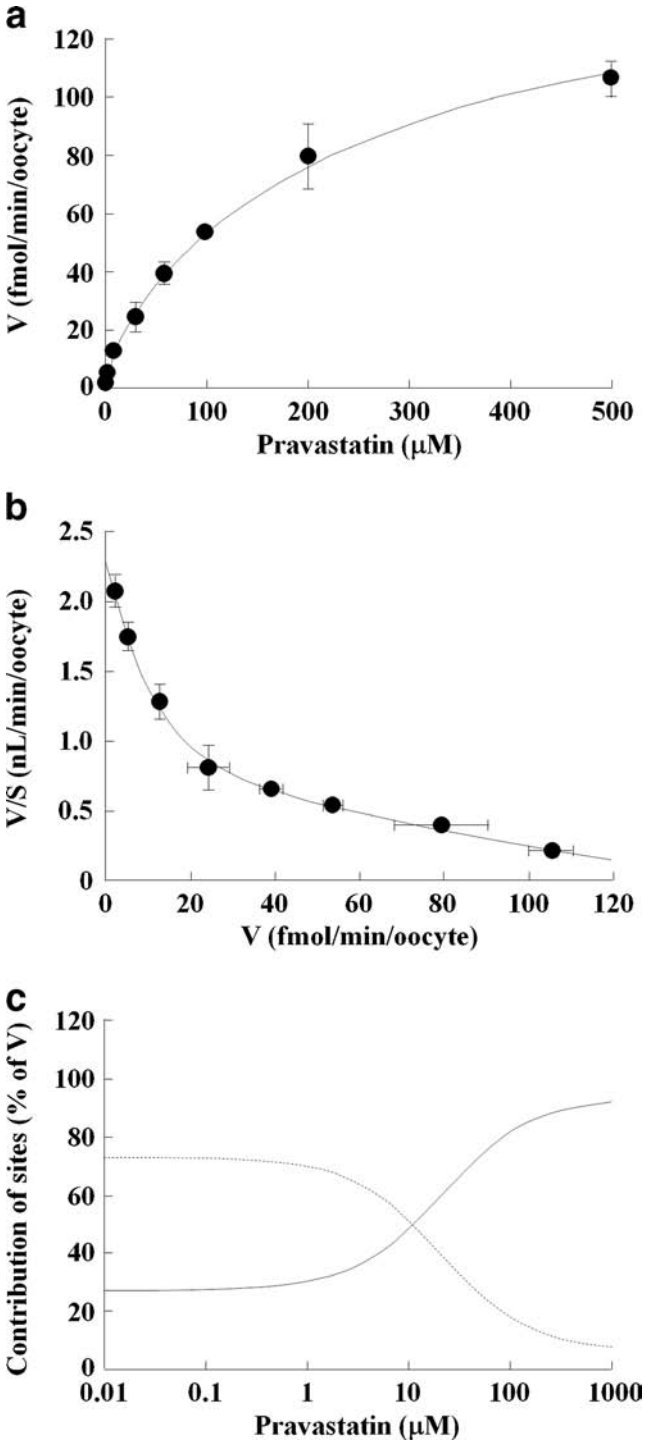

Fig. 3 Concentration dependence of OATP2B1-mediated transport of pravastatin. (**a**) Michaelis-Menten and (**b**) Eadie-Hofstee plots of the concentration dependence of pravastatin uptake by *Xenopus* oocytes expressing OATP2B1. Uptake of pravastatin by water-injected or OATP2B1 cRNA-injected oocytes was measured for 180 min at 25°C and pH 6.5, and OATP2B1-mediated uptake 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). (**c**) Contributions of high-affinity (*dashed line*) and low-affinity sites (*solid line*) to OATP2B1-mediated transport of pravastatin was simulated using the kinetic parameters. The contributions of the high- and low-affinity sites to OATP2B1-mediated transport of fexofenadine and pravastatin are presented as percent of total velocity ($V=V_{high}+V_{low}$).

Table I Kinetic Parameters (K_m and V_{max}) for Transport of Fexofenadine and Pravastatin Mediated by High- and low-Affinity Sites on OATP2B1

Substrates	Affinity site	K_m (μM)	V_{max} (fmol/min/oocyte)	V_{max}/K_m (nL/min/oocyte)
Fexofenadine	High	0.140 ± 0.062	0.183 ± 0.060	1.31
	Low	885 ± 624	288 ± 162	0.325
Pravastatin	High	6.25 ± 1.43	10.5 ± 2.3	1.68
	Low	225 ± 34	142 ± 9	0.631

Uptake of fexofenadine and pravastatin by *Xenopus* oocytes expressing OATP2B1 was measured for 120 and 180 min, respectively, at 25°C and pH 6.5. OATP2B1-mediated uptake was determined by subtracting the uptake by water-injected oocytes from that by OATP2B1 cRNA-injected oocytes. Data are represented as means ± SEM ($n=3$)

pravastatin at two different concentrations (1 μM and 500 μM) were examined using *Xenopus* oocytes expressing OATP2B1. As shown in Fig. 4a and b, GFJ slightly inhibited the OATP2B1-mediated uptake of fexofenadine at the concentration of 1 μM, but strongly inhibited the uptake at 500 μM. Naringin did not affect the OATP2B1-mediated uptake of fexofenadine at either 1 μM or 500 μM (Fig. 4a and b). On the other hand, GFJ strongly inhibited the OATP2B1-mediated uptake of pravastatin at the concentration of 1 μM, while no significant inhibition of the uptake was observed at 500 μM (Fig. 5a and b). Similarly, naringin significantly inhibited the OATP2B1-mediated uptake of pravastatin at the concentration of 1 μM, but not 500 μM (Fig. 5a and b).

Inhibitory Effects of Grapefruit, Orange and Apple Juices on OATP2B1-Mediated Uptake of Fexofenadine

The inhibitory effects of GFJ (20%), OJ (20%) and AJ (20%) on OATP2B1-mediated uptake of fexofenadine at two different concentrations (1 μM and 500 μM) were examined using *Xenopus* oocytes expressing OATP2B1. As shown in Fig. 6a, OJ and AJ strongly inhibited the OATP2B1-mediated uptake of fexofenadine at the concentration of 1 μM, while only slight inhibition of the uptake was observed in the presence of GFJ. On the other hand, all three juices significantly inhibited the OATP2B1-mediated uptake of fexofenadine at the concentration of 500 μM (Fig. 6b).

DISCUSSION

GFJ clinically interacts with intestinal absorption of fexofenadine, talinolol, celiprolol and aliskiren, but not pravastatin, digoxin, etoposide or glibenclamide, although they are also OATP substrate drugs (20,28). In the present study, we aimed to examine the idea that MBS on OATP2B1 can

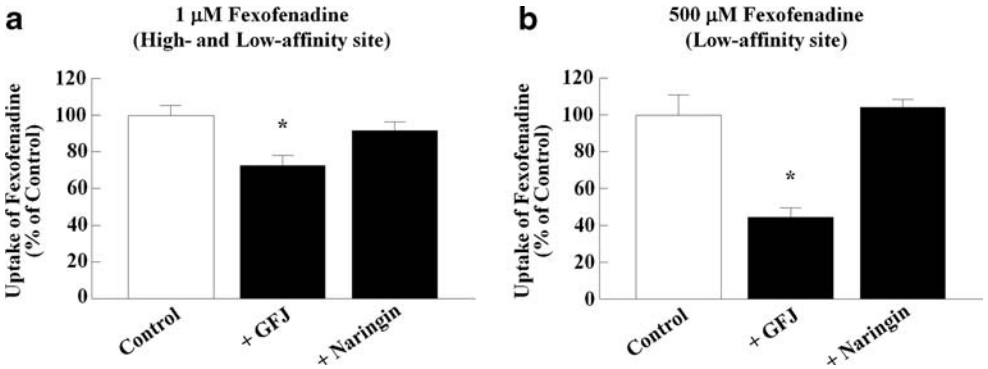

Fig. 4 Inhibitory effects of grapefruit juice and naringin on fexofenadine uptake by *Xenopus* oocytes expressing OATP2B1. OATP2B1-mediated uptake of fexofenadine at the concentrations of 1 μM (**a**) and 500 μM (**b**) was measured in the absence or presence of GFJ (20%) or naringin (1000 μM) for 120 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. The inhibitory effects of GFJ or naringin on OATP2B1-mediated uptake of fexofenadine are presented as percent of control. *$P < 0.05$, significantly different from control (without GFJ and naringin). Data are shown as the mean ± SEM ($n = 8–10$).

account for the substrate- and dose-dependent GFJ-drug interaction in the intestinal absorption process, using fexofenadine and pravastatin as model drugs. OATP2B1-mediated uptake of both fexofenadine and pravastatin exhibited apparently biphasic saturation kinetics (Figs. 2 and 3). As shown in Figs. 2c and 3c, the contribution of the low-affinity site to total transport of both fexofenadine and pravastatin was estimated to be over 90% when the substrate concentration was 500 μM. Therefore, a reduction in OATP2B1-mediated uptake of 500 μM fexofenadine by GFJ may be due to inhibition at the low-affinity site on OATP2B1 (Fig. 4b). On the other hand, it is considered that the absence of a significant effect of GFJ on the uptake of 500 μM pravastatin is due to lack of inhibition at the low-affinity site on OATP2B1 in this case (Fig. 5b). In the reported clinical studies, the gastrointestinal concentrations of fexofenadine and pravastatin after oral administration can be roughly estimated as 750 μM (120 mg fexofenadine in a GFJ volume of 300 mL) and 450 μM (40 mg pravastatin in a GFJ volume of 200 mL), respectively (17,33). Accordingly, it is possible that both fexofenadine and pravastatin after oral administration would have been absorbed mainly via the low-affinity site on OATP2B1, implying that GFJ would interact with OATP2B1-mediated absorption of fexofenadine, but not pravastatin. These speculations are consistent with the clinical observation of a decrease of plasma concentration of fexofenadine, but not pravastatin, upon coadministration with GFJ in humans (17,33).

In Fig. 5b, no significant effect of GFJ on OATP2B1-mediaetd transport of 500 μM pravastatin may result from the attenuation of the inhibitory effect of GFJ on the low-affinity transport of pravastatin due to saturation of the low-affinity site. However, the pravastatin concentration is not sufficiently higher than the K_m value, suggesting that saturation of low-affinity site on OATP2B1 is incomplete (Table I). Therefore, our result can account at least partly for no effect of GFJ on the low-affinity transport of pravastatin. Meanwhile, if GFJ interacts with OATP2B1-mediated pravastatin transport via a noncompetitive inhibition mechanism, the V_{max} value of pravastatin transport must be reduced by GFJ irrespective of saturation status of OATP2B1. In this case, our result shown in Fig. 5b can indicate that GFJ does not affect the low-affinity transport of pravastatin, even if the low-affinity site on OATP2B1 was saturated. To

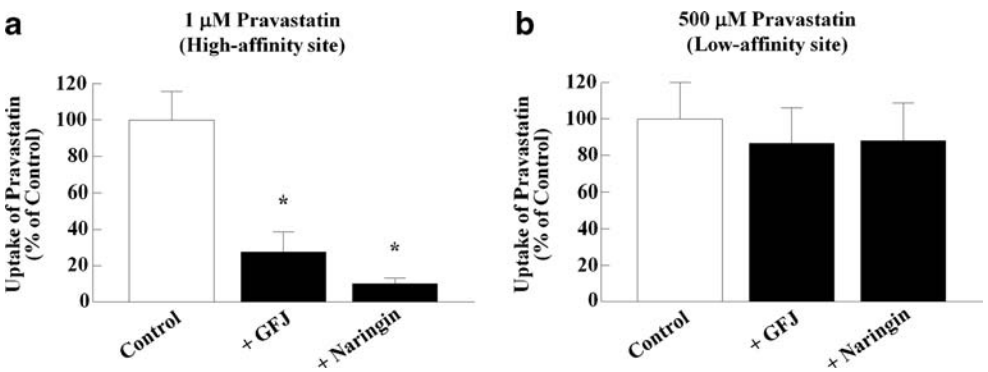

Fig. 5 Inhibitory effects of grapefruit juice and naringin on pravastatin uptake by *Xenopus* oocytes expressing OATP2B1. OATP2B1-mediated uptake of pravastatin at the concentrations of 1 μM (**a**) and 500 μM (**b**) was measured in the absence or presence of GFJ (20%) or naringin (1000 μM) for 180 min at 25°C and pH 6.5. OATP2B1-mediated uptake of pravastatin was determined by subtracting the uptake by water-injected oocytes from that by OATP2B1 cRNA-injected oocytes. The inhibitory effects of GFJ or naringin on OATP2B1-mediated uptake of pravastatin are presented as percent of control. *$P < 0.05$, significantly different from control (without GFJ and naringin). Data are shown as the mean ± SEM ($n = 8–10$).

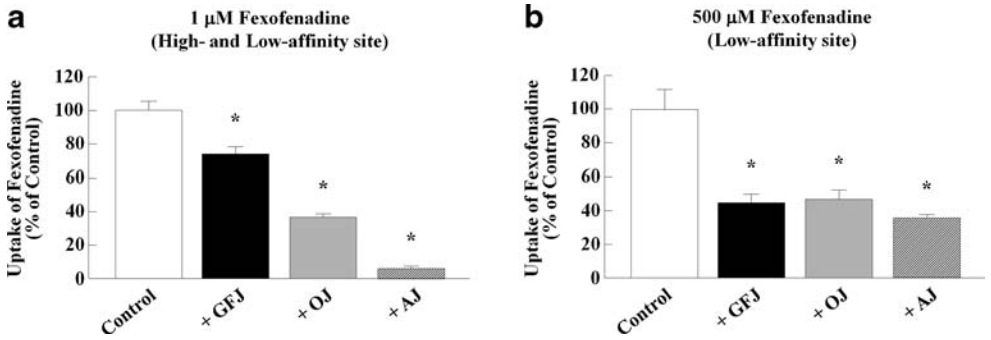

Fig. 6 Inhibitory effects of grapefruit, orange and apple juices on fexofenadine uptake by *Xenopus* oocytes expressing OATP2B1. OATP2B1-mediated uptake of fexofenadine at the concentrations of 1 μM (**a**) and 500 μM (**b**) was measured in the absence or presence of GFJ (20%), OJ (20%) and AJ (20%) juices 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. The inhibitory effects of GFJ, OJ and AJ on OATP2B1-mediated uptake of fexofenadine are presented as percent of control. *$P < 0.05$, significantly different from control (without all fruit juices). Data are shown as the mean ± SEM ($n = 8–10$).

clarify the precise mechanism of the inhibitory effect of GFJ on low-affinity transport of pravastatin via OATP2B1, further studies would be needed. On the other hand, when the substrate concentration was 1 μM, GFJ strongly inhibited OATP2B1-mediated pravastatin uptake, indicating that GFJ has the potential to inhibit high-affinity transport of pravastatin (Fig. 5a). Taken together, these results indicate that OATP2B1-mediated GFJ-pravastatin interaction should be observed when the concentration is sufficiently low that the contribution of the high-affinity site is predominant.

As shown in Fig. 4a, GFJ showed a small but statistically significant inhibition of OATP2B1-mediated uptake of 1 μM fexofenadine. Therefore, it might seem that GFJ can inhibit high-affinity transport of fexofenadine. However, this might be explained by the contribution of low-affinity site, because based on the kinetic parameters, the low-affinity transport still contributes 60% to the total uptake at 1 μM (Figs 2c and 4a). Therefore, GFJ may not affect high-affinity transport of fexofenadine. To examine GFJ-fexofenadine interaction specifically at the high-affinity site on OATP2B1, a substrate concentration of less than 0.1 μM would have to be used. However, the detection limit of fexofenadine in our LC/MS/MS analysis does not permit uptake study at concentrations below 1 μM.

Interestingly, a reduction in the bioavailability of fexofenadine has also been observed after OJ and AJ ingestion in humans (17). In Fig. 6b, OJ and AJ as well as GFJ significantly inhibited OATP2B1-mediated uptake of 500 μM fexofenadine. These findings suggest that the low-affinity site on OATP2B1 is the site of fexofenadine interactions with fruit juices, including GFJ, OJ and AJ. However, as shown in Fig. 6a, OJ and AJ also inhibited the OATP2B1-mediated uptake of 1 μM fexofenadine, while GFJ had a statistically significant but minimal effect on the fexofenadine uptake. Therefore, it is likely that OJ and AJ at least inhibit fexofenadine transport mediated by not only the low-affinity site, but also the high-affinity site on OATP2B1. Recently, we demonstrated that naringin is one of the major OATP2B1 inhibitors in GFJ (26,27). Indeed, naringin significantly inhibited the OATP2B1-mediated uptake of pravastatin at the concentration of 1 μM, but not 500 μM, in accordance with the observations in the presence of GFJ (Fig. 5a and b). However, naringin did not affect OATP2B1-mediated uptake of either 1 μM or 500 μM fexofenadine, whereas significant inhibition in the uptake of 500 μM fexofenadine was observed in the presence of GFJ (Fig. 4a and b). This apparent discrepancy may be explained by inhibitory activity of other GFJ components on OATP2B1-mediated fexofenadine uptake. In our recent study, we concluded that hesperidin as well as naringin contributes to the inhibition of OATP2B1 by GFJ (26). These results suggested that the presence of MBS should be taken into the consideration in seeking to identify the major causal components of OATP2B1-mediated drug interactions with fruit juices.

In addition to transporters, many integral membrane proteins, such as receptors, channels and ATPases, function at the plasma membrane where they interact with their ligands and/or substrates. A receptor is a protein molecule to which a substance (ligand) can bind, resulting in a change in the activity of the cell. The ligand-receptor binding process is theoretically the kinetics of association and dissociation constants for a ligand-receptor interaction based on 3-dimensional structural similarity between ligand and binding site. Meanwhile, drug transport can be considered as a 4-dimensional phenomenon including time (velocity) but not 3-dimensional phenomenon such as a ligand-receptor interaction. Therefore, it is no wonder that the OATP2B1-mediated transport processes of both fexofenadine and pravastatin exhibit multiple pathways with different velocities. It may be a distinct feature for transporters including OATP2B1 to possess MBS with different velocities as well as different affinities for the same substrate.

In conclusion, the present study demonstrated that fruit juices can interfere with OATP2B1-mediated drug absorption, and apparent discrepancies among drugs may be at least

partly explained by the presence of MBS on OATP2B1 with different affinities for substrate drugs, resulting in substrate- and dose-dependent GFJ-drug interactions. Although we did not obtain direct evidence of the involvement of MBS in OATP2B1-mediated drug absorption in this *in vitro* study, it seems likely that drug-drug and beverage-drug interactions occur only when GFJ (or its components) and drugs share the same binding site on OATP2B1. Thus, it is important to identify precisely the interaction sites of GFJ components and substrates at therapeutic concentrations, in order to predict drug-drug and beverage-drug interactions mediated by OATP2B1.

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