ABSTRACT:

Human organic anion-transporting polypeptide B (OATP-B; OATP2B1) is expressed in the human intestinal epithelial cells, and is suggested to be involved in the intestinal absorption of anionic drugs in vivo. Although citrus juices have been shown to inhibit the function of human OATP-A (OATP1A2), the effect of citrus juices on the OATP-B function remains unclear. In this study, we aimed to examine the effects of citrus juices on the function of OATP-B. The effects of citrus juices on the uptake of estrone-3-sulfate, a typical substrate for OATP-B, into human embryonic kidney 293 cells stably expressing OATP-B were evaluated. Juices were diluted with uptake buffer, adjusted to pH 7.4 and approximately 300 mOsm, and used for the experiments. Grapefruit juice (GFJ) and orange juice (OJ) at a concentration of 5% significantly inhibited the OATP-B-mediated uptake of estrone-3-sulfate by 82 and 53%, respectively. Major constituents of GFJ and OJ also significantly inhibited the OATP-B-mediated uptake of estrone-3-sulfate. Glibenclamide, a hypoglycemic drug, was identified for the first time as a substrate for OATP-B with a Kᵢ value of 6.26 μM. GFJ and OJ inhibited the OATP-B-mediated uptake of glibenclamide. These results suggest that citrus juices may inhibit the intestinal absorption of anionic drugs, such as glibenclamide, via the inhibition of OATP-B.

It is well known that grapefruit juice (GFJ) enhances the systemic exposure to a large variety of therapeutic drugs, including calcium channel blockers, antihistamines, hypnotic benzodiazepines, immunosuppressants, human immunodeficiency virus protease inhibitors, and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (Ammer and Weintraub, 1997; Bailey et al., 1998; Fuhr, 1998; Kane and Lipsky, 2000). The interaction is observed when drugs are orally, but not intravenously, administered (Lundahl et al., 1997), indicating that the interaction occurs during the gastrointestinal absorption. Most drugs that undergo interaction with GFJ are substrates for cytochrome P450 (CYP) 3A4, and it has also been reported that GFJ selectively down-regulates CYP3A4 in the small intestine (Lown et al., 1997). P-glycoprotein (P-gp) is localized on the luminal membranes of the intestinal epithelial cells and functions as an efflux pump to limit the absorption of xenobiotics, including structurally and therapeutically unrelated drugs. We have reported previously that orange juice (OJ) and GFJ inhibit the function of P-gp in vitro (Takanaga et al., 1998, 2000). Therefore, OJ and GFJ are likely to influence the bioavailability of P-gp substrates, as well as CYP3A4 substrates.

Recently, GFJ and OJ have been reported to reduce the bioavailability of an anti-histamine, fexofenadine (Banfield et al., 2002; Dresser et al., 2002), and a β₁-receptor antagonist, celpiroprol (Lilja et al., 2003, 2004). Whereas fexofenadine and celpiroprol are substrates for P-gp and not for cytochromes P450 (Milne and Buckley, 1991; Karlsson et al., 1993; Lippert et al., 1995; Cvetkovic et al., 1999), P-gp is not likely to play a role in this interaction, because the inhibition of P-gp may not decrease, but increase the plasma concentration of P-gp substrate. A possible explanation for this interaction is the inhibition of uptake transporter(s) expressed on the luminal membranes of intestinal epithelial cells. The inhibition may cause a decrease in the intestinal absorption of substrate drugs.

The organic anion-transporting polypeptide (OATP) family is a group of membrane-solute carriers that mediate the uptake of various anionic compounds (Tirona and Kim, 2002; Hagenbuch and Meier, 2003). The inhibition of drug uptake mediated by OATPs in the intestine may decrease the plasma concentration of a substrate for OATPs. Recently, it has been reported that GFJ, OJ, and apple juice (AJ) inhibit the function of human OATP-A (OATP1A2, gene symbol SLC21A3/SLCO1A2) in vitro (Dresser et al., 2002). However, OATP-A is predominantly expressed in the brain, but not in the intestine (Kullak-Ublick et al., 1995; Abe et al., 1999; Gao et al., 2000; Tamai et al., 2000). In contrast, human OATP-B (OATP2B1, gene symbol SLC21A9/SLCO2B1) has recently been shown to be expressed on the luminal membranes of intestinal epithelial cells (Kobayashi et al., 2003). OATP-B mediates the transport of anionic compounds such as sulfobromophthalein, estrone-3-sulfate, and dehydroepiandrosterone sulfate (Tamai et al., 2000; Kullak-Ublick et al., 2001; St-Pierre et al., 2002), and may be involved in the intestinal absorption of anionic drugs in vivo. In this study, we aimed to

ABBREVIATIONS: GFJ, grapefruit juice; AJ, apple juice; DMSO, dimethyl sulfoxide; HEK, human embryonic kidney; OATP, organic anion-transporting polypeptide; OJ, orange juice; P-gp, P-glycoprotein; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; ANOVA, analysis of variance.
examine the effects of citrus juices on the function of OATP-B and to identify novel substrate drug(s) for OATP-B.

Materials and Methods

Materials. [14C]Tolbutamide (2.26 GBq/mmol) was purchased from Amersham Biosciences Inc. (Biscatway, NJ). Estrone-3-sulfate potassium salt and glibenclamide were purchased from Sigma-Aldrich (St. Louis, MO). GFJ, OJ, and AJ (Dole brand) from concentrate were commercially obtained from Nippon Milk Community Co., Ltd. (Tokyo, Japan). Naringin (4’,5’,7-trihydroxflavonol-7-rhamnoglucoside), naringenin (4’,5’,7-trihydroxyflavonol), and quercetin (3,3’,4’,5’,7-pentahydroxyflavone) were purchased from Sigma-Aldrich. Bergamottin (4-(3,7-dimethyl-2,6-octadienyl)oxy)psoralen was purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ). Tangeretin (4’,5,6,7,8-pentamethoxyflavone) was purchased from Extrasynthese S.A. (Genay, France). Nobiletin (3’,4’,5,7,8-hexamethoxyflavone) was a kind gift from Kanebo Ltd. (Tokyo, Japan). 6,7-Dihydroxybergamottin (4-[(6,7-dihydroxy-3,7-dimethyl-2-ocetyl)oxy]psoralen) was extracted and purified from GFJ in our laboratory by the method previously reported (Ohnishi et al., 2000). Human embryonic kidney (HEK) 293 cells were purchased from Health Science Research Resources Bank (Osaka, Japan). All other chemicals were commercial products of reagent grade.

Stable Transfection of Human OATP-B into HEK293 Cells. A human cDNA clone, hk07457, including OATP-B cDNA was a kind gift from Kazusa Bioscience Inc. (Nakayama, Japan). This cDNA was authenticated by DNA sequencing that the sequence of the cDNA clone, hk07457, including OATP-B, into HEK/OATP-B cells was confirmed by DNA sequencing that the sequence of the cDNA clone, hk07457, including OATP-B, was identical to the reference sequence (GenBank accession number NM_007256). The pRES/OATP-B construct was then transfected into HEK293 cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA), according to the protocol of the manufacturer. After selection in 800 μg/ml G418 (Sigma-Aldrich) for 2 weeks, single clones were isolated from the transfected cell pool and tested for [14H]estrone-3-sulfate uptake. The clone with the highest uptake activity was designated as HEK/OATP-B. Similarly, a single clone of HEK293 cells transfected with pRESneo vector alone was designated as HEK/Mock, and this cell line was used for an index of nonspecific uptake under 5% CO2/95% air. For uptake experiments, the cells were seeded at a density of 4 × 104 cells per well on 96-well microwell plates (Nalge Nunc International, Rochester, NY). After 2 days cultivation, culture medium was replaced with fresh Eagle’s minimal essential medium as above, but without G418. On the next day, the uptake study was performed.

Uptake Experiments. The culture medium was removed, and the cells were washed three times with 100 μl of uptake buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM tris-glucose, 1.2 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM HEPES, pH 7.4, 37°C) and then preincubated with 200 μl of buffer at 37°C for 10 min. After the preincubation, buffer was replaced with 100 μl of buffer containing a radiolabeled compound to initiate the uptake reaction. In the concentration-dependence experiments, substrate concentrations were adjusted to indicated values by addition of unlabeled compound to the buffer. For examining the effect of extracellular pH, the pH of buffer containing a radiolabeled compound was adjusted to 5.5–7.4 by 25 mM 2-(N-morpholino)ethanesulfonic acid/Tris (pH 5.5–6.5) or 25 mM HEPES/Tris (pH 7.0–7.4). After incubation for the indicated times, 200 μl of ice-cold buffer was added to the well to stop the uptake reaction, and immediately, the cells were washed three times with 100 μl of ice-cold buffer. The cells were solubilized in 200 μl of 1 N NaOH over 4 h and then neutralized with 100 μl of 2 N HCl. A liquid scintillation cocktail (Clear-sol II; Nakalai Tesque) was added to 200-μl aliquots of the solubilized cells, and radioactivity was determined by using a liquid scintillation counter (LSC-3500; Aloka Co., Ltd., Tokyo, Japan). The content of protein in the solubilized cells was measured by the Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard.

Inhibition Experiments. To assure consistency throughout this study, the same lot of purchased juices were divided into appropriate volumes and stored at −20°C. The juices were centrifuged at 10,000 g for 10 min and filtered through a 0.22-μm membrane filter (Milllex GV52; Millipore Corporation, Billerica, MA), and the filtrate was designated as 100% juice. These juices were diluted to 1, 2, or 5% with buffer and adjusted to pH 7.4 and approximately 300 mOsm. Uptake experiments were performed in the absence or presence of 1, 2, or 5% juice according to the procedure described in the section on uptake experiments.

In addition, we investigated the effects of juice constituents, including naringin, naringenin, quercetin, bergamottin, 6,7-dihydroxybergamottin, tangeretin, and nobiletin. Each compound was dissolved in dimethyl sulfoxide (DMSO) and diluted with buffer to give 0.5% final DMSO concentration. Uptake experiments were performed in the absence or presence of 1 or 10 μM juice constituent according to the procedure described in the section on uptake experiments. The buffer containing 0.5% DMSO was used in the control experiment.

Data Analysis. The uptake value (μl/μg protein) was obtained by dividing the radioactivity taken up into the cells per cellular protein content by the concentration of radioactivity of the test compound in the buffer. The OATP-B-mediated uptake was evaluated after subtracting the initial uptake of estrone-3-sulfate (20 s) or glibenclamide (1 min) into HEK/Mock cells from that into HEK/OATP-B cells.

To estimate the kinetic parameters of the OATP-B-mediated uptake of estrone-3-sulfate and glibenclamide, the following equation was fitted to the observed data for the OATP-B-mediated uptake (JIC) at various concentrations of estrone-3-sulfate or glibenclamide by using a nonlinear least-squares regression analysis program, MULTI (Yamaoka et al., 1981): JIC = Jmax(Kc + C), where Jmax, Kc, and C represent the maximum transport rate, the transport affinity, and the concentration of substrate, respectively.

Inhibitory effect (percentage of control) was evaluated as the ratio of the OATP-B-mediated uptake in the presence of juice or juice constituents to that in the absence of juice or juice constituents (control). In statistical analysis, the significance of differences between the mean values was determined by Student's t test or analysis of variance (ANOVA), followed by Dunnett's test, and a p value of less than 0.05 was considered statistically significant.

Results

OATP-B-Mediated Uptake of Estrone-3-sulfate. Expression of OATP-B mRNA was detected in HEK/OATP-B cells, but not in HEK/Mock cells, as assessed by RT-PCR (data not shown). The uptake of 10 nM [14H]estrone-3-sulfate, a typical substrate for OATP-B, into HEK/OATP-B cells was significantly higher than that into HEK/Mock cells (Fig. 1A). Since this uptake increased linearly over 20 s, the initial uptake rate was determined at 20 s. We examined the effect of extracellular pH on the uptake of estrone-3-sulfate. The uptake of estrone-3-sulfate mediated by OATP-B was not affected by extracellular pH between 5.5 and 7.4 (data not shown), and the extracellular pH was adjusted to pH 7.4 in the following experiments. The OATP-B-mediated initial uptake of estrone-3-sulfate was saturable (Fig. 1B) with kinetic parameters, Kc and Jmax, of 7.14 ± 2.25 μM and 184 ± 53.5 pmol/mg protein/20 s, respectively.

Effects of Citrus Juices and Their Constituents on OATP-B-Mediated Uptake of Estrone-3-sulfate. GFJ and OJ significantly
inhibited the OATP-B-mediated uptake of estrone-3-sulfate, whereas AJ did not affect the uptake (Fig. 2). The inhibitory potency of GFJ was higher than that of OJ: GFJ (5%) and OJ (5%) significantly inhibited the uptake by 82 and 53%, respectively (Fig. 2). The effects of several citrus juice constituents on the OATP-B-mediated uptake of estrone-3-sulfate were evaluated. GFJ constituents, naringin, naringenin, quercetin, bergamottin, and 6',7'-dihydroxybergamottin, and OJ constituents, tangeretin and nobiletin, at the concentration of 10 μM significantly inhibited the OATP-B-mediated uptake of estrone-3-sulfate by 39, 28, 21, 60, 43, 42, and 60%, respectively (Fig. 3).

**Substrate Specificity of Human OATP-B.** We investigated whether OATP-B transports anionic compounds such as estrone-3-sulfate, dehydroepiandrosterone sulfate, taurocholic acid, salicylic acid, ibuprofen, glibenclamide, and tolbutamide. The uptake of [3H]estrone-3-sulfate, [3H]dehydroepiandrosterone sulfate, and [3H]glibenclamide into HEK/OATP-B cells was significantly higher than that into HEK/Mock cells (Table 1). In contrast, the uptake of [3H]taurocholic acid, [14C]salicylic acid, [14C]ibuprofen, and [14C]tolbutamide into HEK/OATP-B cells did not differ from that into HEK/Mock cells (Table 1).

**OATP-B-Mediated Uptake of Glibenclamide.** We investigated the OATP-B-mediated uptake of glibenclamide in detail. The uptake of 10 nM [3H]glibenclamide into HEK/OATP-B cells was significantly higher than that into HEK/Mock cells (Fig. 4A). Since this uptake increased linearly over 1 min, the initial uptake rate was measured at 1 min. The OATP-B-mediated initial uptake of glibenclamide was saturable (Fig. 4A).
4B), and its kinetic parameters, $K_t$ and $J_{\text{max}}$, were $6.26 \pm 3.15 \mu M$ and $112 \pm 52.2 \, \text{pmol/mg protein/min}$, respectively.

**Effects of Citrus Juices and Their Constituents on OATP-B-Mediated Uptake of Glibenclamide.** GFJ and OJ significantly inhibited the OATP-B-mediated uptake of glibenclamide, whereas AJ did not affect the uptake (Fig. 5). The OATP-B-mediated uptake of glibenclamide was significantly inhibited by 6',7'-dihydroxybergamotin and tangeretin, and moderately inhibited by naringin, naringenin, and nobiletin (Fig. 6). In contrast, no inhibitory effect of quercetin or bergamottin was observed in this study (Fig. 6).

**Discussion**

Human OATP-B has been shown to mediate the uptake of estrone-3-sulfate when expressed in *Xenopus laevis* oocytes (Kullak-Ublick et al., 2001), transiently expressed in HEK293 cells (Tamai et al., 2001), and stably expressed in Chinese hamster ovary cells (Pizzagalli et al., 2003) with affinity constant values of 6.3, 9.04, and 5 \mu M, respectively. The HEK/OATP-B cell line established in this study took up estrone-3-sulfate with a transport affinity ($K_t$) of 7.14 \mu M (Fig. 1B), and this affinity is in accordance with previously reported values (Kullak-Ublick et al., 2001; Tamai et al., 2001; Pizzagalli et al., 2003). The uptake of dehydroepiandrosterone sulfate into HEK/OATP-B cells was much higher than that into HEK/Mock cells, whereas the uptake of taurocholic acid did not differ from that into HEK/Mock cells (Table 1). These results are also consistent with previous observations using *X. laevis* oocytes expressing OATP-B (Kullak-Ublick et al., 2001). Therefore, the HEK/OATP-B cell line established in this study is considered to express OATP-B with a function equivalent to that in previous studies.

In our experiment, the extracellular pH did not affect the OATP-B-mediated uptake of estrone-3-sulfate. In contrast, Kobayashi et al. (2003) and Nozawa et al. (2004) have reported that the OATP-B-mediated uptake of estrone-3-sulfate was increase under acidic conditions. This contradictory finding may be possibly explained by the characteristics of the cells used. We used adherent cells, HEK293, and established a stably expressing cell line, whereas Kobayashi et al. (2003) and Nozawa et al. (2004) have examined a transiently expressing system, and in suspension. However, the cause of this discrepancy in detail remains unclear. To prevent the potential damage of the cells, we adjusted the pH of buffer to 7.4. Indeed, Dresser et al. (2002) used concentrations up to 5% to examine the effect of juices on the function of OATP-A. Therefore, we investigated the effect of juices up to a concentration of 5%. We have confirmed that the effect of 5% juices on the osmolarity of buffer was negligible (data not shown). Under this condition, GFJ and OJ significantly inhibited the OATP-B-

### TABLE 1

<table>
<thead>
<tr>
<th>Anionic Compound (Concentration)</th>
<th>HEK/Mock</th>
<th>HEK/OATP-B</th>
<th>OATP-B-Mediated</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H] Estrone-3-sulfate (12.8 nM)</td>
<td>6.45 ± 0.19</td>
<td>211 ± 3</td>
<td>204</td>
</tr>
<tr>
<td>[3H] DHEAS (17.4 nM)</td>
<td>5.92 ± 0.25</td>
<td>12.7 ± 0.6</td>
<td>6.8</td>
</tr>
<tr>
<td>[3H] Taurocholic acid (41.9 nM)</td>
<td>2.55 ± 0.14</td>
<td>2.95 ± 0.35</td>
<td>0.40</td>
</tr>
<tr>
<td>[3H] Salicylic acid (1.43 \mu M)</td>
<td>9.52 ± 0.76</td>
<td>11.2 ± 0.2</td>
<td>1.7</td>
</tr>
<tr>
<td>[3H] Ibuprofen (11.3 \mu M)</td>
<td>12.6 ± 0.5</td>
<td>12.4 ± 0.2</td>
<td>−0.2</td>
</tr>
<tr>
<td>[3H] Glibenclamide (10.1 nM)</td>
<td>82.0 ± 4.3</td>
<td>135 ± 1</td>
<td>53</td>
</tr>
<tr>
<td>[3H] Folbutamide (8.82 \mu M)</td>
<td>7.25 ± 0.19</td>
<td>7.50 ± 0.39</td>
<td>0.25</td>
</tr>
</tbody>
</table>

DHEAS, dehydroepiandrosterone sulfate; N.S., not statistically significant.
that human OATP-D and OATP-E mRNAs are also expressed in the human intestine (Tamai et al., 2000). Contributions of these transporters cannot be excluded.

GFJ contains high levels of bioflavonoids, including naringin, its aglycone naringenin, and quercetin (Bailey et al., 2000; Ross et al., 2000). Furanocoumarins, such as bergamottin and 6',7'-dihydroxybergamottin, are also contained in GFJ and are considered to be major constituents responsible for inhibition of CYP3A4 (Guo et al., 2000). Polymethoxyflavones, such as tangeretin and nobiletin, are contained in OJ (Rouseff et al., 1979; Sendra et al., 1988) and inhibit the activity of P-gp in vitro (Takanaga et al., 1998, 2000). In this study, these constituents were demonstrated to inhibit the function of OATP-B. GFJ contains very high levels of naringin (up to 1000 μM) in comparison with other constituents (Ross et al., 2000). However, the content of each constituent depends upon the brand, origin, picking season, and other factors, and we did not quantify the content of these constituents in this study. It is conceivable that constituents investigated in this study, at least in part, are responsible for the inhibitory effect of GFJ on OATP-B function. Because the contents of tangeretin and nobiletin in OJ are as low as 0.36 to 1.6 μM and 2.5 to 11 μM, respectively (Rouseff et al., 1979; Sendra et al., 1988), these constituents cannot fully account for the inhibitory effect of OJ.

OATPs display broad substrate selectivity and transport bile salts, sulfated or glucuronated endogenous and exogenous compounds, anionic peptides, and even some cationic compounds (Tirona and Kim, 2002; Hagenbuch and Meier et al., 2003). However, only estrone-3-sulfate, sulfobromophthalein, and dehydroepiandrosterone sulfate have been identified as substrates for OATP-B, suggesting that it has high substrate selectivity (Kullak-Ublick et al., 2001). We aimed to investigate whether OATP-B mediates the transport of therapeutic drugs. Although OATP-B has been shown to transport pravastatin under an acidic extracellular condition, it does not do so at physiological pH (7.4) (Kobayashi et al., 2003; Nozawa et al., 2004). In this study, we first identified a hypoglycemic drug, glibenclamide, as a substrate for OATP-B at physiological pH (K, 6.26 μM; Fig. 4B). This is the first example of a therapeutic drug transported by OATP-B at physiological pH. The OATP-B-mediated uptake of glibenclamide as well as estrone-3-sulfate, was significantly inhibited by GFJ and OJ (Fig. 5) and moderately inhibited by constituents of the juices (Fig. 6). It remains to be examined whether clinically significant interactions occur between glibenclamide and citrus juices in humans, because the contribution of OATP-B-mediated uptake and passive diffusion to the intestinal absorption of glibenclamide is unknown.

Citrus juices are well known to cause drug interactions by inhibiting the activity of CYP3A4 and/or P-gp. In such cases, the plasma concentrations of the target drugs are increased (Ameer and Weintraub, 1997; Lown et al., 1997; Bailey et al., 1998; Fuhr, 1998; Takanaga et al., 1998; Kane and Lipsky, 2000). On the other hand, the plasma concentrations of OATP-B substrates are expected to be reduced by citrus juices that inhibit the function of OATP-B, because OATP-B is expressed on the apical membranes of intestinal epithelial cells and functions to take up substrates into the cells.

In conclusion, GFJ and OJ inhibit the function of human OATP-B at a concentration of 5%. Moreover, glibenclamide was identified for the first time as a novel substrate for human OATP-B.

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**Fig. 5.** Effects of grapefruit, orange, and apple juices on the OATP-B-mediated uptake of glibenclamide. Uptake of 10 nM [3H]glibenclamide into HEK/OATP-B cells and HEK/Mock cells was measured for 1 min in the absence (control, open column) or presence of 1 μM (gray columns) or 10 μM (closed columns) constituents at 37°C and pH 7.4. OATP-B-mediated uptake was determined by ANOVA followed by Dunnett’s test (*, p < 0.05; **, p < 0.01).

**Fig. 6.** Effects of constituents in citrus juices on the OATP-B-mediated uptake of glibenclamide. Uptake of 10 nM [3H]glibenclamide into HEK/OATP-B cells and HEK/Mock cells was measured for 1 min in the absence (control, open column) or presence of 1 μM (gray columns) or 10 μM (closed columns) constituents at 37°C and pH 7.4. OATP-B-mediated uptake was determined by ANOVA followed by Dunnett’s test (*, p < 0.05; **, p < 0.01).
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