



# Investigation of Fluorescent Substrates and Substrate-Dependent Interactions of a Drug Transporter Organic Anion Transporting Polypeptide 2B1 (OATP2B1)

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## ABSTRACT

**Purpose** In this study, we investigated organic anion transporting polypeptide 2B1 (OATP2B1)-mediated uptake of fluorescent anions to better identify fluorescent substrates for *in vitro* OATP2B1 assays. The OATP2B1 is involved in the intestinal absorption and one of the pharmacokinetic determinants of orally administered drugs.

**Methods** A microplate reader was used to determine the cellular accumulation of the fluorescent compounds into the OATP2B1 or the empty vector-transfected HEK293 cells.

**Results** Two types of derivatives were found to be OATP2B1 substrates: heavy halogenated derivatives, such as 4',5'-dibromofluorescein (DBF), and carboxylated derivatives, such as 5-carboxyfluorescein (5-CF). The DBF and 5-CF were transported in a time and concentration-dependent manner. The DBF was transported at a broad pH (pH 6.5–8.0) while 5-CF was transported at an acidic pH (pH 5.5–6.5). The  $K_m$  values were  $0.818 \pm 0.067 \mu\text{M}$  at pH 7.4 for DBF and  $8.56 \pm 0.41 \mu\text{M}$  at pH 5.5 for 5-CF. The OATP2B1 inhibitors, including atorvastatin, bromosulphophthalein, glibenclamide, sulfasalazine, talinolol, and estrone 3-sulfate, inhibited the DBF and the 5-CF transport. Contrastively, testosterone, dehydroepiandrosterone sulfate, and progesterone inhibited the DBF transport but stimulated the 5-CF transport. Natural flavonoid aglycones, such as naringenin and baicalein, also exhibited substrate-dependent effects in this manner.

**Conclusion** We found two fluorescein analogs, DBF and 5-CF as the OATP2B1 substrates that exhibited substrate-dependent interactions.

**KEY WORDS** drug–drug interaction (DDI) · fluorescent substrate · food–drug interaction (FDI) · inhibition assay · organic anion transporting polypeptide 2B1 (OATP2B1)

## ABBREVIATIONS

5-AF	5-aminofluorescein
5-CF	5-carboxyfluorescein
6-CF	6-carboxyfluorescein
AUC	Area under the curve
BSP	bromosulphophthalein
CDCF	5-carboxy-2',7'-dichlorofluorescein
CDCSF	5-carboxy-2',7'-dichlorosulfonfluorescein
COR	Corticosterone
E <sub>1</sub> S	Estrone 3-sulfate
E <sub>2</sub> G	Estradiol-17 $\beta$ -glucuronide
DBF	2',7'-dibromofluorescein
DCF	2',7'-dichlorofluorescein
DDI	Drug–drug interaction
DEX	Dexamethasone
DHEAS	Dehydroepiandrosterone sulfate
EY	Eosin Y
FDI	Food–drug interaction
FL	Fluorescein
OG	Oregon Green
OATP	Organic anion transporting polypeptide
PRO	Progesterone
RB	Rose bengal
Rho123	Rhodamine123
SF	sulfonfluorescein
TET	Testosterone

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## INTRODUCTION

The organic anion transporting polypeptide (OATP) family of transporters are expressed in various tissues of the body, recognize a variety of endogenous substances (such as conjugates of steroids and bilirubin) and numerous drugs, and are deeply involved in maintaining homeostasis and pharmacokinetics (1). In humans, 11 isoforms of OATP have been identified and function as uptake transporters (1). The localization varies depending on the isoform. The locally expressed isoforms include the liver-specific OATP1B1 and the OATP1B3 (encoded by *SLCO1B1* and *SLCO1B3*, respectively) and the kidney-specific OATP4C1 (2).

The ubiquitously expressed isoforms include the OATP2B1 (small intestine, liver, placenta, lung, kidney, heart, and brain) (2). In the small intestine, the expression of OATP1A2 and OATP2B1 has been reported; however, based on their expression level, the OATP2B1 is considered as the major functional isoform in the small intestine (3).

The OATP2B1 transports diverse organic anions and zwitterions, including the clinically important drugs (fexofenadine, lipid-lowering statins, and glibenclamide), and the endogenous compounds (taurocholate, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and steroids) (4). While many substrates, such as fexofenadine, montelukast, and atorvastatin, overlap with other OATP isoforms such as OATP1B1 and OATP1B3, aliskiren, celirolol, and pemetrexed are selective for OATP2B1 (5). As OATP2B1 substrates are expected to improve gastrointestinal absorption, the OATP2B1 is an interesting transporter for drug discovery and clinical practice.

The OATP2B1 inhibition could affect the efficacy and toxicity of its substrates and is involved in food-drug and drug-drug interactions (FDI/DDI). With the information about the FDI, patients can avoid side effects. Therefore, the FDI has attracted attention for successful drug treatment in recent years. A well-known OATP2B1-targeted FDI, for example, is interaction with the fruit juice. Common juices, such as grapefruit, orange, and apple, inhibit OATP2B1, reduce absorption, and decrease the area under the curve (AUC) of substrates fexofenadine, aliskiren, and celirolol (6). The OATP2B1 inhibition by natural compounds in fruits, including naringin and hesperidin, has been reported *in vitro* and has been demonstrated in clinical studies (7–10). Additionally, the OATP2B1-mediated uptake of SN-38, a biliary-excreted metabolite of irinotecan hydrochloride (CPT-11), causes gastrointestinal toxicity, but concomitant use of drugs containing the OATP2B1 inhibitors reduces this toxicity and prevents late-onset diarrhea (11–13). In this way, FDI/DDI targeting OATP2B1 affects the efficacy and toxicity of orally administered drugs. Therefore, the information about OATP2B1 ligands and their inhibition profile is important for a successful pharmacotherapy through the prediction and prevention of FDI/DDI. For this purpose, to evaluate the OATP2B1-

mediated FDI/DDIs, a fluorescence-based *in vitro* assay system is useful because it is simple, highly sensitive, and has a high throughput.

In this study, we investigated an OATP2B1-mediated uptake of fluorescent anions to identify better the fluorescent substrates for *in vitro* OATP2B1 assay systems. Finally, we evaluated the effects of flavonoids, which are a well-known class of natural compounds included in vegetables and fruits, on the transport activity of OATP2B1 using two of the fluorescent substrates that we found.

## MATERIALS AND METHODS

### Materials

The following materials were used in the study: Dulbecco's Modified Eagle's Medium (DMEM, Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan); fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA); plasmid vectors pTCN-empty and pTCN-hOATP2B1 (transOMIC, Huntsville, AL, USA); fluorescein (FL), 2',7'-dibromofluorescein (DBF), 5-carboxyfluorescein (5-CF), 6-carboxyfluorescein (6-CF), estrone 3-sulfate (E<sub>1</sub>S), estradiol-17 $\beta$ -glucuronide (E<sub>2</sub>G), naringenin, and naringin (Sigma-Aldrich, St. Louis, MO, USA); 5-carboxy-2',7'-dichlorofluorescein (CDCF) and 5-carboxy-2',7'-dichlorosulfonfluorescein (CDCSF) (PromoKine, Heidelberg Germany); eosin Y (EY) (Alfa Aesar, Heysham, UK); 2',7'-dichlorofluorescein (DCF), sulfonfluorescein (SF), 5-aminofluorescein (5-AF), atorvastatin, and phlorizin (Tokyo Chemical Industries, Tokyo, Japan); Oregon Green (OG) (Life Technologies, Carlsbad, CA, USA); Rose Bengal (RB), glibenclamide, penicillin G, dehydroepiandrosterone sulfate (DHEAS), testosterone (TET), progesterone (PRO), corticosterone (COR), dexamethasone (DEX), quercetin, kaempferol, rutin, quercitrin, apigenin, luteolin, baicalein, baicalin, daidzein, daidzin, phloretin, glycyrrhetic acid (GA), and glycyrrhizin (GL) (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan); bromosulfophthalein (BSP) (MP Biomedicals, Heidelberg, Germany); montelukast and sulfasalazine (LKT Laboratories, St. Paul, MN, USA); talinolol (Cayman Chemical, Ann Arbor, MI, USA); sodium taurocholate (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA); and puerarin (Koshiro Company, Osaka, Japan). All other chemicals used were commercial products of analytical grade.

### Cell Culture

The HEK293 cells (CRL-1573, lot number 60920959) were obtained from the American Type Culture Collection

(ATCC, Manassas, VA, USA). The HEK293 cells were cultured in DMEM, supplemented with 10% FBS. The cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>–95% air and routinely subcultured by TrypLE Express (Life Technologies, CA, USA).

### Uptake Assay

The cellular accumulation of fluorescent anions was measured using a method similar to our previous reports for other transporters, but with some modification (14,15). Briefly, the HEK293 cells ( $8.0 \times 10^4$  cells/well) were seeded. For transient expression, the pTCN-OATP2B1 was transfected with polyethylenimine (PEI) MAX (Polysciences Inc. Warrington, PA, USA) according to the manufacturer's instructions. The empty vector pTCN-transfected cells (Mock) were used as controls. The cells were washed twice with 100  $\mu$ L Waymouth buffer (in mM: 135 NaCl, 5 KCl, 1.2 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 0.8 MgSO<sub>4</sub>, 28 D-glucose, and 13 HEPES-NaOH, pH 7.4) 48 h after transfection and pre-incubated in the same buffer without the test compounds for 10 min at 37°C. Next, the cells were incubated for 2 min in an uptake buffer (Waymouth buffer containing substrate and test compounds) at 37°C and washed twice with 100  $\mu$ L ice-cold PBS. When the effect of extracellular pH was assessed, Waymouth buffers were prepared with MES-NaOH (pH 5.5–6.5), HEPES-NaOH (pH 7.0–7.4), or Tricine-NaOH (pH 8.0). The cells were lysed with 100  $\mu$ L 1% SDS, and the fluorescence intensity (ex 485 nm and em 535 nm) was measured using a microplate reader Spark 10 M (Tecan Group Ltd., Männedorf, Switzerland). The protein concentrations were determined using the Takara BCA Protein Assay Kit (Takara Bio Inc., Shiga, Japan) utilizing the bovine serum albumin as a standard.

### Data Analysis

All the nonlinear kinetic regression analyses and statistical analyses were conducted using KaleidaGraph (Synergy Software, Reading, PA, USA). The Michaelis–Menten kinetic parameters,  $K_m$  and  $V_{max}$ , were determined using the following equation:

$$v = \frac{V_{max} \times [S]}{K_m + [S]},$$

where  $v$  is the measured rate of the cellular accumulation,  $[S]$  is the substrate concentration, and  $V_{max}$  and  $K_m$  are the maximal rates of transport and substrate concentration at the half-maximal rate, respectively. The  $IC_{50}$  values were determined using the four-parameter log-logistic equation (16):

$$v = v_{max} + \frac{v_{max} - v_{min}}{1 + 10^{(\log[I] - \log IC_{50}) \times n}},$$

where  $v_{min}$  and  $v_{max}$  are the lowest and highest uptake, respectively,  $n$  is the Hill slope, and  $[I]$  is the inhibitor concentration. The data are expressed as mean  $\pm$  the standard error of the mean (SE). The statistical differences between two or multiple groups were examined using the unpaired Student's  $t$ -tests or the Dunnett's multiple comparison test, respectively. The differences were regarded as statistically significant with a  $p$  value  $< 0.05$ .

## RESULTS

### Uptake of Fluorescent Compounds

First, we investigated the cellular accumulation of commercially available fluorescein analogs (Figs. 1 and 2). Because OATP2B1 activity is higher and its ligand recognition profile is wider at an acidic pH (3), we performed the uptake assay with both a physiological pH 7.4 and an acidic pH 5.5. At 1  $\mu$ M, fluorescein and its positively charged analogs rhodamine 123 are not transported significantly. However, DCF, DBF, and EY at pH 7.4 and 5-CF, 6-CF, CDCF, and CDCSF at pH 5.5 were remarkably accumulated in the HEK293-OATP2B1 cells.

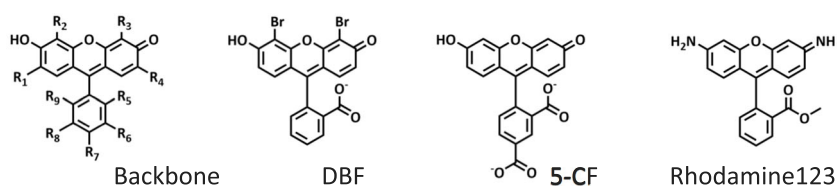
### Kinetic Analysis of the Fluorescein Derivatives

To investigate the recognition of the fluorescein analogs by OATP2B1, we further conducted the kinetic analysis (Fig. 3, Table 1). The FL, OG, DCF, DBF, and EY were transported by OATP2B1 at pH 7.4 in Michaelis–Menten kinetics. Similarly, 5-CF, 6-CF, and CDCSF were transported at a pH of 5.5. Lastly, SF at pH 7.4 and 5-AF at pH 5.5 didn't exhibit any dose-dependent active accumulation (data not shown).

In a comparison between FL and OG at pH 7.4, the halogen at xanthene moiety, 2', 7'-fluorine, a bioisostere of hydrogen atom, increased  $K_m$  about 4-fold (FL:  $19.8 \pm 3.1 \mu$ M to OG:  $87.1 \pm 8.4 \mu$ M), whereas they increased  $V_{max}$  about 3-fold (FL:  $72.1 \pm 5.5$  pmol/mg/5 min to OG:  $207 \pm 11$  pmol/mg/5 min, Table 1). In contrast, from the comparison between FL and DBF, 4', 5'-bromine, heavy and lipophilic atom drastically decreased in  $K_m$  (DBF:  $0.818 \pm 0.067 \mu$ M, Table 1). Also, in the comparison between DBF and EY, an addition of 2', 7'-bromine increased the  $V_{max}$  (DBF:  $44.7 \pm 0.9$  pmol/mg/5 min to EY:  $70.2 \pm 3.0$  pmol/mg/5 min, Table 1).

At pH 5.5, in the comparison between 5-CF and 6-CF, for carboxyl group at benzoic acid moiety, moving it from position R<sub>7</sub> to R<sub>8</sub> decreases the  $V_{max}$  (5-CF:  $36.4 \pm 0.4$  pmol/mg/5 min to 6-CF:  $7.12 \pm 0.31$  pmol/mg/5 min) while the  $K_m$  were unchanged ( $8.56 \pm 0.41$  and  $9.92 \pm 0.37 \mu$ M, respectively, Table 1).

**Fig. 1** Chemical structures, names, and abbreviations of the tested fluorescein analogs. FL: fluorescein, SF: sulfonfluorescein, OG: Oregon Green, DCF: 2',7'-dichlorofluorescein, DBF: 2',7'-dibromofluorescein, EY: eosin Y, RB: rose bengal, 5-AF: 5-aminofluorescein, 5-CF: 5-carboxyfluorescein, 6-CF: 6-carboxyfluorescein, CDCF: 5-carboxy-2',7'-dichlorofluorescein, CDCSF: 5-carboxy-2',7'-dichlorosulfonfluorescein, Rho123: rhodamine123.

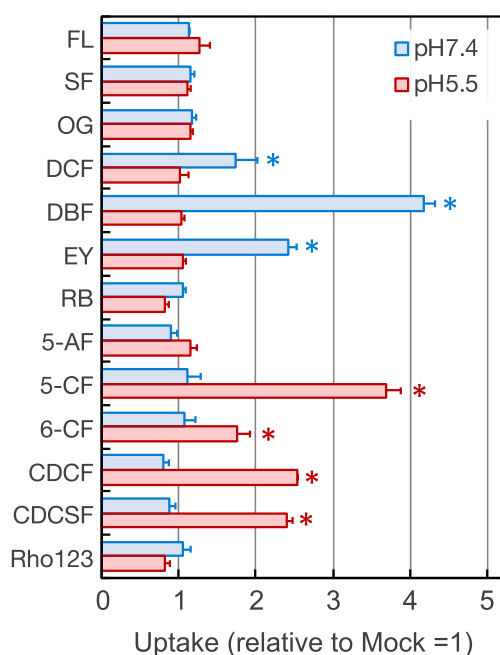


Compounds	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	R <sub>6</sub>	R <sub>7</sub>	R <sub>8</sub>	R <sub>9</sub>
FL	H	H	H	H	COOH	H	H	H	H
SF	H	H	H	H	SO <sub>3</sub> H	H	H	H	H
OG	F	H	H	F	COOH	H	H	H	H
DCF	Cl	H	H	Cl	COOH	H	H	H	H
DBF	H	Br	Br	H	COOH	H	H	H	H
EY	Br	Br	Br	Br	COOH	H	H	H	H
RB	I	I	I	I	COOH	Cl	Cl	Cl	Cl
5-AF	H	H	H	H	COOH	H	NH <sub>2</sub>	H	H
5-CF	H	H	H	H	COOH	H	COOH	H	H
6-CF	H	H	H	H	COOH	H	H	COOH	H
CDCF	Cl	H	H	H	COOH	H	COOH	H	H
CDCSF	Cl	H	H	Cl	SO <sub>3</sub> H	H	COOH	H	H

### Characteristics of DBF and 5-CF Transport

We then selected representative substrates based on a high OATP2B1/Mock accumulation ratio and low Mock

accumulation. Because EY was more accumulated than DBF in the Mock cells (DBF:  $8.2 \pm 0.2$  pmol/mg/5 min, EY:  $35.8 \pm 3.0$  pmol/mg/5 min), DBF and 5-CF were selected as representative substrates due to their high OATP2B1/Mock accumulation ratio and low Mock accumulation (Fig. 2). Next, we characterized the DBF and 5-CF transport (Fig. 4). The uptake of DBF and 5-CF was time- and concentration-dependent. The DBF was transported at a broad pH (6.5–8.0), while 5-CF was transported at an acidic pH (5.5–6.5). The DBF at pH 7.4 showed smaller  $K_m$  value than that of 5-CF at pH 5.5 ( $0.818 \pm 0.067$  and  $8.56 \pm 0.41$   $\mu$ M, respectively, Table 1).

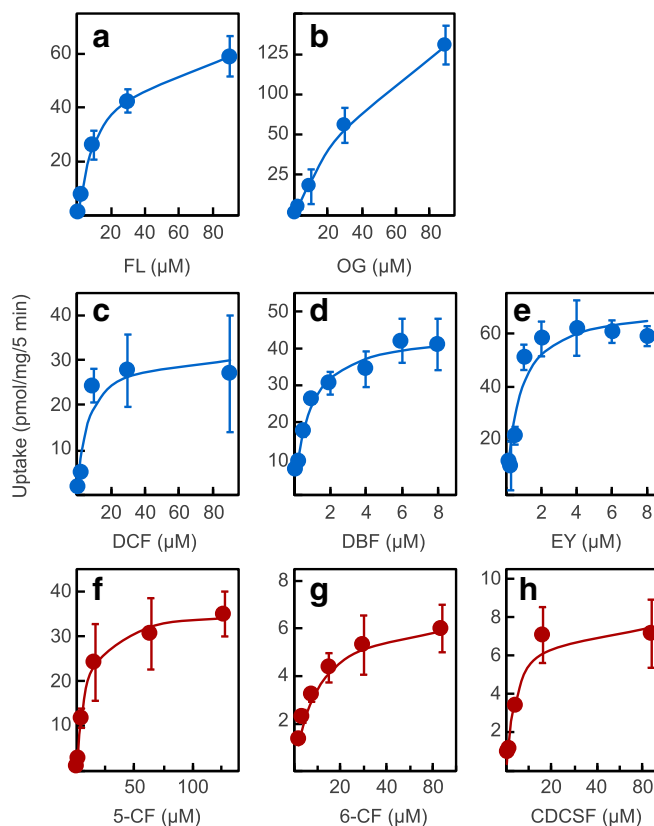


**Fig. 2** Uptake of fluorescein analogs by OATP2B1 at pH 7.4 and 5.5. Uptake study was performed at 1  $\mu$ M of represented compounds, 37°C for 5 min. Data are expressed as mean  $\pm$  SE ( $n = 3$ ) of accumulation ratio (OATP2B1/Mock). \*:  $p < 0.05$  vs. Mock.

### Interaction Profile with Drugs and Endogenous Compounds

Subsequently, we investigated the effect of known OATP2B1 substrates and/or inhibitors at 100  $\mu$ M on the transport of DBF and 5-CF (Fig. 5). It is known that OATP2B1 has at least two substrate-binding sites with different affinities (17,18). Shirasaka *et al.* classified inhibitors due to their selectivity for binding sites (*viz.*, high-affinity, low-affinity, and both-affinity site inhibitors) (17). The inhibitors for high-affinity sites (pravastatin, talinolol, and taurocholate) inhibited both the DBF and 5-CF transports. Among the inhibitors for low-affinity sites, testosterone inhibited DBF but stimulated 5-CF. Among inhibitors of both sites, atorvastatin, BSP, and E<sub>1</sub>S

**Fig. 3** Kinetic analysis on the uptake of fluorescein analogs mediated by OATP2B1. Uptake assay was performed at pH 7.4 for FL (a), OG (b), DCF (c), DBF (d), and EY (e) and at pH 5.5 for 5-CF (f), 6-CF (g), and CDCSF (h). The specific uptake was calculated by subtracting the uptake of Mock from that of HEK293-OATP2B1 and plotted as mean  $\pm$  SE ( $n = 3-6$ ).



inhibited both the DBF and 5-CF transports. However, DHEAS inhibited DBF but stimulated 5-CF.

Since testosterone and DHEAS exhibited substrate-dependent interactions, we further investigated the concentration dependence of the effects of steroids on the DBF and 5-CF transports when mediated by OATP2B1 (Fig. 6, Table 2). The E<sub>1</sub>S and DEX inhibited both transports with sigmoidal inhibition curves with estimated IC<sub>50</sub><sup>DBF</sup> of 2.86  $\pm$  0.46 and > 100 and IC<sub>50</sub><sup>5-CF</sup> of 1.61  $\pm$  0.14 and 50.7  $\pm$  8.5  $\mu$ M for E<sub>1</sub>S and DEX, respectively (Table 2). The E<sub>2</sub>G and COR also

exhibited sigmoidal dose-response curves. However, the low concentrations of E<sub>2</sub>G on DBF uptake and COR on 5-CF uptake exhibited  $v$  values of >100% with IC<sub>50</sub><sup>DBF</sup> of 86.5  $\pm$  21.3 and 21.7  $\pm$  1.8  $\mu$ M and IC<sub>50</sub><sup>5-CF</sup> of 50.3  $\pm$  2.0  $\mu$ M and not determined for E<sub>2</sub>G and COR, respectively (Table 2). The effect of COR on DBF uptake was weaker than that of other steroids. Furthermore, DHEAS and PRO inhibited DBF transport with IC<sub>50</sub><sup>DBF</sup> of 63.4  $\pm$  5.1 and 31.5  $\pm$  0.6  $\mu$ M, respectively (Table 2), while they clearly stimulated the 5-CF transport with non-sigmoidal dose-response curves. Therefore, the IC<sub>50</sub><sup>5-CF</sup> could not be determined. Testosterone stimulated 5-CF transport with sigmoidal stimulation curves with EC<sub>50</sub> of 5.18  $\pm$  2.05  $\mu$ M.

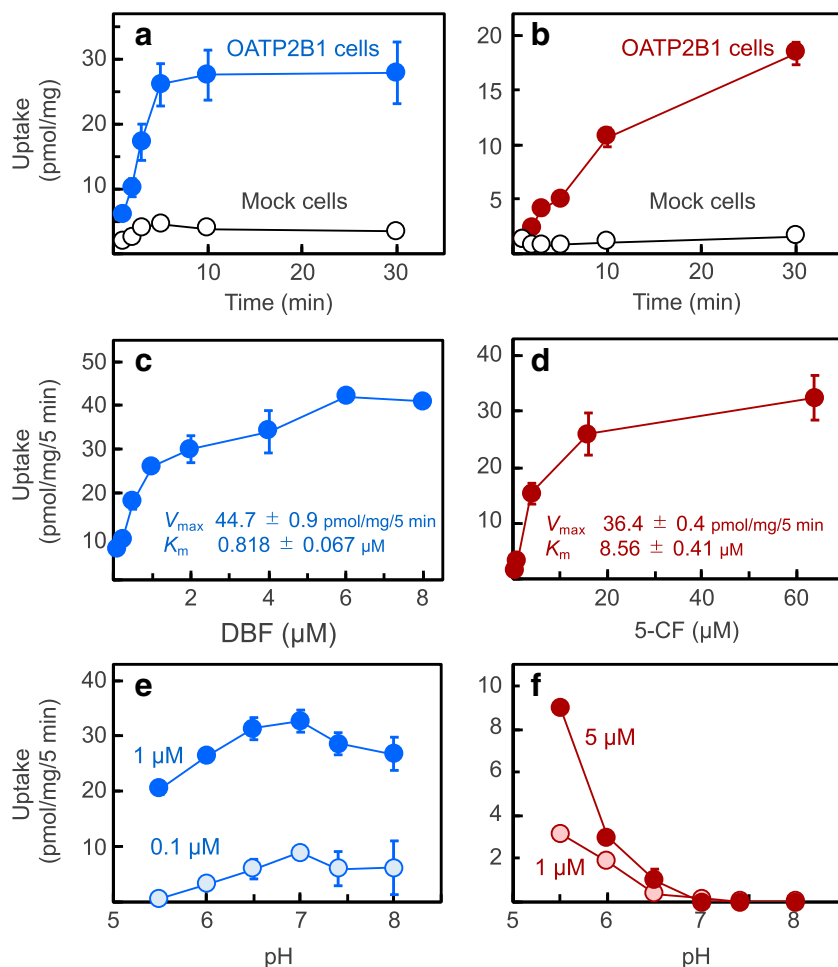
Furthermore, we examined the effects of flavonoids and other natural compounds in food on the DBF and 5-CF transports mediated by OATP2B1 (Fig. 7, Table 2). The flavonol aglycone quercetin and kaempferol, the flavanone aglycone hesperetin and naringenin, and the flavone aglycone baicalin, apigenin, and luteolin showed substrate-dependent modulation in a similar way to that of steroids. The concentration-effect curves of 5-CF showed non-sigmoidal curves with both inhibitory and stimulatory effects. More so, the flavonol glycoside rutin and quercitrin, the flavanone glycoside hesperidin and naringin, and flavone aglycone baicalin only showed inhibitory effects. Among the tested flavonoids, the smallest IC<sub>50</sub> was 1.39  $\pm$  0.16  $\mu$ M for DBF and 0.152  $\pm$  0.161  $\mu$ M for 5-CF by baicalin (Table 2).

**Table 1** Kinetic Parameters of Uptake by OATP2B1

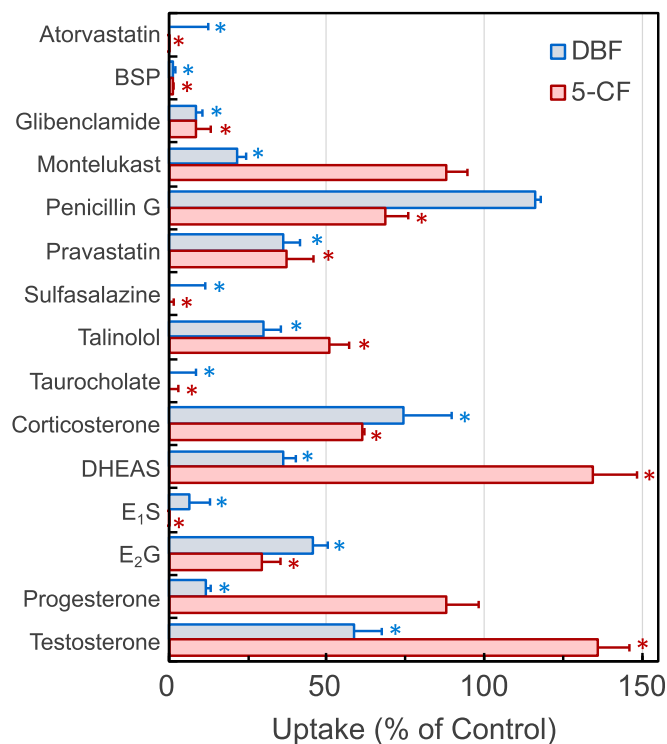
pH	Substrate	V <sub>max</sub>	K <sub>m</sub>
		pmol/mg/5 min	$\mu$ M
7.4	FL	72.1 $\pm$ 5.5	19.8 $\pm$ 3.1
7.4	OG	207 $\pm$ 11	87.1 $\pm$ 8.4
7.4	DCF	32.3 $\pm$ 2.4	6.79 $\pm$ 1.91
7.4	DBF	44.7 $\pm$ 0.9	0.818 $\pm$ 0.067
7.4	EY	70.2 $\pm$ 3.0	0.694 $\pm$ 0.115
5.5	5-CF	36.4 $\pm$ 0.4	8.56 $\pm$ 0.41
5.5	6-CF	7.12 $\pm$ 0.31	9.92 $\pm$ 0.37
5.5	CDCSF	8.09 $\pm$ 0.44	5.25 $\pm$ 1.06

Kinetic parameters were obtained from Fig. 3 as described in the Materials and Methods Section.

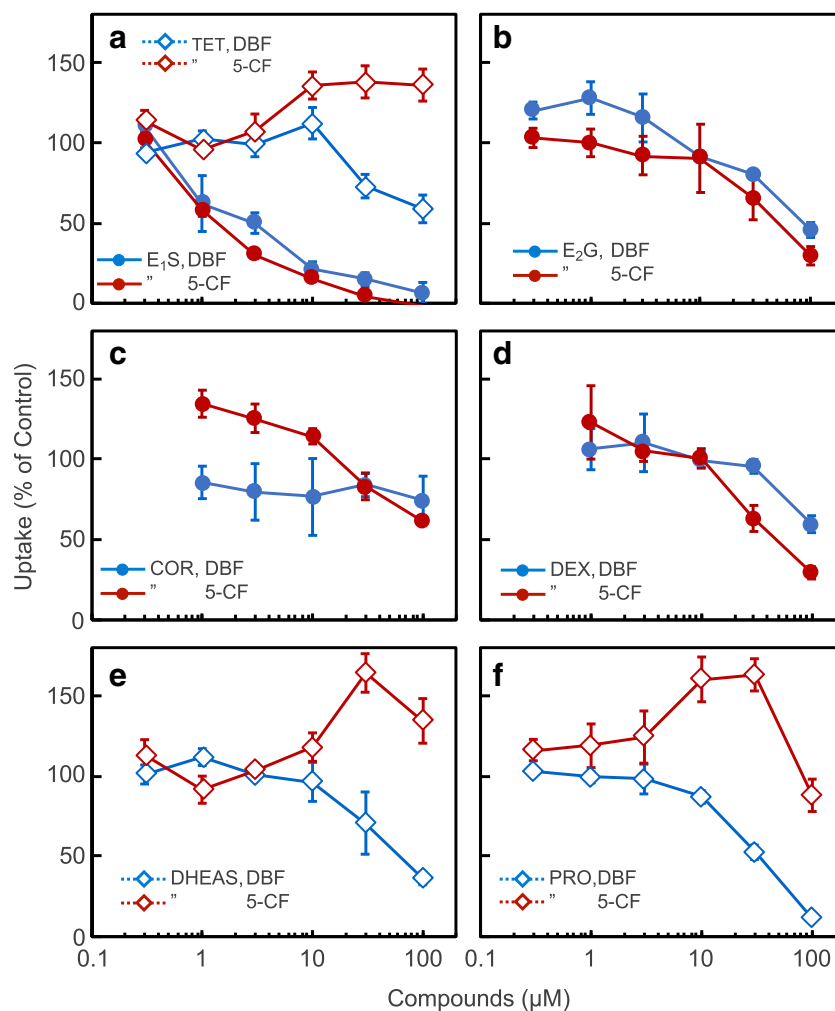
**Fig. 4** Characterization of DBF and 5-CF transport. The uptake study was performed at 0.1  $\mu\text{M}$  of DBF at pH 7.4 or 1  $\mu\text{M}$  of 5-CF at pH 5.5, 37°C for 5 min unless otherwise indicated. Time-dependent uptake of DBF (a) and 5-CF (b). Concentration-dependent uptake of DBF (c) and 5-CF (d). Effects of extracellular pH on the uptake of DBF (e) and 5-CF (f). The specific uptake was calculated by subtracting the uptake of Mock from that of HEK293-OATP2B1 and plotted for c, d, e, and f. Data are expressed as mean  $\pm$  SE ( $n = 3-6$ ).



**Fig. 5** *cis*-Inhibition of DBF and 5-CF transport. The uptake of DBF (0.1  $\mu\text{M}$ ) at pH 7.4 or 5-CF (1  $\mu\text{M}$ ) at pH 5.5 was measured for 5 min, at 37°C in the presence of the indicated compounds (100  $\mu\text{M}$ ). The specific uptake was calculated as in Fig. 4 and plotted as a percentage of control. The average of the control values for DBF and 5-CF uptake were  $3.29 \pm 0.92$  and  $6.69 \pm 0.30$  (pmol/mg/5 min), respectively. Data are expressed as mean  $\pm$  SE ( $n = 3-6$ ). \*:  $p < 0.05$  vs. control. BSP: bromosulphthalein, DHEAS: dehydroepiandrosterone sulfate, E<sub>1</sub>S: estrone 3-sulfate, E<sub>2</sub>G: estradiol-17 $\beta$ -glucuronide.



**Fig. 6** Effects of steroids on DBF and 5-CF transport. The uptake of DBF (0.1  $\mu\text{M}$ ) at pH 7.4 or 5-CF (1  $\mu\text{M}$ ) at pH 5.5 was measured for 5 min, at 37°C in the presence of (a) E<sub>1</sub>S (0.3–100  $\mu\text{M}$ ) or TET (0.3–100  $\mu\text{M}$ ), (b) E<sub>2</sub>G (0.3–100  $\mu\text{M}$ ), (c) COR (1–100  $\mu\text{M}$ ), (d) DEX (1–100  $\mu\text{M}$ ), (e) DHEAS (0.3–100  $\mu\text{M}$ ), and (f) PRO (0.3–100  $\mu\text{M}$ ). The specific uptake was calculated as in Fig. 4 and plotted as a percentage of control. The average of the control values for DBF and 5-CF uptake were  $4.14 \pm 0.06$  and  $7.05 \pm 0.30$  (pmol/mg/5 min), respectively. Data are expressed as mean  $\pm$  SE ( $n = 3$ –6). E<sub>1</sub>S: estrone 3-sulfate, TET: testosterone, E<sub>2</sub>G: estradiol-17 $\beta$ -glucuronide, COR: corticosterone, DEX: dexamethasone, DHEAS: dehydroepiandrosterone sulfate, PRO: progesterone.



Since the stimulatory effect on 5-CF disappeared with flavonoid glycoside, other combinations of aglycone and glycoside of natural compounds were also examined. Isoflavone is a B-ring isomer of flavone. An isoflavone aglycone daidzein and its glycoside daidzin exhibited weak but similar interaction properties. Dihydrochalcone has a structure in which the C-ring of the flavonoid is cleaved. Dihydrochalcone aglycone phloretin and its glycoside phlorizin only exhibited an inhibitory effect. Triterpenoids are different from the above compounds and so exhibited a different tendency of interaction. Triterpenoid aglycone GA inhibited both DBF and 5-CF transport, and its glycoside GL weakly stimulated and inhibited DBF transport.

### The Inhibitory and Stimulatory Effect of Naringenin

As representatives, we focused on naringenin to examine the mechanism of modulation (Fig. 8). For 5-CF transport, naringenin stimulated at 0.3 to 30  $\mu\text{M}$  and inhibited at 100  $\mu\text{M}$ . For DBF transport, it acts as an inhibitor. These kinetic analyses reveal that 30  $\mu\text{M}$  naringenin does not change  $K_m$  for

DBF or 5-CF ( $0.931 \pm 0.129$  to  $1.34 \pm 0.49$   $\mu\text{M}$  for DBF and  $5.96 \pm 0.94$  to  $6.47 \pm 1.04$   $\mu\text{M}$  for 5-CF). However, it decreased  $V_{max}$  of transport for DBF ( $34.2 \pm 0.8$  to  $22.0 \pm 1.1$  pmol/mg/5 min) while increased for 5-CF ( $36.2 \pm 1.9$  to  $129 \pm 4$  pmol/mg/5 min).

## DISCUSSION

The OATP family members are uptake transporters and are deeply involved in pharmacokinetics (19). In particular, the liver OATP1B1 and OATP1B3 are designated by regulatory authorities, such as the US Food and Drug Administration, as transporters with which to conduct drug interaction studies (20). The OATP2B1 is also considered as an important drug transporter with strong evidence of clinically-significant interactions (5). Izumi *et al.* reported that DCF, DBF, and OG are good fluorescent substrates of OATP1B1 (21). However, they investigated only at a pH 7.4, and carboxylated derivatives are not found to be substrates of OATP2B1. In this study, carboxylated fluorescein analogs are found to be good fluorescent

**Table 2** IC<sub>50</sub> Values of Tested Compounds

Classification	Compounds	IC <sub>50</sub> <sup>DBF</sup> μM	IC <sub>50</sub> <sup>5-CF</sup> μM
Flavonol	Kaempferol	2.38 ± 0.14	stimulation
	Quercetin	3.24 ± 0.35	stimulation
	Quercitrin	17.6 ± 3.1	43.6 ± 3.94
	Rutin	60.2 ± 0.8	22.8 ± 2.0
Flavanone	Hesperetin	12.9 ± 1.0	stimulation
	Hesperidin	6.21 ± 0.46	8.26 ± 0.89
	Naringenin	13.4 ± 1.4	stimulation
	Naringin	30.0 ± 0.3	10.3 ± 0.2
Flavone	Apigenin	3.06 ± 0.55	stimulation
	Baicalein	2.52 ± 0.15	stimulation
	Baicalin	1.39 ± 0.16	0.152 ± 0.161
	Luteolin	1.51 ± 0.33	stimulation
Isoflavone	Daidzein	12.2 ± 0.87	stimulation
	Daidzin	>100	>100
	Puerarin	>100	>100
Dihydrochalcone	Phloretin	1.98 ± 0.11	2.87 ± 0.82
	Phlorizin	25.8 ± 1.1	7.53 ± 0.83
Triterpenoid	Glycyrrhetic acid	stimulation	31.9 ± 7.1
	Glycyrrhizin	>100	26.5 ± 1.2

The IC<sub>50</sub> values were obtained from Fig. 7 as described in the [Materials and Methods](#) Section. Stimulation: IC<sub>50</sub> was not obtained because the dose-response curve was not a sigmoidal shape.

substrates for OATP2B1. Since acidic pH broadens substrate recognition and facilitates OATP2B1 transport, the local acidic environment of the small intestinal epithelium is convenient for OATP2B1. Therefore, 5-CF may be a useful tool for research on OATP2B1-mediated oral absorption.

The uptakes of FL and OG are not significant as accumulation ratio OATP2B1/Mock, at the concentration below about 1/20 of  $K_m$  (i.e. 1 μM), suggesting that the contribution of passive diffusion and/or intrinsic transporter-mediated uptake is higher than that of OATP2B1-mediated uptake at this concentration. As the concentration increases up to 90 μM, the difference of accumulation between cellular accumulation into Mock and HEK293-OATP2B1 is also increased, which suggests that the contribution of OATP2B1 to cellular accumulation is also increased. Consequently, the transporter-mediated concentration-dependent uptake is observed clearly, even with the relatively high Mock accumulation. The differences of accumulation into Mock and that into HEK293-OATP2B1 are fitted with simple Michaelis-Menten kinetics, which suggests that the contribution of intrinsic transporters of HEK293 is negligible in our assay system.

Understanding structure-activity relationships between substrates/inhibitors and transporters allows for a more rational design of selective ligands and control of their pharmacokinetic characteristics. We explore OATP-selective

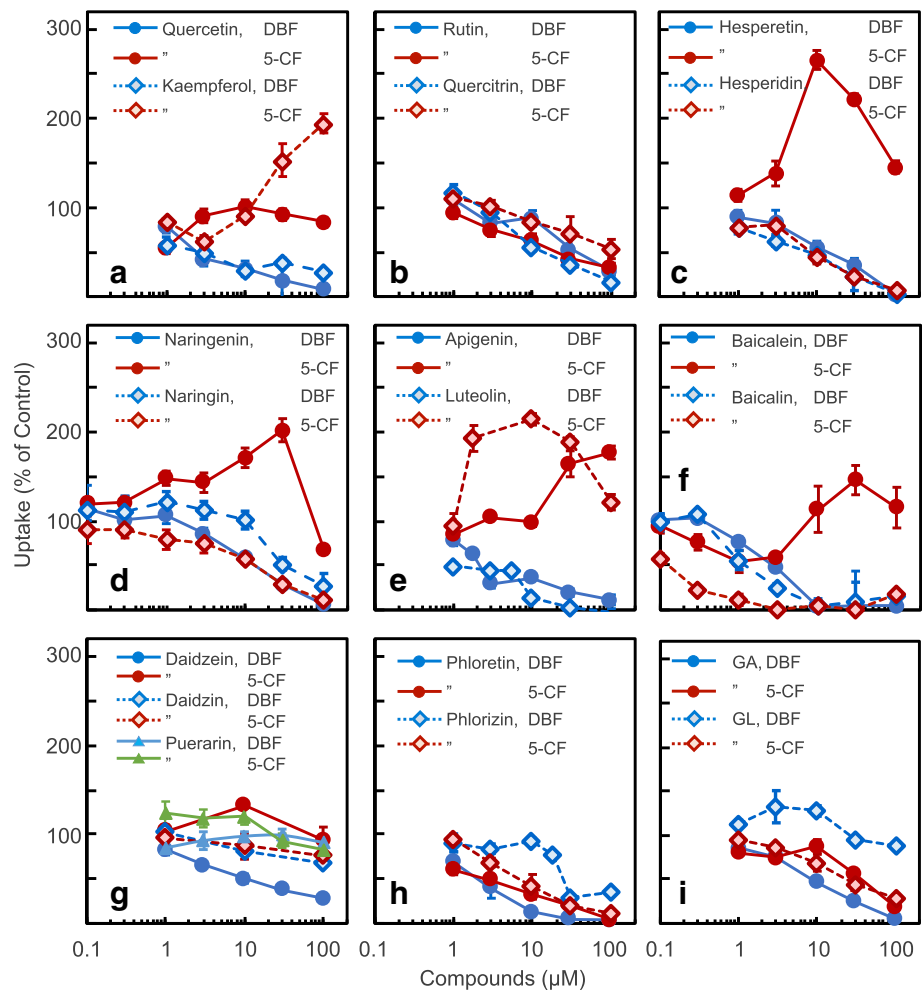
substitutions of fluorescein analogs. The kinetic analysis suggests that bromines at R<sub>2</sub> and R<sub>3</sub> drastically increase the affinity to OATP2B1. Similar to DBF, EY is also a good fluorescent substrate with nM order  $K_m$ , but it is highly liposoluble and easily accumulates in cells by passive diffusion. Therefore, DBF is superior as a probe substrate. Bromine-containing compound (BSP) is a well-known substrate of OATP2B1 with a  $K_m$  of nM order (22). Taken together, OATP2B1 prefers heavy halogenated (brominated) compounds, and bromine substitution may increase not only metabolic resistance but also oral bioavailability by improving selectivity to OATP2B1. At pH 5.5, two carboxylated analogs, 5-CF and 6-CF, have similar physicochemical profiles, such as charge or lipophilicity, and have a similar  $K_m$ , which suggests that the carboxyl group at R<sub>7</sub> and R<sub>8</sub> positions are similarly recognized by OATP2B1. However, 6-CF slows down turnover, which suggests that the substitution of R<sub>8</sub> interferes with conformational changes along the transport cycle of OATP2B1. Furthermore, 5-AF is not transported. These results suggest that OATP2B1 prefers an R<sub>7</sub>-negative charge.

Fluorescein analogs can form an equilibrium between quinoid and lactone species, while non-substrate sulfonylfluorescein analog does not form lactone species because they have dissociated sulfate groups instead of carboxylate group at the R<sub>5</sub> position. Thus, OATP2B1 may prefer lactone-form microspecies than a quinoid-form at pH 7.4. When the pH is decreased from pH 7.4 to 5.5 to an acidic pH, lactone species increase in number. The uptake by OATP2B1 of a non-lactone CDCSF is comparable to that of 6-CF at pH 5.5. Taken together, OATP2B1 could transport non-lactones with the presence of side-chain carboxyl groups at pH 5.5. These results may be useful in constructing a probe substrate with higher selectivity for OATP2B1 than other OATP subfamily members.

Among tested steroids, E<sub>1</sub>S and E<sub>2</sub>G show only inhibitory effects on OATP2B1 transport. These two compounds have large structures, such as a 3-sulfate group and 17-glucuronic acid, respectively. Although the number of tested compounds is small to explore a clear structure-activity relationship, these results suggest that a relatively large substitution at 3- and 17-position increases the inhibitory effect. Contrastively, three unconjugated androgens (testosterone, DHEAS, and progesterone) show concentration-dependent stimulatory effect on 5-CF transport while they showed an inhibitory effect on DBF transport by OATP2B1. OATP2B1 exhibits biphasic saturation kinetics for E<sub>1</sub>S and fexofenadine transport (17,23). These transports have pH-independent high-affinity and pH-dependent low-affinity components, which have different inhibitor and/or stimulator sensitivity. Grube *et al.* reported that testosterone inhibits the E<sub>1</sub>S transport by OATP2B1 (24). Later, Shirasaka *et al.* reported that testosterone stimulates pH-independent



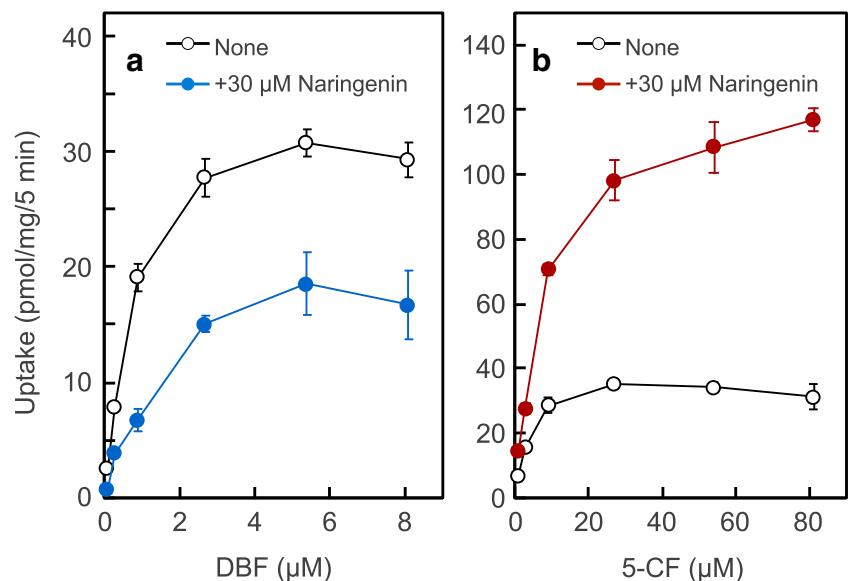
**Fig. 7** Effects of natural compounds on DBF and 5-CF transport. The uptake of DBF (0.1  $\mu\text{M}$ ) at pH 7.4 or 5-CF (1  $\mu\text{M}$ ) at pH 5.5 was measured for 5 min, at 37°C in the presence of (a) quercetin (1–100  $\mu\text{M}$ ) or kaempferol (1–100  $\mu\text{M}$ ), (b) rutin (1–100  $\mu\text{M}$ ) or quercitrin (1–100  $\mu\text{M}$ ), (c) hesperetin (1–100  $\mu\text{M}$ ) or hesperidin (1–100  $\mu\text{M}$ ), (d) naringenin (0.1–100  $\mu\text{M}$ ) or naringin (1–100  $\mu\text{M}$ ) or luteolin (1–100  $\mu\text{M}$ ), (f) baicalein or baicalin (0.1–100  $\mu\text{M}$ ), (g) daidzein (1–100  $\mu\text{M}$ ), daidzin (1–100  $\mu\text{M}$ ) or puerarin (1–100  $\mu\text{M}$ ), (h) phloretin (1–100  $\mu\text{M}$ ) or phlorizin (1–100  $\mu\text{M}$ ), and (i) GA (1–100  $\mu\text{M}$ ) or GL (1–100  $\mu\text{M}$ ). The specific uptake was calculated as in Fig. 4 and plotted as a percentage of control. The average of the control values for DBF and 5-CF uptake were  $3.61 \pm 0.88$  and  $8.21 \pm 1.29$  (pmol/mg/5 min), respectively. Data are expressed as mean  $\pm$  SE ( $n = 3$ –9). GA: glycyrrhetic acid, GL: glycyrrhizin.



high-affinity  $E_1S$  transport and inhibited pH-dependent low-affinity  $E_1S$  transport by OATP2B1 and proposed the presence of multiple binding sites on OATP2B1 (23).

Similarly, Hoshino *et al.* reported that progesterone modulates two  $E_1S$  transport components in the same manner and they proposed a model of OATP2B1 with multiple

**Fig. 8** Effects of naringenin on DBF and 5-CF transport. Michaelis–Menten curves showing the kinetics of DBF or 5-CF. The uptake of DBF (0.1, 0.3, 0.9, 2.7, 5.4, and 8.1  $\mu\text{M}$ ) and 5-CF (1, 3, 9, 27, 54, and 81  $\mu\text{M}$ ) was measured for 5 min in the presence or absence of the 30  $\mu\text{M}$  naringenin. The specific uptake was calculated as in Fig. 4 and plotted as mean  $\pm$  SE ( $n = 3$ ).



distinct binding sites of two E<sub>1</sub>S, some statins, PGE<sub>2</sub>, and fexofenadine (25). Although precise spatial location remains unclear, they predicted that two E<sub>1</sub>S sites are located around His579 and His618. By considering the pH-dependency, DBF and 5-CF binding sites may also be located around these histidine residues. Interestingly, DBF and 5-CF are the novel substrates with consistent pH-dependency and reversed testosterone/progesterone sensitivities to that of two E<sub>1</sub>S transport components. For future work, we shall combine the two concentrations of E<sub>1</sub>S, DBF, and 5-CF which are useful for identifying testosterone/progesterone binding sites and the underlying mechanisms of their substrate-dependent modulation.

From the viewpoint of clinical safety, the understanding of DDI/FDI potentials as the OATP inhibitors/modulators is crucial. Because natural compounds are components in food and often taken orally, they may affect the intestinal absorption of drugs. Therefore, we further investigate natural compounds as modulators of OATP2B1. As representatives, naringenin shows substrate-dependent interaction. Recently, similar biphasic effects of flavonoids on transporters were reported. Among them, the molecular mechanism for glucose transporter 1 (GLUT1) has been investigated extensively (26). Ojejabí *et al.* observed three flavonoids act as *cis*-allosteric activators and competitive inhibitors and proposed the mechanism involving multiple binding sites on GLUT1 tetramer (26). Similarly, multiple binding sites may be involved in the biphasic effect of naringenin. However, detailed mechanisms remain unclear and further investigations are required for precise prediction of substrate- and concentration-dependent interactions mediated by OATP2B1.

Substrate-dependent interactions that inhibit DBF and stimulate 5-CF are common properties to aglycone of flavanone, flavone, and flavonol. According to kinetic analysis, naringenin does not share a binding site with DBF nor 5-CF and is an allosteric modulator. Flavonoids are also modulators of other drug transporters. However, their structure-modulation relationship varies and has the potential to be used to predict drug pharmacokinetics and DDIs. Similar to OATP2B1, OATP1B1 has been reported as substrate-dependent interactions affected by the presence or absence of sugar moiety (27), which suggests that the modulator binding pockets of OATP isoforms are similar and overlap. For other drug transporter families, Multidrug and toxin extrusion 1 (encoded by *SLC47A1*) is only inhibited to some extent, and the position of the functional group, such as hydroxyl, highly effects inhibitory potencies (28). Also, P-glycoprotein (encoded by *ABCB1*) is inhibited, but the position and numbers of the functional group have little effect, and inhibitory potencies are corresponded with the partition

coefficients of flavonoids between *n*-octanol and PBS (29). Due to these variations of the effects of flavonoids on many drug transporters, careful handling is required to extrapolate *in vitro* results to *in vivo* because the same compounds have inhibitory, stimulatory, and/or inducing effects on multiple drug transporters.

There are limitations to the interpretation of the results obtained in this study. First, the dose-response curve is thought to be influenced by both stimulation and inhibition, and only an apparent effect, after offsetting both effects, can be observed. Second, the cytoplasmic/membrane surface localization of OATP2B1 molecule may be altered by the tested compounds (30), but this study does not examine the change of localization. Although a transport assay that can separate stimulation and inhibition, as well as localization change, will be required. The combination of various substrates and modulators with different selectivities including steroids and flavonoids would be useful for further *in vitro* studies on the binding pocket of OATP2B1 to better understand and predict DDI/FDIs at OATP2B1. In this context, two fluorescent substrates DBF and 5-CF are promising compounds that facilitate or help in identifying the responsible amino acid residues of multiple binding pockets on OATP2B1.

## CONCLUSIONS

In conclusion, DBF and 5-CF are fluorescent substrates of OATP2B1, which exhibit different profiles for pH dependency and inhibitor/modulator sensitivity. Several steroids and flavonoids modulate OATP2B1 substrate- and concentration-dependent manners. These results suggest the importance of using multiple substrates to predict DDI/FDIs mediated by OATP2B1. Taking advantage of their fluorescent nature, DBF and 5-CF are promising substrates for further investigation of multiple binding sites of OATP2B1.

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## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest** The authors state that they have no conflicts of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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