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Research Paper

Concentration-Dependent Effect of Naringin on Intestinal Absorption of β_1-Adrenoceptor Antagonist Talinolol Mediated by P-Glycoprotein and Organic Anion Transporting Polypeptide (Oatp)

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Purpose. The purpose of this study is to clarify the impact of P-gp and Oatp on intestinal absorption of the β_1-adrenoceptor antagonist talinolol.
Methods. P-gp-mediated transport was measured in LLC-PK1/MDR1 cells. Oatp-mediated uptake was evaluated with *Xenopus* oocytes expressing Oatp1a5. Rat intestinal permeability was measured by the *in situ* closed loop method. *In vivo* absorption was pharmacokinetically assessed by measuring plasma concentration after oral administration in rats.
Results. In LLC-PK1/MDR1 cells, the permeability of talinolol was markedly higher in the secretory direction than in the absorptive one. The uptake of talinolol by *Xenopus* oocytes expressing Oatp1a5 was significantly increased compared with that by water-injected oocytes. Naringin inhibited talinolol uptake by Oatp1a5 (IC_{50}=12.7 μM). The reported IC_{50} value of naringin for P-gp-mediated transport of talinolol is approximately 2,000 μM. Rat intestinal permeability of talinolol was significantly decreased in the presence of 200 μM naringin, but was significantly increased by 2,000 μM naringin. Similar results were obtained in *in vivo* absorption studies in rats.
Conclusion. The absorption behavior of talinolol can be explained by the involvement of both P-gp and Oatp, based on characterization of talinolol transport by Oatp1a5 and P-gp, and the effects of naringin.

KEY WORDS: intestinal absorption; naringin; Oatp; P-gp; talinolol.

INTRODUCTION

Intestinal absorption of drugs is not easily predicted, because it is affected by many physicochemical and physiological factors (1,2). Among the physiological factors, intestinal transporters, including both influx and efflux transporters, play an important role in the oral absorption of various drugs (3–5). In particular, P-glycoprotein (P-gp/MDR1 or ABCB1), an ATP-dependent efflux transporter which is located in the apical (AP) membrane of enterocytes, significantly affects the absorption of various compounds, including xenobiotics and clinically used drugs (6–10). These drugs also have the potential to exhibit drug–drug interactions (DDI) in the absorption process through the inhibition or induction of P-gp. Impairment of P-gp-mediated efflux owing to DDI might lead to potentially hazardous increases in the plasma levels of substrate drugs (11–13).

Talinolol (weak base; pK_a 9.4; log D=1.1 at pH 7.4 and 37°C; high water solubility at acidic pH; human bioavailability, 55±15%) is a long-acting and highly selective β_1-adrenergic antagonist, and is marketed as a racemate (14–16). S(−)-talinolol is slightly less well absorbed and faster eliminated than R(+)-talinolol, and the absolute bioavailability of the R(+) enantiomer is slightly higher than that of the S(−) enantiomer (17,18). Finally, it was concluded that stereoselectivity is of minor importance in talinolol disposition. Because both enantiomers are P-gp substrates, an increase in oral absorption of talinolol racemate is expected to be caused by DDI or drug–food interactions that lead to inhibition of P-gp (15,16). Our group previously reported a doubled maximum plasma concentration, an enhanced area under the plasma concentration–time curve (AUC), and a decreased apparent oral clearance for talinolol when it was coadministered with grapefruit juice (GFJ) in rats (19). Although GFJ is known to affect the pharmacokinetics of various drugs by inhibition of cytochrome P3A4 (CYP3A4) and/or P-gp, the

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ABBREVIATIONS: AP, Apical; AUC, Area under the plasma concentration–time curve; BL, Basolateral; DDI, Drug–drug interactions; GFJ, Grapefruit juice; MDR, Multidrug resistance; OATP/Oatp, Organic anion transporting polypeptide; P_{app}, Apparent permeability; P-gp, P-glycoprotein.

increase in absorption of talinolol was presumed to be mainly due to inhibition of P-gp-mediated efflux, because talinolol shows little first-pass metabolism in man, and less than 1% is excreted into urine in the form of hydroxylated metabolites, though the importance of other metabolic or excretory pathways (e.g., biliary, intestinal) remains to be established (14).

On the other hand, in a human clinical study, Schwarz *et al.* reported that GFJ ingestion markedly reduced the oral bioavailability of talinolol (20). Since recent investigations have shown that naringin, the main constituent flavonoid of GFJ, has a significant inhibitory effect on not only P-gp-mediated drug efflux, but also organic anion transporting polypeptide (OATP)-mediated uptake of drugs in intestine, the effect of GFJ on talinolol absorption might be complex, arising from effects on both P-gp and intestinal OATP-mediated transport of talinolol (21–23). We have shown that OATP/Oatp transporters influence the intestinal absorption of various drugs (5,24–27). Furthermore, we have reported that the interaction of influx and efflux transporters can lead to complex nonlinear patterns of intestinal absorption (28–30). Therefore, the apparently inconsistent effects of GFJ ingestion on talinolol absorption between human and rat may be due to differential effects of naringin or other constituents of GFJ on P-gp and OATP/Oatp transporters.

Similarly inconsistent results have been reported for DDI in talinolol absorption (31,32). The oral bioavailability of talinolol was reported to be increased after concomitant erythromycin administration in humans, presumably due to inhibition of intestinal P-gp (31). We have reported that verapamil increased the AUC of talinolol when the two compounds were orally coadministered in rats (32,33). In contrast, it has been found that coadministration of verapamil reduced the AUC of talinolol in humans (34). Because verapamil is known to be a potent inhibitor not only of P-gp, but also of OATP/Oatp, these findings indicate that intestinal P-gp and OATP/Oatp may both influence the intestinal absorption of talinolol (23,35).

The purpose of this study is to clarify the roles of P-gp and Oatp in the intestinal absorption of talinolol. By means of pharmacokinetic analysis, we also examined the concentration dependence of the effects of naringin on P-gp and Oatp-mediated absorption of talinolol in rats.

MATERIALS AND METHODS

Materials

Talinolol racemate was kindly provided by Arzneimittelwerk Dresden (AWD, Radebeul, Germany). Naringin was purchased from Chromadex (Irvine, CA, USA). MDR1-transfected LLC-PK1 (LLC-PK1/MDR1) and mock-transfected LLC-PK1 (LLC-PK1/mock) cells were from GenoMembrane, Inc. (Yokohama, Japan) and were used between passage numbers 7 and 10. Medium 199 was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). Transwells (12 well/plate, 3.0 μm pores, 0.9 cm^2 membrane surface area) were purchased from Nippon Becton Dickinson Company, Ltd. (Tokyo, Japan). Benzylpenicillin, streptomycin, G418 and gentamicin were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other compounds and reagents were obtained from Nacalai Tesque, Inc. (Kyoto, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma-Aldrich Co, Bio-Rad Laboratories (Hercules, CA), or Applied Biosystems (Foster City, CA).

LLC-PK1 Cell Culture

LLC-PK1/MDR1 and LLC-PK1/mock cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air using Medium 199 supplemented with 14.3 mM $NaHCO_3$, 10% FBS, 100 U/mL benzylpenicillin, 100 μg/mL streptomycin and 500 μg/mL G418. Cells were routinely subcultured at 90% confluency with trypsin (0.25%) EDTA (1 mM) and seeded onto plastic culture dishes (10 cm).

For transport studies, cells were seeded onto Transwell filter membrane inserts at a density of 3.6×10^5 cells/cm^2. Medium was replaced with fresh medium after 3 and 5 days, and cell monolayers were used for transport studies at 6 days after seeding.

Transport Experiments

The cell monolayers were preincubated in transport medium (Hanks' balanced salt solution; 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM NaCl, 0.385 mM Na_2HPO_4, 25 mM D-glucose, and 10 mM HEPES, pH 7.4) for 30 min at 37°C. After preincubation, the transepithelial electrical resistance was measured routinely before and after each experiment with a Millicell®-ERS system (Millipore Corporation, Bedford, MA) to ensure cell monolayer integrity. LLC-PK1/MDR1 and LLC-PK1/mock cells with transepithelial electrical resistance values >150 $\Omega\cdot cm^2$ and >200 $\Omega\cdot cm^2$ were used for experiments, respectively. Transport measurement was initiated by adding talinolol (10 μM) to the donor side and transport medium to the receiver side. Transport of talinolol was observed in two directions (apical [AP] to basal [BL]) and BL to AP. Samples were obtained from the donor side at 5 min (for measurement of initial concentration) and from the receiver side at 30, 60, 80, 100 and 120 min. Transport experiments were performed in the absence of a pH gradient (apical pH=basal pH=7.4). All experiments were performed at 37°C.

The apparent permeability (P_{app}, cm/s) of talinolol across cell monolayers was calculated using the following equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot C_D} \quad (1)$$

where Q is the amount of talinolol transported over time t (therefore dQ/dt is the amount of talinolol transported within a given time period [μmol/s]). C_D is the initial concentration of talinolol added to the donor compartment (μM), and A is the membrane surface area (0.9 cm^2).

Uptake Experiments in *Xenopus laevis* Oocytes

Preparation of oocytes, *in vitro* synthesis of Oatp1a5 (SLCO1a5) cRNA, and uptake experiments were conducted as described previously (26). In brief, for standard experi-

ments, defolliculated oocytes were injected with 50 nL of water containing 50 ng cRNA, then cultured 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. To initiate uptake experiments, the oocytes were transferred to a 12-well culture plate and 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) containing talinolol at room temperature for the designated time. The uptake was terminated by washing three times with ice-cold modified Barth's solution.

Kinetic parameters were estimated by means of nonlinear least-squares analysis using Kaleida Graph (Synergy Software, Reading, PA). The affinity of talinolol for Oatp1a5 (K_m) and the maximal velocity of Oatp1a5-mediated talinolol uptake (V_{max}) were obtained by fitting to the following equation:

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

where V is the initial uptake rate of talinolol (pmol/min/oocyte), and C is the concentration of talinolol in the medium (μM).

The inhibitory effect of naringin on talinolol uptake was expressed as percent of control, and the naringin concentration giving half-maximum inhibition (IC_{50}) was obtained by application of the following equation:

$$\% \text{ of control} = \frac{100 \times IC_{50}}{IC_{50} + [I]} \quad (3)$$

where $[I]$ is the naringin concentration (μM).

In Situ Intestinal Closed Loop Experiment

Male Wistar rats (220±20 g body weight) were housed three per cage with free access to commercial chow and tap water, and were maintained on a 12 h dark/light cycle (8:00 A.M.–8:00 P.M. light) in an air-controlled room (temperature, 24.0±1°C; humidity, 55±5%). All animal experimentation was carried out in accordance with the Declaration of Helsinki and with the Guide of Tokyo University of Science for the Care and Use of Laboratory Animals.

The permeability of rat intestinal membrane was evaluated by the *in situ* intestinal closed loop method. Male Wistar rats (body weight, 220±20 g) fasted overnight were anesthetized with pentobarbital. The abdominal cavity was opened and an intestinal loop (length: 10 cm) was made at the upper jejunum by cannulation with silicone tubing (i.d., 3 mm). The intestinal contents were removed by slow infusion of saline and air. The test solution (phosphate-buffered solution, adjusted to pH 6.5) containing talinolol (10 μM) in the absence or presence of naringin (20, 50, 200 or 2,000 μM) was introduced into the intestinal loop and both ends of the loop were ligated. After a certain period (usually 15 min), the luminal solution in the loop was collected. The permeability of talinolol was evaluated in terms of the percentage of dose absorbed, calculated by subtracting the remaining amount of talinolol from the administered amount. The following equation was used to calculate the permeability:

$$\text{Permeability} = \frac{k_a \cdot V}{2\pi rl} \quad (4)$$

where k_a is the first-order absorption rate constant of talinolol estimated from the percentage of the dose absorbed during the defined period. V is the volume of talinolol solution introduced to the loop, and r and l represent the radius and length of the used segment of intestine, respectively; thus, $2\pi rl$ corresponds to its surface area. The length was 10 cm, and the values of the radius of each intestinal segment reported by Fagerholm *et al*. were used (0.18 cm for jejunum) (36).

Pharmacokinetic Study

The right jugular vein of male Wistar rats (220±20 g body weight) was cannulated. The animals were allowed to recover before experiments. Groups of rats (n=4) were administered an oral dose of talinolol solution (10 mg/kg, 2 mg/mL, pH 7.0) in the absence or presence of naringin (0.0581, 0.145, 0.581 and 5.81 mg/kg [corresponding to 20, 50, 200 and 2,000 μM, respectively]) by gavage. Blood samples (250 μL) were collected from the cannula into heparinized tubes before and 30, 60, 90, 120, 150, 180, 240 and 360 min after talinolol oral administration. Each blood sample was replaced with an equal volume of saline and heparinized saline was used to maintain patency of the catheter. Blood samples were centrifuged at 750 ×g for 10 min. The resultant plasma was stored at −30°C until analysis.

Plasma concentrations of talinolol were analyzed using a non-compartmental method. Area under the plasma concentration–time curve from 0 to 6 h (AUC_{0-6}) was calculated using the linear trapezoidal rule. The maximum plasma drug concentration (C_{max}) and time to reach maximum plasma concentration (t_{max}) were obtained directly from the experimental data. The apparent elimination half-life of the log–linear phase ($t_{1/2}$) was calculated based on the terminal elimination rate constant determined by log–linear regression of the final data points (at least 3).

LC/MS/MS Analysis

The concentration of talinolol in all samples was quantified with a liquid chromatography–tandem mass spectrometry (LC/MS/MS) system consisting of MDS-Sciex API 3200™ triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) coupled with a LC-20AD high-performance liquid chromatography system (Shimadzu Co., Kyoto, Japan). A Capcellpak MGII (C_{18}, 100 mm×4.6 mm, 5 μm, Shiseido, Tokyo, Japan) or Mercury MS (C_{18}, 10×4.0 mm, 5 μm, Phenomenex, Torrance, CA) was used as the analytical column, and the mobile phase was composed of 0.1% formic acid in water and acetonitrile. In the LC/MS/MS system, electrospray ionization in the positive ion mode was employed. Mass transitions (Q1/Q3) of m/z 364.3/100.2 (or 363.9/309.3) and 260.2/116.1 were used for talinolol and propranolol, respectively. The limit of quantitation was 1 ng/mL for both analytes. 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 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

Effect of P-gp on Transcellular Transport of Talinolol

The effect of P-gp on transcellular transport of talinolol was examined by using LLC-PK1/MDR1 cells. Table I summarizes the absorptive (AP to BL) and secretory (BL to AP) permeability of talinolol (10 μM) across monolayers of LLC-PK1/MDR1 and LLC-PK1/mock cells. In LLC-PK1/MDR1 cells, the permeability of talinolol in the BL to AP direction was markedly higher than that in the AP to BL direction, while the permeabilities in LLC-PK1/mock cells were comparable in both directions, with permeability ratios (BL to AP/AP to BL) of approximately 4.9 and 1.3 in LLC-PK1/MDR1 and LLC-PK1/mock cells, respectively. In the presence of cyclosporin A (10 μM), an inhibitor of P-gp, on both sides, the permeability ratio of talinolol was decreased to 2.6 from 4.9 in LLC-PK1/MDR1 transport studies. These findings confirm that talinolol is a substrate of P-gp, and that its permeability is significantly affected by P-gp (37).

Uptake of Talinolol by *Xenopus laevis* Oocytes Expressing Oatp1a5

To investigate whether talinolol is transported by Oatp1a5, the time course of talinolol uptake was examined using *Xenopus* oocytes expressing Oatp1a5. As shown in Fig. 1, the uptake of talinolol (100 μM) by *Xenopus* oocytes expressing Oatp1a5 was significantly increased compared with that by water-injected oocytes, suggesting that talinolol is a substrate of Oatp1a5. Since the Oatp1a5-mediated uptake of talinolol increased linearly up to at least 90 min, the uptake at 60 min was routinely used for the measurement of the uptake rate in subsequent studies. The Oatp1a5-mediated uptake of talinolol was found to be saturable, with K_m and V_{max} values of 2,000±819 μM and 59.9±9.0 pmol/oocyte/60 min, respectively (Fig. 2).

Next, the inhibitory effect of naringin, the main constituent of GFJ, on Oatp1a5-mediated uptake of talinolol (100 μM) was investigated. As shown in Fig. 3, naringin inhibited Oatp1a5-mediated uptake of talinolol (100 μM) with IC_{50} value of 12.7±3.1 μM.

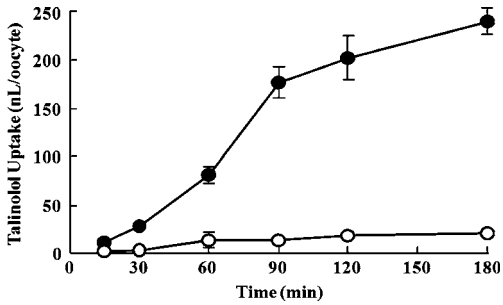

Fig. 1. Time course of talinolol uptake by *Xenopus* oocytes expressing Oatp1a5. Uptake of talinolol (100 μM) by water-injected (*open circles*) or Oatp1a5-cRNA-injected (*filled circles*) oocytes was measured at room temperature and pH 6.5. Data are shown as means± SEM (n=8–10).

Concentration-Dependent Effect of Naringin on Intestinal Permeability of Talinolol in Rats

To evaluate the concentration dependence of the effect of naringin on intestinal absorption of talinolol, rat intestinal permeability of talinolol was measured by means of the *in situ* closed loop method. It was reported that the expression level of P-gp in ileum is higher than that in jejunum of rats, so we expected that the impact of influx transporters such as Oatp1a5 on the intestinal absorption of talinolol would be more clearly observable in jejunum (10). Therefore, in the present study, the rat intestinal permeability of talinolol was measured in the jejunum.

As shown in Fig. 4, the permeability of talinolol in rat jejunum in the absence of naringin was 1.29×10^{-4} cm/s. In the presence of 200 μM naringin, the permeability was significantly decreased to 0.73×10^{-4} cm/s. In the presence of 2,000 μM naringin, the permeability was increased to 2.26×10^{-4} cm/s.

Concentration-Dependent Effect of Naringin on Talinolol Absorption in Rats

When talinolol was administered orally to rats (10 mg/kg, 5 mL/kg) together with 200 μM or 2,000 μM naringin, the AUC_{0-6} of talinolol was increased by 193 and 187%, respectively. The maximum plasma concentration (C_{max}) of talinolol was also increased by 181 and 168%, respectively (Table II). In contrast, naringin at 50 μM significantly lowered

Table I. Impact of P-gp on Transcellular Transport of Talinolol in LLC-PK1/MDR1 and LLC-PK1/mock Cells

Cell lines	Talinolol[a]	P_{app} ($\times10^{-7}$ cm/s) AP to BL	P_{app} ($\times10^{-7}$ cm/s) BL to AP	Ratio[c]
LLC-PK1/MDR1	Alone	1.09±0.10	5.33±0.15	4.91
	+Cyclosporin A[b]	1.57±0.06	4.08±0.17	2.60
LLC-PK1/mock	Alone	1.15±0.07	1.52±0.08	1.32

[a] Initial donor concentration of talinolol was 10 μM
[b] Applied concentration of cyclosporin A was 10 μM
[c] The permeability ratio (BL to AP/AP to BL). Data are represented as means±SEM (n=3)

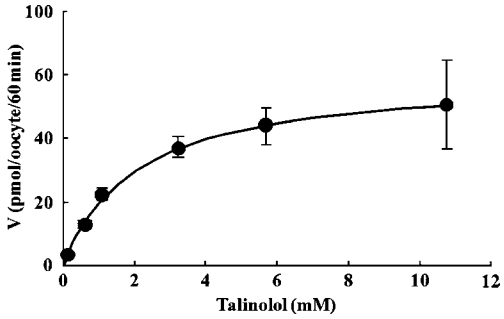

Fig. 2. Concentration dependence of Oatp1a5-mediated talinolol uptake by *Xenopus* oocytes expressing Oatp1a5. Uptake of talinolol by water-injected or Oatp1a5-cRNA-injected oocytes was measured at various concentrations for 60 min at room temperature and pH 6.5. Oatp1a5-mediated uptake was determined by subtracting the uptake by water-injected oocytes from that by Oatp1a5-cRNA-injected oocytes. Data are represented as means±SEM (n=8–10).

the C_{max} and AUC_{0-6} values of talinolol, while there were no significant changes in C_{max} or AUC_{0-6} values of talinolol in the presence of 20 μM naringin (Fig. 5). Naringin at 50 μM decreased the mean AUC_{0-6} and C_{max} values of talinolol to 43.4% and 43.5% of the control, respectively, suggesting that an influx transport process may be inhibited (Table II). On the other hand, naringin at all concentrations tested here did not alter the time to reach maximum plasma concentration (t_{max}) or the apparent elimination half-life ($t_{1/2}$) of talinolol (Table II).

DISCUSSION

Intestinal OATP has recently been suggested to influence the pharmacokinetic profiles of several orally administered drugs. The β_1-adrenergic antagonist talinolol was shown to be a P-gp substrate in various *in vitro* studies, and in agreement with that finding, the bioavailability of talinolol was found to be enhanced upon coadministration with GFJ in rats (19). However, in a human clinical study, it was found that GFJ ingestion lowered talinolol absorption (20). Because naringin, the main constituent of GFJ, affects both P-gp and OATP/Oatp located on the apical membrane of enterocytes, the apparently inconsistent findings may be explained by differences in the interactions of naringin and talinolol at P-gp

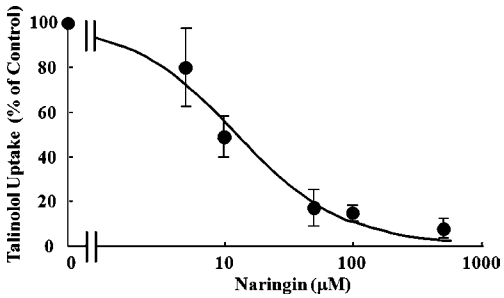

Fig. 3. Inhibitory effects of naringin on talinolol uptake by *Xenopus* oocytes expressing Oatp1a5. Uptake of talinolol (100 μM) by water-injected or Oatp1a5-cRNA-injected oocytes was measured in the absence or presence of various concentrations of naringin for 60 min at room temperature and pH 6.5. Oatp1a5-mediated uptake was determined by subtracting the uptake by water-injected oocytes from that by Oatp1a5-cRNA-injected oocytes. Data are shown as means± SEM (n=8–10).

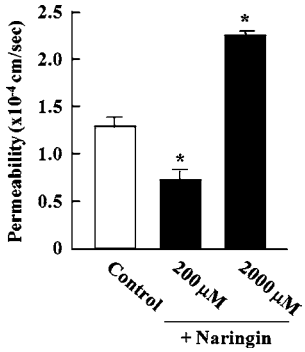

Fig. 4. Concentration-dependent effects of naringin on talinolol absorption in rat small intestine (jejunum). Permeability of talinolol (10 μM, pH 6.5) in rat small intestine (jejunum) was determined by means of an *in situ* closed loop method in the absence or presence of naringin (200 and 2,000 μM) for 15 min at 37°C. *P<0.05, significantly different from permeability without naringin. Data are shown as means±SEM (n=3).

and OATP/Oatp in the two species (19,21–23). In the present study, we performed various *in vitro*, *in situ* and *in vivo* talinolol–naringin interaction studies in order to understand the impact of P-gp and Oatp on intestinal absorption of talinolol. Although intestinal mRNA and/or protein expression of various Oatp family members (e.g. Oatp1a1, Oatp1a4, Oatp1a5, Oatp2b1 and Oatp4a1) has been reported in rats, the localizations of these proteins have not been fully clarified yet, except for Oatp1a5 (38–40). We hypothesized that Oatp1a5, which is known to be expressed at the apical membrane, contributes to intestinal talinolol absorption in rats (40).

As shown in Table I, we confirmed that talinolol is a substrate of P-gp. However, even in the presence of cyclosporin A, the permeability ratio was 2.60 and BL to AP transport was still predominant in LLC-PK1/MDR1 cells, implying the involvement of other efflux transporter(s). Since the permeability ratio of talinolol in LLC-PK1/mock cells was around unity in the absence of cyclosporin A, it is reasonable to consider that no endogenous transporters affected talinolol transport. Thus, the results of inhibition studies with LLC-PK1/MDR1 cells are considered to reflect only the role of P-gp. As shown in Figs. 1 and 2, talinolol was transported by *Xenopus* oocytes expressing Oatp1a5. Thus, talinolol is a substrate of both P-gp and Oatp1a5. Naringin inhibited the Oatp1a5-mediated uptake of talinolol with an IC_{50} value of 12.7 μM (Fig. 3). In contrast, it has been reported to inhibit P-gp-mediated transport of talinolol with an IC_{50} value of approximately 2,000 μM (21). The difference in the IC_{50} values of naringin for P-gp and Oatp1a5-mediated talinolol transport may account for the complex concentration-dependent effect of naringin on the intestinal absorption process of talinolol.

At a moderate concentration of naringin (e.g. 200 μM), inhibition of Oatp1a5 would predominate over that of P-gp, leading to a decrease of talinolol absorption. In contrast, at a high naringin concentration, both P-gp and Oatp1a5 would be inhibited, so that intestinal absorption of talinolol would be increased, mainly due to the inhibition of P-gp-mediated efflux. In the present study, the rat intestinal permeability of talinolol measured by the *in situ* closed loop method was indeed significantly decreased in the presence of 200 μM naringin, while a significant increase was observed in the

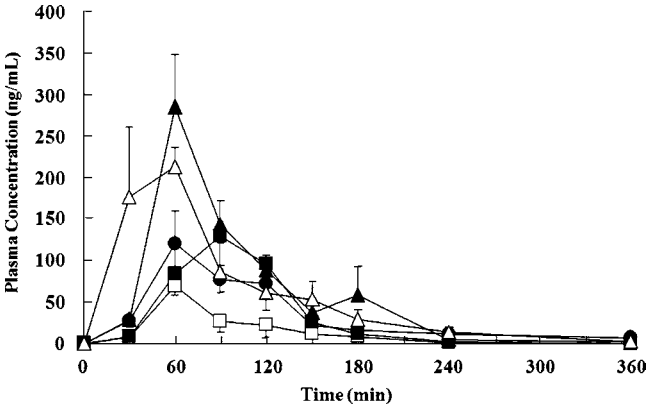

Fig. 5. Mean plasma concentration–time profiles of talinolol in rats after oral administration. Talinolol was administered at a dose of 10 mg/kg (5 mL/kg) in saline (pH 7.0) in the absence (●) or presence of naringin at 20 (■), 50 (□), 200 (▲) and 2,000 μM (Δ). Data are shown as means±SEM (n=4).

presence of 2,000 μM naringin (Fig. 4). Although we did not obtain direct evidence of the involvement of Oatp1a5 in the absorption of talinolol in this *in situ* study, these results are consistent with a role of Oatp1a5 in talinolol uptake. However, we cannot rule out the possibility that other influx transporters susceptible to naringin might be involved in the absorption of talinolol. On the other hand, de Castro *et al.* reported that naringin content was highly variable (from 174 to 1492 μM) among 14 brands of GFJ (41). Therefore, the inhibitory effect of GFJ on P-gp and Oatp1a5-mediated talinolol absorption might well depend on the naringin concentration in ingested GFJ.

To confirm these findings, we performed an *in vivo* pharmacokinetic study of talinolol in rats with coadministration of naringin at various concentrations (Fig. 5 and Table II). The plasma concentration of talinolol after oral administration was significantly increased by coadministration of 200 μM naringin, as well as 2,000 μM naringin, while intestinal permeability of talinolol measured by the *in situ* closed loop method was significantly decreased in the presence of 200 μM naringin (Fig. 5 and Table II). In a recent study, the luminal drug concentration was measured directly in each segment of the GI tract after oral administration in fasted rats (42). This study revealed that only 20–25% of ingested water remained in the lower part of the small intestine and further demonstrated that due to the absorption of water, luminal concentration of drugs with low permeability (e.g. atenolol) was elevated and exceeded the initial dosing concentration. Because naringin is a poorly permeable compound (permeability of 8.1×10^{-8} cm/s in Caco-2 cells), its local luminal concentration may exceed the dosing concentration (43). This simply means that when 200 μM naringin is orally administered to rats, the luminal concentration might be higher than 200 μM, so that not only Oatp1a5, but also P-gp may be inhibited, resulting in an increase of the plasma concentration of talinolol (Fig. 5 and Table II). A decrease of plasma talinolol concentration by coadministration of 50 μM naringin would be consistent with this hypothesis if the luminal concentration of naringin reached as much as 200 μM, where Oatp1a5 rather than P-gp might be predominantly inhibited (Fig. 5 and Table II).

In this discussion, we have assumed that the systemic clearance of talinolol is not affected by naringin. Although we did not examine the influence of naringin on talinolol disposition (by administration of intravenous talinolol with naringin) in the present study, the lack of any significant difference between $t_{1/2}$ of talinolol after oral administration with and without naringin supports the view that naringin has no effect on the systemic clearance of talinolol. However, if talinolol is highly distributed to the organ mainly responsible for clearance (probably kidney), $t_{1/2}$ of talinolol may not be changed, because the distribution volume as well as clearance would both be decreased. We have little information about the distribution volume of talinolol in the kidney, so further studies will be needed to exclude the possible inhibition of the systemic clearance of talinolol by naringin.

In our previous study, the concentration-dependent uptake curves in the presence of two counteracting transporters were simulated using a bidirectional membrane transport model. With respect to permeation through the cellular membrane and the influence of inhibitors, the present findings indicate that qualitatively and/or quantitatively different observations may be explained on the basis of concentration-dependent interaction with two oppositely directed transport systems (30). The present *in situ* and *in vivo* observations of the concentration-dependent effects of naringin on P-gp and Oatp1a5-mediated absorption of talinolol in rats are consistent with the previous considerations.

Table II. Pharmacokinetic Parameters of Talinolol after Oral Administration in the Absence and Presence of Naringin at Various Concentrations in Rats

Naringin Concentration (μM)	Pharmacokinetics Data of Talinolol[a]			
	AUC_{0-6} (ng·h/mL)	C_{max} (ng/mL)	t_{max} (hr)	$t_{1/2}$ (h)
0	175±22	157±24	1.25±0.25	0.682±0.003
20	193±32	154±12	1.67±0.29	0.674±0.002
50	76.0±16.0*	68.3±4.0*	1.00±0.00	0.670±0.009
200	338±65*	284±55*	1.00±0.00	0.679±0.005
2,000	326±24**	264±37*	1.25±0.43	0.684±0.002

AUC_{0-6} Area under plasma concentration–time curve from 0 to 6 h, *C_{max}* peak plasma drug concentration, *t_{max}* time to reach maximum plasma concentration, *$t_{1/2}$* elimination half-life

[a] Talinolol was administered as a racemic mixture at 10 mg/kg; 5 mL/kg. *P<0.05, significantly different from values without naringin (0 μM naringin). **P<0.01, significantly different from values without naringin (0 μM naringin). Data are represented as means±SEM (n=4)

Assuming that talinolol absorption is regulated by P-gp and Oatp1a5, the difference between the permeability in the absence (control) and presence of 200 μM naringin can be regarded as representing Oatp1a5-mediated absorption of talinolol, and the difference between the permeability in the presence of 200 and 2,000 μM naringin can be regarded as representing P-gp-mediated excretion of talinolol. Therefore, from the results obtained in Fig. 4, it appears that P-gp activity plays a significantly more important role than Oatp1a5 activity in the intestinal absorption of talinolol in rats. However, Oatp1a5-mediated transport must contribute substantially to the high intestinal permeability of talinolol in rats, because 50 μM naringin decreased the mean AUC_{0-6} value of talinolol to approximately 40% of the control, as shown in Table II.

In conclusion, although contributions of other intestinal Oatp members and/or other influx transporters to talinolol absorption cannot be ruled out, our findings support that Oatp1a5 and P-gp are both major determinants of the intestinal absorption of talinolol. In addition, the inhibitory effect of GFJ on P-gp and Oatp1a5-mediated talinolol absorption may depend on the naringin concentration in particular lots of ingested GFJ.

REFERENCES

1. P. Macheras, and P. Argyrakis. Gastrointestinal drug absorption: is it time to consider heterogeneity as well as homogeneity. *Pharm. Res*. **14**:842–847 (1997). doi:10.1023/A:1012183313218.
2. H. Zhou. Pharmacokinetic strategies in deciphering atypical drug absorption profiles. *J. Clin. Pharmacol*. **43**:211–227 (2003). doi:10.1177/0091270002250613.
3. A. Tsuji, and I. Tamai. Carrier-mediated intestinal transport of drugs. *Pharm. Res*. **13**:963–977 (1996). doi:10.1023/A:1016086003070.
4. K. Naruhashi, I. Tamai, N. Inoue, H. Muraoka, Y. Sai, N. Suzuki, and A. Tsuji. Active intestinal secretion of new quinolone antimicrobials and the partial contribution of P-glycoprotein. *J. Pharm. Pharmacol*. **53**:699–709 (2001). doi:10.1211/0022357011775820.
5. T. Tani, L. K. Gram, H. Arakawa, A. Kikuchi, M. Chiba, Y. Ishii, B. Steffansen, and I. Tamai. Involvement of organic anion transporting polypeptide 1a5 (Oatp1a5) in the intestinal absorption of endothelin receptor antagonist in rats. *Pharm. Res*. **25**:1085–1091 (2008). doi:10.1007/s11095-007-9472-4.
6. Y. Tanigawara. Role of P-glycoprotein in drug disposition. *Ther. Drug. Monit*. **22**:137–140 (2000). doi:10.1097/00007691-200002000-00029.
7. A. H. Schinkel. P-Glycoprotein, a gatekeeper in the blood–brain barrier. *Adv. Drug Deliv. Rev*. **36**:179–194 (1999). doi:10.1016/S0169-409X(98)00085-4.
8. M. F. Fromm. P-glycoprotein: a defense mechanism limiting oral bioavailability and CNS accumulation of drugs. *Int. J. Clin. Pharmacol. Ther*. **38**:69–74 (2000).
9. Y. Shirasaka, T. Sakane, and S. Yamashita. Effect of P-glycoprotein expression levels on the concentration-dependent permeability of drugs to the cell membrane. *J. Pharm. Sci*. **97**:553–565 (2008). doi:10.1002/jps.21114.
10. Y. Shirasaka, Y. Masaoka, M. Kataoka, S. Sakuma, and S. Yamashita. Scaling of *in vitro* membrane permeability to predict P-glycoprotein-mediated drug absorption *in vivo*. *Drug Metab. Dispos*. **36**:916–922 (2008). doi:10.1124/dmd.107.020040.
11. M. Verschraagen, C. H. Koks, J. H. Schellens, and J. H. Beijnen. P-glycoprotein system as a determinant of drug interactions: the case of digoxin–verapamil. *Pharmacol. Res*. **40**:301–306 (1999). doi:10.1006/phrs.1999.0535.
12. D. K. Yu. The contribution of P-glycoprotein to pharmacokinetic drug–drug interactions. *J. Clin. Pharmacol*. **39**:1203–1211 (1999). doi:10.1177/00912709922012006.
13. S. V. Ambudkar, S. Dey, C. A. Hrycyna, M. Ramachandra, I. Pastan, and M. M. Gottesman. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu. Rev. Pharmacol. Toxicol*. **39**:361–398 (1999). doi:10.1146/annurev.pharmtox.39.1.361.
14. B. Trausch, R. Oertel, K. Richter, and T. Gramatté. Disposition and bioavailability of the beta 1-adrenoceptor antagonist talinolol in man. *Biopharm. Drug Dispos*. **16**:403–414 (1995). doi:10.1002/bdd.2510160505.
15. K. Westphal, A. Weinbrenner, T. Giessmann, M. Stuhr, G. Franke, M. Zschiesche, R. Oertel, B. Terhaag, H. K. Kroemer, and W. Siegmund. Oral bioavailability of digoxin is enhanced by talinolol: evidence for involvement of intestinal P-glycoprotein. *Clin. Pharmacol. Ther*. **68**:6–12 (2000). doi:10.1067/mcp.2000.107579.
16. W. Weitschies, A. Bernsdorf, T. Giessmann, M. Zschiesche, C. Modess, V. Hartmann, C. Mrazek, D. Wegner, S. Nagel, and W. Siegmund. The talinolol double-peak phenomenon is likely caused by presystemic processing after uptake from gut lumen. *Pharm. Res*. **22**:728–735 (2005). doi:10.1007/s11095-005-2588-5.
17. U. Wetterich, H. Spahn-Langguth, E. Mutschler, B. Terhaag, W. Rösch, and P. Langguth. Evidence for intestinal secretion as an additional clearance pathway of talinolol enantiomers: concentration- and dose-dependent absorption *in vitro* and *in vivo*. *Pharm. Res*. **13**:514–522 (1996). doi:10.1023/A:1016029601311.
18. M. Zschiesche, G. L. Lemma, K. J. Klebingat, G. Franke, B. Terhaag, A. Hoffmann, T. Gramatté, H. K. Kroemer, and W. Siegmund. Stereoselective disposition of talinolol in man. *J. Pharm. Sci*. **91**:303–311 (2002). doi:10.1002/jps.10054.
19. H. Spahn-Langguth, and P. Langguth. Grapefruit juice enhances intestinal absorption of the P-glycoprotein substrate talinolol. *Eur. J. Pharm. Sci*. **12**:361–367 (2001). doi:10.1016/S0928-0987(00)00191-3.
20. U. I. Schwarz, D. Seemann, R. Oertel, S. Miehlke, E. Kuhlisch, M. F. Fromm, R. B. Kim, D. G. Bailey, and W. Kirch. Grapefruit juice ingestion significantly reduces talinolol bioavailability. *Clin. Pharmacol. Ther*. **77**:291–301 (2005). doi:10.1016/j.clpt.2004.11.111.
21. W. V. de Castro, S. Mertens-Talcott, H. Derendorf, and V. Butterweck. Grapefruit juice–drug interactions: grapefruit juice and its components inhibit P-glycoprotein (ABCB1) mediated transport of talinolol in Caco-2 cells. *J. Pharm. Sci*. **96**:2808–2817 (2007). doi:10.1002/jps.20975.
22. G. K. Dresser, D. G. Bailey, B. F. Leake, U. I. Schwarz, P. A. Dawson, D. J. Freeman, and R. B. Kim. Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to decrease the oral availability of fexofenadine. *Clin. Pharmacol. Ther*. **71**:11–20 (2002). doi:10.1067/mcp.2002.121152.
23. D. G. Bailey, G. K. Dresser, B. F. Leake, and R. B. Kim. Naringin is a major and selective clinical inhibitor of organic anion-transporting polypeptide 1A2 (OATP1A2) in grapefruit juice. *Clin. Pharmacol. Ther*. **81**:495–502 (2007). doi:10.1038/sj.clpt.6100104.
24. I. Tamai, J. Nezu, H. Uchino, Y. Sai, A. Oku, M. Shimane, and A. Tsuji. Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. *Biochem. Biophys. Res. Commun*. **273**:251–260 (2000). doi:10.1006/bbrc.2000.2922.
25. D. Kobayashi, T. Nozawa, K. Imai, J. Nezu, A. Tsuji, and I. Tamai. 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 (2003). doi:10.1124/jpet.103.051300.
26. T. Nozawa, K. Imai, J. Nezu, A. Tsuji, and I. Tamai. Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. *J. Pharmacol. Exp. Ther*. **308**:438–445 (2004). doi:10.1124/jpet.103.060194.
27. T. Maeda, K. Takahashi, N. Ohtsu, T. Oguma, T. Ohnishi, R. Atsumi, and I. Tamai. Identification of influx transporter for the quinolone antibacterial agent levofloxacin. *Mol. Pharm*. **4**:85–94 (2007). doi:10.1021/mp060082j.
28. I. Tamai, A. Saheki, R. Saitoh, Y. Sai, I. Yamada, and A. Tsuji. Nonlinear intestinal absorption of 5-hydroxytryptamine receptor antagonist caused by absorptive and secretory transporters. *J. Pharmacol. Exp. Ther*. **283**:108–115 (1997).

29. A. Kikuchi, T. Nozawa, T. Wakasawa, T. Maeda, and I. Tamai. Transporter-mediated intestinal absorption of fexofenadine in rats. *Drug. Metab. Pharmacokinet.* **21**:308–314 (2006). doi:10.2133/dmpk.21.308.
30. M. Ofer, P. Langguth, and H. Spahn-Langguth. Bidirectional membrane transport: simulations of transport inhibition in uptake studies explain data obtained with flavonoids. *Eur. J. Pharm. Sci.* **29**:251–258 (2006). doi:10.1016/j.ejps.2006.06.010.
31. U. I. Schwarz, T. Gramatté, J. Krappweis, R. Oertel, and W. Kirch. P-glycoprotein inhibitor erythromycin increases oral bioavailability of talinolol in humans. *Int. J. Clin. Pharmacol. Ther.* **38**:161–167 (2000).
32. A. Hanafy, P. Langguth, and H. Spahn-Langguth. Pretreatment with potent P-glycoprotein ligands may increase intestinal secretion in rats. *Eur. J. Pharm. Sci.* **12**:405–415 (2001). doi:10.1016/S0928-0987(00)00195-0.
33. H. Spahn-Langguth, G. Baktir, A. Radschuweit, A. Okyar, B. Terhaag, P. Ader, A. Hanafy, and P. Langguth. P-glycoprotein transporters and the gastrointestinal tract: evaluation of the potential *in vivo* relevance of *in vitro* data employing talinolol as model compound. *Int. J. Clin. Pharmacol. Ther.* **36**:16–24 (1998).
34. U. I. Schwarz, T. Gramatté, J. Krappweis, A. Berndt, R. Oertel, O. von Richter, and W. Kirch. Unexpected effect of verapamil on oral bioavailability of the beta-blocker talinolol in humans. *Clin. Pharmacol. Ther.* **65**:283–290 (1999). doi:10.1016/S0009-9236(99)70107-4.
35. Y. Shitara, D. Sugiyama, H. Kusuhara, Y. Kato, T. Abe, P. J. Meier, T. Itoh, and Y. Sugiyama. Comparative inhibitory effects of different compounds on rat oatpl (slc21a1)- and Oatp2 (Slc21a5)-mediated transport. *Pharm. Res.* **19**:147–153 (2002). doi:10.1023/A:1014264614637.
36. U. Fagerholm, A. Lindahl, and H. Lennernäs. Regional intestinal permeability in rats of compounds with different physicochemical properties and transport mechanisms. *J. Pharm. Pharmacol.* **49**:687–690 (1997).
37. C. Hilgendorf, H. Spahn-Langguth, M. Rhedin, C. G. Regårdh, B. Löwenadler, and P. Langguth. Selective downregulation of the MDR1 gene product in Caco-2 cells by stable transfection to prove its relevance in secretory drug transport. *Mol. Pharm.* **2**:64–73 (2005). doi:10.1021/mp049931y.
38. L. M. Augustine, R. J. Markelewicz, K. Boekelheide, and N. J. Cherrington. Xenobiotic and endobiotic transporter mRNA expression in the blood–testis barrier. *Drug. Metab. Dispos.* **33**:182–189 (2005). doi:10.1124/dmd.104.001024.
39. Y. Koitabashi, T. Kumai, N. Matsumoto, M. Watanabe, S. Sekine, Y. Yanagida, and S. Kobayashi. Orange juice increased the bioavailability of pravastatin, 3-hydroxy-3-methylglutaryl CoA reductase inhibitor, in rats and healthy human subjects. *Life Sci.* **78**:2852–2859 (2006). doi:10.1016/j.lfs.2005.11.006.
40. H. C. Walters, A. L. Craddock, H. Fusegawa, M. C. Willingham, and P. A. Dawson. Expression, transport properties, and chromosomal location of organic anion transporter subtype 3. *Am. J. Physiol. Gastrointest. Liver Physiol.* **279**:G1188–1200 (2000).
41. W. V. de Castro, S. Mertens-Talcott, A. Rubner, V. Butterweck, and H. Derendorf. Variation of flavonoids and furanocoumarins in grapefruit juices: a potential source of variability in grapefruit juice–drug interaction studies. *J. Agric. Food. Chem.* **54**:249–255 (2006). doi:10.1021/jf0516944.
42. Y. Masaoka, Y. Tanaka, M. Kataoka, S. Sakuma, and S. Yamashita. Site of drug absorption after oral administration: assessment of membrane permeability and luminal concentration of drugs in each segment of gastrointestinal tract. *Eur. J. Pharm. Sci.* **29**:240–250 (2006). doi:10.1016/j.ejps.2006.06.004.
43. F. Tourniaire, M. Hassan, M. André, O. Ghiringhelli, C. Alquier, and M. J. Amiot. Molecular mechanisms of the naringin low uptake by intestinal Caco-2 cells. *Mol. Nutr. Food Res.* **49**:957–962 (2005). doi:10.1002/mnfr.200500088.