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Transporter-mediated oral absorption of celiprolol

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Abbreviations: OATP, organic anion transporting polypeptide; P-gp, p-glycoprotein; FD-4, FITC-dextran (Mw. 4,000); IS, internal standard; E3S, estrone-3-sulfate; BSP, bromosulfophthalein

ABSTRACT

Gastrointestinal absorption of several ß-blockers is inhibited by citrus juices, although molecular mechanism(s) lying on their small intestinal absorption has not yet been identified. Here, we attempted to demonstrate involvement of both influx and efflux transporters *in vivo* in gastrointestinal absorption of celiprolol in mice. Plasma concentration of celiprolol (3 mg/kg) after oral administration was mostly under the limit of quantification in wild mice, whereas that in *mdr1a/b* knockout (*mdr1a/b*$^{(-/-)}$) mice was much more obvious, indicating P-glycoprotein-mediated efflux. Then, the oral absorption of celiprolol in *mdr1a/b*$^{(-/-)}$ mice was further examined to investigate influx transport mechanism with avoiding effect of P-glycoprotein. Coadministration of bromosulfophthalein (BSP), an inhibitor of various influx transporters including organic anion transporting polypeptide (OATP) reduced plasma celiprolol concentration. Inhibition by BSP of celiprolol uptake from apical membranes was confirmed in Ussing-type chamber of small intestinal tissues. Uptake of celiprolol by human small intestinal transporter OATP-A/1A2 was also confirmed in *Xenopus Laevis* oocytes. Interestingly, OATP-A/1A2 accepts various ß-blockers including acebutolol, atenolol and sotalol, oral absorption of which is inhibited by coadministration of citrus juice or telithromycin in human. Taken together, these findings have suggested fundamental role of influx transport system(s) in oral absorption of celiprolol.

INTRODUCTION

ß-blockers have been clinically used for the treatment of various types of cardiovascular diseases including hypertension, myocardial infarction, angina pectoris and arrhythmia. They are also recently prescribed for chronic heart failure, although such application is still under the clinical trial in Japan [1,2]. In most cases, ß-blockers are orally administered to the patients because of their adequate absorption. The mechanism for the gastrointestinal absorption of ß-blockers has not yet been fully identified, but the earlier research done by Taylor et al. (1985) has proposed two different types of absorption mechanism for hydrophilic (atenolol, nadolol, practolol and sotalol with logP values from -0.79 to 0.76) and lipophilic (alprenolol, metoprolol, oxprenolol, pindolol, propranolol and timolol with logP values from 1.75 to 3.65) ß-blockers: The absorption rate constant for hydrophilic ones is almost identical among the compounds, whereas that for lipophilic ones depends on octanol-water partition and is consistent with pH-partition theory [3].

The unique properties in gastrointestinal absorption of ß-blockers include possible involvement of efflux transporter(s) in their small intestinal absorption.

Bioavailability in human of acebutolol and celiprolol increases as the increase in dose [4,5], probably due to the saturation of the efflux transport system in small intestine. Identification of the active efflux systems for ß-blockers in small intestine was first proposed in our laboratory by the previous observation that cyclosporine A, an inhibitor for P-glycoprotein (P-gp) increased absorption rate constant of several ß-blockers including acebtolol, atenolol, celiprolol and nadolol in rat *in situ* jejunum loop [6]. Among the ß-blockers, celiprolol is one of the best characterized P-gp substrates and has also been suggested to be actively pumped out by P-gp in rat small intestine [7] and human intestinal Caco-2 cells [8-10]. In human, oral absorption of celiprolol was reported to increase by itraconazole [11], and this effect could be accounted for by the inhibition of efflux transport system, possibly P-gp, if we consider minor contribution of metabolism to systemic elimination of celiprolol.

On the other hand, recent clinical investigations have clarified that grapefruit juice decreased oral absorption of acebutolol, atenolol, celiprolol and talinolol [11-14]. Oral absorption of atenolol and celiprolol was also reduced by orange juice [13,15]. Coadministration of therapeutic agents including verapamil and telithromycin has also

been reported to reduce oral bioavailability of talinolol and sotalol, respectively [16,17]. One of feasible hypotheses may be that a certain constituents in the juice inhibits uptake process of these ß-blockers from apical side in the small intestinal epithelial cells, although such drug-food interactions may also be explained by other possibilities including inhibitory effect on gastric emptying rate and/or lowering effect on intestinal pH, leading to decrease in unionized form of ß-blockers. Possible involvement of the influx transporters has already been suggested for gastrointestinal absorption of fexofenadine, which could be mediated by organic anion transporting polypeptide (OATP)-A (OATP1A2) [18,19], although the influx transporters for ß-blockers has not yet been identified.

The drug-food interaction for ß-blockers decreases their systemic exposure after oral administration. Therefore, molecular identification of intestinal transporter(s) responsible for the oral absorption should be important to avoid any unexpected drug-drug or drug-food interaction. However, limited information is available especially on influx transporters for ß-blockers from apical membranes in small intestine. The purpose of the present study is to demonstrate involvement of influx (uptake)

transporter(s) for celiprolol, since inhibitory effect of grapefruit juice on oral absorption of celiprolol was most obviously reported among the ß-blockers [11-14]. After oral absorption, celiprolol is mainly excreted into urine as an unchanged form [1,20,21], suggesting minor contribution of metabolism to the systemic elimination. Kirby and Unadkat (2007) have already referred to in vitro data for celiprolol and talinolol being substrates for influx and efflux transporters in humans [22]. However, there has been no direct demonstration regarding the involvement of both types of transporters in human small intestine possibly due to the limited availability of experimental systems in humans. On the other hand, the present study was aimed to use experimental animals to demonstrate the involvement of transporters in small intestinal tissues. For such purpose, involvement of the efflux transporter (such as P-gp) in small intestine may hinder the analysis of influx transporter since inhibition of the efflux transporter may compensate the inhibition of influx transporters. Then, the *mdr1a/b*$^{(-/-)}$ mice were further used to focus on the influx transporter(s) for celiprolol to avoid any interference by P-gp. To demonstrate involvement of influx transporter(s) in small intestine, bromosulfophthalein (BSP) was used as an inhibitor of various influx transporters

including OATP in both *in vivo* and Ussing-type chamber system. To further clarify possible involvement of influx transporters, uptake of celiprolol and other ß-blockers was demonstrated and characterized in *Xenopus laevis* oocytes expressing human intestinal transporters OATP-A and OATP-B (OATP2B1), both of which are the influx transporters localized on apical membranes of human small intestine [19,23].

MATERIALS AND METHODS

Materials

Celiprolol hydrochloride was gifts from Nichi-iko Pharmaceutical Co., Ltd. (Toyama, Japan). [^{3}H]Estrone-3-sulfate (E3S) ammonium salt (1.59 TBq/mmol) was purchased from PerkinElmer Life and Analytical Sciences, Inc. (Boston, MA). The pcDNA3 vector was obtained from Invitrogen (Carlsbad, CA). FITC-dextran with an average molecular weight of 4,000 (FD-4) was purchased from Sigma-Aldrich Inc. (St. Louis, MO). All other reagents were commercial products of reagent grade.

Animals

Six- to eight-week old male FVB/NJcl and *mdr1a/b* knockout mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and Taconic Farms Inc. (Germantown, NY, USA), respectively. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kanazawa University.

Pharmacokinetic Studies

Mice were fasted overnight with free access to water and anesthetized with diethylether during drug administration. Celiprolol (3.0 mg/5 mL/kg body weight) alone, or with BSP (30 mg/5 mL/kg body weight) dissolved in saline was orally administered by gavage, the concentration of each compound in the dosing solutions being 1.58 and 7.16 mM, respectively. At various intervals up to 360 min after administration, aliquots of about 20 μL of blood were collected through the caudal vein. All blood samples were immediately centrifuged to obtain plasma. The plasma samples (8 μL) were mixed with sterile distilled water containing of acebutolol as an IS (16 μL) and acetonitrile (32 μL). All the mixed solutions were centrifuged at 19,000 g for 15 min at 4°C. The supernatants were diluted with an equal volume of mobile phase and centrifuged at 19,000 g for 15 min at 4°C. The supernatants were subjected to LC-MS/MS analysis.

Transport experiments in Ussing-type chamber

Intestinal tissue sheets were prepared as described previously [24]. The muscle layer was removed with fine tweezers and vertically mounted in Ussing-type chambers

that provided an exposed area of 0.25 cm^2. The volume of bathing solution on each side was 1.2 mL, and the solution temperature was maintained at 37 °C in a water-jacketed reservoir. The test solution was composed of 128 mM NaCl, 5.1 mM KCl, 1.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 21 mM $NaHCO_3$, 1.3 mM KH_2PO_4, 10 mM NaH_2PO_4 and 5 mM D-glucose (adjusted at pH 6.0 or 7.4 for apical or basal side, respectively), and gassed with 95% O_2/5% CO_2 before and during the transport experiment. The pH value at the luminal side in Ussing-type chamber was set to be 6.0 according to our previous studies [24-26]. The concentration of celiprolol and FD-4 in the apical side was set to be 100 μM. At the designated times, a 250 μL aliquot of basal side buffer was sampled and replaced with an equal volume of fresh buffer. The apical side buffer was also collected after finish of the incubation. To determine the concentration of celiprolol, the sample solutions (20 μL) were mixed with mobile phase containing of timolol as an IS (20 μL) and acetonitrile (40 μL). All the mixed solutions were centrifuged at 19,000 g for 15 min at 4°C. After centrifugation, the supernatants (70 μL) were dried under nitrogen at 55°C. The residues were reconstituted with mobile phase (70 μL) and centrifuged at 19000 g for 15 min at 4°C. The supernatants were subjected to LC-MS/MS analysis.

The sample solutions (150 μL) were diluted with sterile distilled water (150 μL). FD-4 concentrations in samples were determined by a spectrofluorometer (Spectrafluor Plus, Tecan Group Ltd., Zurich, Switzerland) at excitation and emission wavelength of 485 and 535 nm, respectively.

Uptake study with *Xenopus laevis* oocytes transfected with cRNA encoding OATP-A

The full-length cDNA of OATP-A was purchased from GenoMembrane, Inc. (Kanagawa, Japan) and subcloned into EcoRV and Xho I sites of pcDNA3. The full-length cDNA of OATP-B was previously obtained [27] and subcloned into HindIII site of pGEMHE vector (kind gift from Prof. Takaaki Abe in Tohoku University Graduate School of Medicine). The capped cRNA of OATP-A was synthesized by means of a mCAPTM RNA capping kit (Stratagene, La Jolla, CA). *Xenopus laevis* oocytes were prepared and injected with 50 nL of cRNA (25 ng) or water as described previously [28]. Three days after the cRNA injection, the oocytes were transferred to ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ $6H_2O$, 5 mM

HEPES, pH 7.4) and pre-incubated at 25˚C for 15 min. Uptake was initiated by replacing the solution with fresh ND96 solution with 5 mM MES instead of HEPES (pH 6.0) at 25°C containing each test compounds. In the cassette dosing studies, the oocytes injected with cRNA for OATP-A or water alone were incubated with a mixture of 50 μM of celiprolol, acebutolol, atenolol, nadolol and sotalol, 1 μM of labetalol and oxprenolol, and 0.1 μM of alprenolol, metoprolol, pindolol and propranolol. To assess the concentration dependence of celiprolol uptake, oocytes were incubated with 1~3000 μM of celiprolol. At the designated times, the oocytes were rinsed five times with ice-cold ND96 solution. Individual oocytes were added with 50% methanol containing of timolol as an IS (35 μL) and acetonitrile (35 μL). The samples were then disrupted with a tip sonicator and the lysate was cleared at 15,000g for 15 min at 4°C. The supernatants were diluted with an equal volume of sterile distilled water and centrifuged at 19,000 g for 15 min at 4°C. The supernatants were subjected to LC-MS/MS analysis. Similarly, the oocytes injected with cRNA for OATP-A, OATP-B or water alone were incubated with 1.0 μM of celiprolol or 9.4 nM of [^{3}H]E3S (positive control). The uptake of [^{3}H]E3S was determined as the radioactivity associated with the oocytes in a liquid

scintillation counter, LSC-5100 (Aloka, Tokyo, Japan) with Clearsol I (Nacalai Tesque, Inc., Kyoto, Japan) as the scintillation fluid.

Measurement of celiprolol by LC-MS/MS analysis

Celiprolol was measured with a LC/MS/MS system equipped with a constant flow pump (Agilent 1200 series G1312A, Agilent Technologies, Tokyo, Japan), an automatic sample injector (G1367B; Agilent Technologies), a column oven (G1316A; Agilent Technologies) and a mass spectrometer (API 3200, Applied Biosystems, Tokyo, Japan). In pharmacokinetic study, the analytical column was COSMOSIL® AR-II (2.0 mm x 150 mm; Nacalai Tesque, Kyoto, Japan). The mobile phase consisted of 18:82 (v/v) methanol and 0.1% formate. Chromatography was isocratically performed at a flow rate of 0.2 mL/min at 40°C. The analytes were detected by a tandem mass spectrometer (MS/MS) with a TIS interface in positive ionization mode. The multiple reaction monitor was set at 337.2 to 116.0 m/z for acebutolol and 380.3 to 74.0 m/z for celiprolol. In Ussing-type chamber and oocyte studies, the analytical column was COSMOSIL® MS-II (2.0 mm x 50 mm; Nacalai Tesque, Kyoto, Japan). The mobile

phase A was 0.01 M ammonium formate and the mobile phase B was methanol. The gradient elution time program was set as follows: 0-2 min, B 7-70%; 2-10 min, B, 70%; 10.1-20 min, B, 7%. The flow rate was 0.2 mL/min. Separation was performed at 40 °C. The multiple reaction monitor was set at 337.2 to 116.0, 250.3 to 116.1, 267.2 to 145.0, 380.3 to 74.0, 329.3 to 162.0, 266.3 to 72.1, 310.3 to 254.3, 266.3 to 72.0, 249.3 to 116.1, 260.2 to 116.3, 273.1 to 132.8 and 317.2 to 261.0 m/z for acebutolol, alprenolol, atenolol, celiprolol, labetalol, metoprolol, nadolol, oxprenolol, pindolol, propranolol, sotalol and timolol, respectively.

Statistical Analysis and Calculation of Kinetic Parameters

Data were expressed as mean ± S.E.M. Student's t-test was used for statistical analysis, with $p < 0.05$ as the criterion of significance.

The rate constant for terminal phase in pharmacokinetic studies was calculated as a slope using the last two data points. The calculation was performed for individual animals.

Intestinal membrane permeability ($\mu L/cm^2$) assessed in Ussing-type chamber

was estimated by dividing the amount appeared in the acceptor side by both surface area (1 cm^2) and initial concentration in the donor side. The permeability of celiprolol was obtained by subtracting the permeability of FD-4 from that of celiprolol.

In the Uptake study with *Xenopus laevis* oocytes, cell-to-medium ratio was obtained by dividing the cellular uptake amount by the concentration of test compound in the medium. Kinetic parameters for transport activity were estimated by nonlinear least-squares fitting of all the concentration-dependent data to the following equation using the MULTI program:

$$V = V_{max} \times S/(K_m + S) + K_{ns} \times S \quad (1)$$

where V, S, V_{max}, K_m and K_{ns} represent the initial uptake velocity, substrate concentration, maximum uptake velocity, Michaelis constant and non-saturable uptake clearance, respectively. Concentration-dependent inhibition of celiprolol uptake by BSP was fitted to the following equation:

$$R = \frac{IC_{50}}{IC_{50} + I} \quad (2)$$

where R is the uptake normalized by the control (without BSP) value, I is the BSP concentration and IC_{50} is the inhibition constant.

RESULTS

Comparison of plasma concentration profile of celiprolol after oral administration between wild and *mdr1a/b*$^{(-/-)}$ mice

Involvement of P-gp as an efflux transport system for celiprolol has already been suggested by using P-gp inhibitor in rats [6], but there has been no direct demonstration using gene knockout mice. Therefore, we first compared plasma concentration profile of celiprolol after oral administration between wild and *mdr1a/b*$^{(-/-)}$ mice. In wild mice, plasma concentration of celiprolol after oral administration at 3 mg/kg was under the limit of quantification (< 3 ng/mL) at most of sampling points, whereas that in *mdr1a/b*$^{(-/-)}$ mice was much higher (Fig. 1).

Inhibitory effect of BSP on intestinal absorption of celiprolol in *mdr1a/b*$^{(-/-)}$ mice

We next attempted to inhibit oral absorption of celiprolol by coadministration of transporter inhibitor to demonstrate involvement of influx (uptake) transporter(s) in small intestine. Since such inhibitor may also inhibit P-gp, the *mdr1a/b*$^{(-/-)}$ mice were used to avoid effect of P-gp on the intestinal absorption. BSP is a quite potent inhibitor

of various OATP family members with an inhibition constant of 0.3-20 μM [29], whereas BSP would stably exist inside the intestinal lumen due to its low gastrointestinal absorption demonstrated in rats [30]. Coadministration of BSP at 30 mg/kg body wt decreased plasma concentration of celiprolol after oral absorption in *mdr1a/b*$^{(-/-)}$ mice (Fig. 1). This dose of BSP was chosen because of its maximum solubility in saline. On the other hand, BSP exhibited minimal effect on slope of the terminal phase (Fig. 1). The rate constant for terminal phase of celiprolol with or without BSP were 9.05 ± 0.84 x 10^{-3} and 6.98 ± 1.19 x 10^{-3} min^{-1}, respectively (mean ± S.E.M., n = 3-5), with no significant difference between the two groups. However, these values were based on only two data points, and further studies may be necessary for validation of the absolute values. In the present study, we did not calculate other pharmacokinetic parameters because only four data points were available in wild mice.

BSP inhibits small intestinal permeability of celiprolol

To demonstrate that inhibition of oral absorption of celiprolol by BSP occurs at the permeability process in small intestinal tissues, similar inhibition study was also

performed in Ussing-type chamber. Permeability of celiprolol from apical to basal side of small intestinal tissues obtained from the *mdr1a/b*$^{(-/-)}$ mice was much higher than that of FD-4, and reduced in the presence of BSP (Fig. 2). Appearance of FD-4 was also measured as a paracellular marker, and permeability of celiprolol was obtained by subtracting that of FD-4. The permeability of FD-4 in the absence or presence of BSP was 4.41 ± 1.58 and 5.81 ±1.30 μL/cm^2 at 120 min, respectively.

Celiprolol and other ß-blockers are substrates of small intestinal transporter OATP-A

OATP-A was recently clarified to be expressed on apical membranes of small intestine and proposed to be involved in intestinal absorption of therapeutic agents [18,19]. On the other hand, OATP-B was also reported to be expressed on apical membranes of human small intestine and Caco-2 cells [23,31,32]. As a candidate of oral absorption mechanism for celiprolol *in vivo*, we examined possible transport of celiprolol and other ß-blockers in *Xenopus Laevis* oocytes injected with cRNA for OATP-A or water. First, uptake of a mixture of the 11 ß-blocker (50 μM of each) by the oocytes was screened,

and then the initial concentration was decreased either to be 0.1, 1 or 50 μM, based on the limit of quantification of each compound, so that apparent uptake of each compound by oocytes injected with water can be detected (Fig. 3). The uptake of acebutolol, atenolol, celiprolol, nadolol, labetalol and sotalol by oocytes injected with cRNA for OATP-A increased in a time-dependent manner and was much higher than that in oocytes injected with water (Fig. 3).

Concentration-dependent uptake of celiprolol alone was then measured in the oocytes. The uptake of celiprolol by OATP-A-injected oocytes was much higher than that in water-injected oocytes (Fig. 4A). Eadie-Hofstee plot for OATP-A-mediated uptake, which was obtained by subtracting the uptake in the water-injected oocytes from that in OATP-A-injected oocytes, exhibited biphasic saturation (Fig. 4B). The Km, Vmax and Kns were estimated to be 20.5 ± 7.1 μM, 2.83 ± 0.69 pmol/oocyte/90 min and 9.12 ± 1.81 nL/oocyte/90 min, respectively. The cell-to-medium ratio of celiprolol at 60 μM was 39.7 nL/oocytes/90 min in OATP-A-injected oocytes (Fig. 4A), which was comparable with that observed in the cassette dosing study shown in Fig. 3 (~40.2 nL/oocyte/90 min). The OATP-A-mediated uptake of celiprolol was concentration-

dependently decreased in the presence of BSP with the IC_{50} value of 32.5 ± 5.5 μM (Fig. 4C). The uptake of celiprolol by water-injected oocytes also exhibited saturation, and this would be the reason for the discrepancy in absolute values for the uptake by water-injected oocytes between Fig. 3A (~ 4 nL/oocyte/90 min at 50 μM) and Table 1 (~ 34 nL/oocyte/90 min at 1 μM). Although the reason for such saturation was unknown, there may be some endogenous binding sites and/or transporters for celiprolol in the oocytes.

The uptake of celiprolol by oocytes injected with cRNA for another small intestinal OATP, OATP-B was much lower than in OATP-A-injected oocytes (Table 1). On the other hand, the uptake of [^{3}H]E3S was much higher in OATP-A and OATP-B-injected oocytes, compared with water-injected ones (Table 1).

DISCUSSION

Possible involvement of influx transporters in gastrointestinal absorption has recently been suggested for several therapeutic agents. Especially, both OATP-A and OATP-B are expressed on apical membrane of small intestine in human and proposed to be involved in uptake of several anionic compounds [18,19,23]. On the other hand, molecular mechanism involved in uptake of ß-blockers from apical side has not yet been identified despite that they have been widely used as oral drugs. Recent observation that citrus juice inhibits oral absorption of acebutolol, atenolol, celiprolol and talinolol may imply similar involvement of OATP transporter(s) in the absorption of ß-blockers [11-15], as suggested for fexofenadine case [18,19]. However, such inhibition study *in vivo* may be affected by other factors than the membrane permeation process. Therefore, more detailed studies focusing on the uptake process of ß-blockers from apical membranes of small intestine is also important for understanding the molecular mechanism lying on their gastrointestinal absorption, and experimental animal models could be one of useful tools for such purpose. In the present study we used *mdr1a/b*$^{(-/-)}$ mice to analyze the influx process of ß-blockers with avoiding any effect of P-gp. This

would probably be appropriate because plasma concentration of celiprolol was much higher in *mdr1a/b*$^{(-/-)}$ mice, compared with wild mice, after oral administration (Fig. 1), indicating predominant role of P-gp as an efflux transport system for celiprolol. The dose of celiprolol (3 mg/kg) is comparable with the clinical dose in human (100~400 mg/body). This finding could be compatible with the clinical observation of the drug-drug interactions for ß-blockers via the small intestinal P-gp [11,33]. However, P-gp is expressed in various tissues other than the small intestine. Celiprolol is mainly excreted into the urine as an unchanged form after oral absorption[1,20,21], with minor contribution of metabolism to the systemic clearance. Therefore, not only the intestinal absorption, but also the distribution and/or renal elimination of celiprolol may be affected by *mdr1a/b* gene knockout.

In *mdr1a/b*$^{(-/-)}$ mice, coadministration of transporter inhibitor, BSP substantially decreased oral absorption of celiprolol (Fig. 1). Since anesthesia might affect intestinal absorption [34], and experimental results obtained *in vivo* alone does not necessarily lead to any conclusion regarding the inhibition of celiprolol transport by BSP, Ussing-type chamber studies were then performed as another intestinal absorption

experiment with an aim to more directly assess the effect of BSP on intestinal permeability of celiprolol. BSP also inhibited intestinal membrane permeability of celiprolol in Ussing-type chamber (Fig. 2), suggesting the influx transport process for celiprolol in mouse small intestine. It would be noteworthy that anionic compound BSP inhibits the uptake of a cationic compound celiprolol. Intestinal transport mechanism for celiprolol may thus have unique recognition specificity.

Because BSP substantially inhibited gastrointestinal absorption of celiprolol (Figs. 1, 2), we speculated that OATP family would be one of candidate transporters responsible for the uptake of celiprolol. Immunohistochemical analysis has revealed that both OATP-A and OATP-B are localized on apical membrane of human intestinal epithelial cells or Caco-2 cells [19,23,31]. Therefore, *Xenopus Laevis* oocytes injected with cRNA for each transporter were used to clarify whether celiprolol is their substrate or not. The present finding demonstrates that celiprolol is efficiently transported by OATP-A, compared with OATP-B (Fig. 3, 4, 5, Table 1). The OATP-A-mediated uptake of celiprolol exhibited saturation and biphasic transport (Fig. 4). Although the reason for biphasic transport is unknown, one of the possible explanations could be the presence of

multiple recognition sites within OATP-A molecule. Actually, similar biphasic uptake has been reported in the case of OATP-C mediated estron-3-sulfate transport [35]. Interestingly, other ß-blockers including acebutolol, atenolol, labetalol, nadolol and sotalol were also identified to be the substrates of OATP-A in the present study (Fig. 3), and citrus juice inhibited oral absorption of at least acebutolol, atenolol and celiprolol in human [11-15]. It was also reported that gastrointestinal absorption of sotalol was inhibited by coadministration of thlithromycin [17]. Thus, substantial transport by OATP-A of ß-blockers (Fig. 3) seems well compatible with the drug-food or drug-drug interaction, and OATP-A would be one of the possible transporters involved in influx of celiprolol from apical membrane in human small intestine. All the ß-blockers transported by OATP-A have basic pKa values (9~10) except that both labetalol and sotalol also have pKa of 7~8. Therefore, OATP-A could have broad substrate specificity, not only for anionic compounds, but also cationic ones.

The uptake study in OATP-A-injected oocytes revealed the presence of saturable transport component for celiprolol by OATP-A with Km of ~ 20 μM (Fig. 4B). On the other hand, the concentration of celiprolol (100 μM) in the Ussing-type chamber

studies was higher than the Km value of OATP-A-mediated celiprolol uptake. Therefore, the uptake system for celiprolol in mouse small intestine might be saturated if the Km value for celiprolol uptake in mouse small intestine is similar to that of human OATP-A. This would be one of the possible reasons for the present finding that inhibitory effect of BSP was not so obvious, the effect at 100 μM BSP being not significant in the Ussing-type chamber studies (Fig. 2).

Up to now, BSP is known to be recognized by various human OATPs such as OATP-A, OATP-B, OATP-C, OATP-8 and OATP-F [36]. BSP is preferentially transported by OATP-C rather than OATP-8 [37,38]. Therefore, BSP may affect these OATPs expressed in various tissues other than small intestine, and further studies are required to clarify possible effect of BSP on distribution and/or elimination of celiprolol after oral absorption. Uptake transporters such as OATP-C and OATP-8 are thought to be liver-specific, whereas OATP-A and OATP-B are expressed in the intestine [19]. Therefore, uptake studies in *Xenopus Laevis* oocytes were performed for OATP-A and OATP-B in the present study. However, the liver-specific expression alone does not necessarily exclude the possibility that small level of intestinal expression may be enough to be

involved in intestinal handling of therapeutic agents. It is essentially important to clarify which OATP member is actually involved in intestinal absorption of celiprolol in humans, but the demonstration of such contribution of each transporter protein is quite difficult due to the limitation of experimental systems using freshly isolated human intestinal tissues. Therefore, the present finding alone has limitation to discuss the possible contribution of OATP-A to intestinal absorption of celiprolol in human, and further studies are required to finally demonstrate involvement of the transporter(s).

In the present study, oocytes were incubated with a mixture of several ß-blockers as a first screening to identify OATP-A substrates (Fig. 3). Doing such a cassette incubation for transporters studies, however, raises the concern for possible interactions. In the present study, some of ß-blockers with higher detection sensitivity in LC-MS/MS were set to be lower (0.1 or 1 μM) concentration with an aim to minimize possible interactions via the transporter. The cell-to-medium ratio of celiprolol at 60 μM in single dosing study was 39.7 nL/oocye/90 min in OATP-A-injected oocytes (Fig. 4A), which was comparable with that observed in the cassette dosing study at 50 μM of celiprolol (~ 40.2 nL/oocyte/90 min, Fig. 3), suggesting minimal interaction for the

uptake of celiprolol. However, this does not necessarily exclude the possibility of the interaction among the other ß-blockers. Further uptake studies using a single ß-blocker are needed to compare the absolute transport activity by OATP-A among the ß-blockers. In addition, it would be noteworthy that interaction due to the cassette dosing may hinder the identification of transporter substrates.

Taylor et al. (1985) reported that hydrophilic ß-blockers including acebutolol, nadolol, atenolol and sotalol (with logP values from 1.87 to -0.79) exhibit almost similar absorption rate constants in rats, and their oral absorption does not follow pH-partition theory [3]. They have proposed characteristics of aqueous phase transport, possibly via membrane pores, for such hydrophilic ß-blockers. Although the contribution of such paracellular route cannot be fully neglected, the present finding demonstrates involvement of influx transporters at least for celiprolol. Actually, BSP minimally affected intestinal permeability of FD-4 (see legends to Fig. 2), suggesting minimal effect of BSP on the paracellular route.

In conclusion, the present study has suggested that P-gp is involved in gastrointestinal absorprion of celiprolol in mice. We have also obtained direct evidence

that gastrointestinal absorption of celiprolol is mediated by influx transporter(s). The *mdr1a/b*$^{(-/-)}$ mice could be an useful tool to identify and characterize the influx transport system(s) for therapeutic agents with avoiding effect of P-gp-mediated efflux. As one of intestinal influx transporter, OATP-A transports several ß-blockers.

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FOOTNOTES

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LEGENDS TO FIGURES

Fig. 1 Plasma concentration-time profile of celiprolol in wild (closed circles) and *mdr1a/b*$^{(-/-)}$ mice with (open triangles) or without (open circles) BSP after an oral dose (3 mg/kg) of celiprolol.

Celiprolol (3 mg/5 mL/kg body wt) with or without BSP (30 mg/5 mL/kg body wt) was orally administered in wild and *mdr1a/b*$^{(-/-)}$ mice, and plasma concentration of celiprolol was determined by LC-MS/MS. Plasma concentration in wild mice at 5, 240 and 360 min was also measured, but was under the limit of quantification (< 3 ng/mL). Each value represents the mean ± S.E.M. (n = 3-5).

*, Significantly different from *mdr1a/b*$^{(-/-)}$ mice in the absence of BSP ($p < 0.05$).

Fig. 2 Effect of BSP on permeability of celiprolol across isolated mouse intestinal tissues.

Celiprolol (100 μM) with FD-4 (100 μM) was added into apical side of small intestinal tissues in Ussing-type chamber, and appearance in basal side was measured by LC-MS/MS in the absence (closed circles) or presence of 100 μM (open circles) or 7.5

mM (open triangles) of BSP. Appearance of FD-4 was also measured as a paracellular marker, and permeability of celiprolol was obtained by subtracting that of FD-4. The permeability of FD-4 in the absence or presence of BSP was 4.41 ± 1.58 and 5.81 ± 1.30 μL/cm^2 at 120 min, respectively. Each value represents the mean ± S.E.M. (n = 4).

*, Significantly different from *mdr1a/b*$^{(-/-)}$ mice in the absence of BSP ($p < 0.05$).

Fig. 3 Time profiles of the uptake of ß-blockers by *Xenopus laevis* oocytes expressing OATP-A.

The oocytes injected with cRNA for OATP-A (closed circles) or water alone (open circle) were incubated with a mixture of 50 μM of celiprolol (A), acebutolol (B), atenolol (C), nadolol (D) and sotalol (E), 1 μM of labetalol (F) and oxprenolol (G), 0.1 μM of alprenolol (H), metoprolol (I), pindolol (J) and propranolol (K) at 25ºC. Uptake of each compound was simultaneously measured by LC-MS/MS. Each value represents the mean ± S.E.M. (n = 7-8).

*, Significantly different from oocytes injected with water ($p < 0.05$).

Fig. 4 Concentration dependence of celiprolol uptake (A, B) and inhibition by BSP of the celiprolol uptake (C) by *Xenopus laevis* oocytes expressing OATP-A.

In panel A, uptake of celiprolol (1 – 3,000 μM) for 90 min was determined at 25ºC in oocytes injected with cRNA for OATP-A (closed circles) or water (open circle). In panel B, OATP-A-mediated uptake was obtained by subtracting the uptake by water-injected oocytes from that by oocytes expressing OATP-A, and shown as Eadie-Hofstee plot in which the straight line indicates fitted one. In panel C, the OATP-A-mediated uptake was measured in the presence of various concentrations of BSP. Each value represents the mean ± S.E.M. (n = 5-8).

Table 1 Uptake of celiprolol and [^{3}H]E3S by OATP-A and OATP-B

Compound	Uptake (nL / oocyte / 90 min)		
	OATP-A	OATP-B	Water
Celiprolol	108 ± 19*	45.7 ± 1.6	33.8 ± 4.8
[^{3}H]E3S	5.26 ± 1.4 x 10^3*	7.05 ± 0.69 x 10^3*	388 ± 41

Oocytes injected with cRNA for OATP-A, OATP-B or water were incubated with celiprolol (1.0 μM) or [^{3}H]E3S (9.4 nM) for 90 min, and uptake was determined. Data were expressed as mean ± SEM (n=8). *, Significantly different from oocytes injected with water ($p < 0.05$).

Figure 1

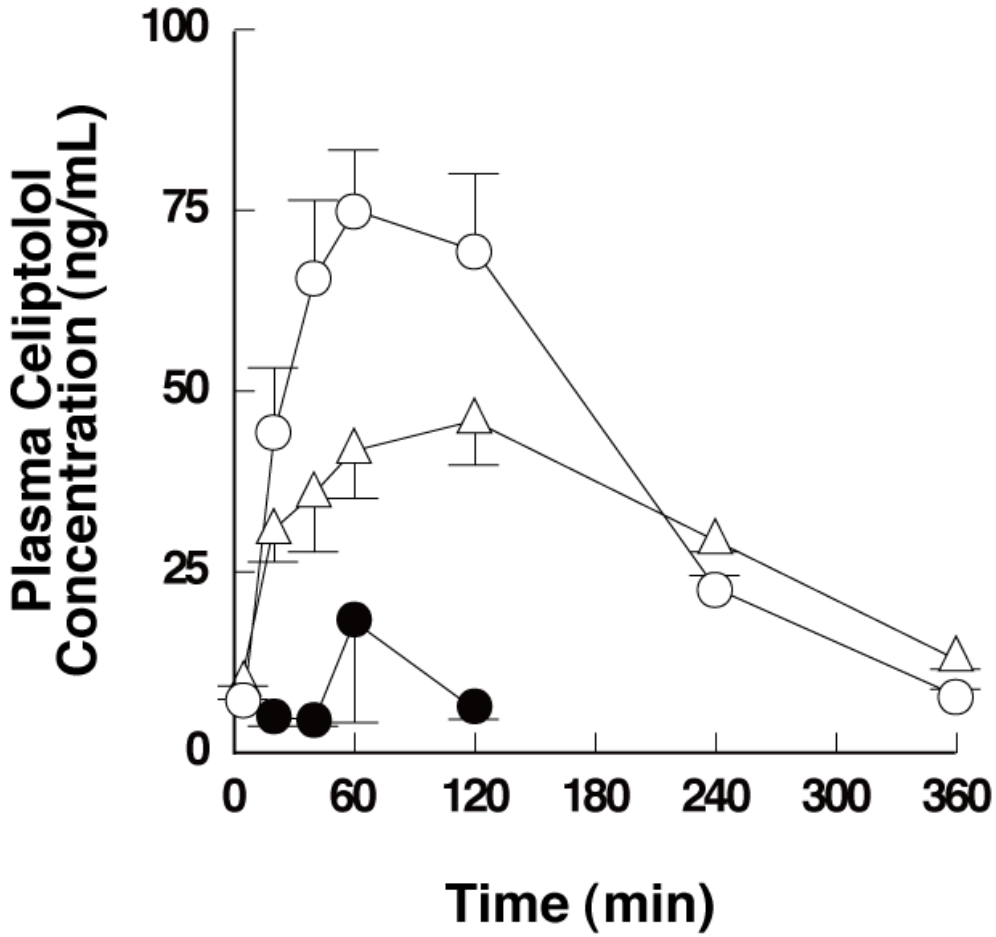

Plasma Celiptolol Concentration (ng/mL)
100
75
50
25
0
0
60
120
180
240
300
360
Time (min)

Figure 2

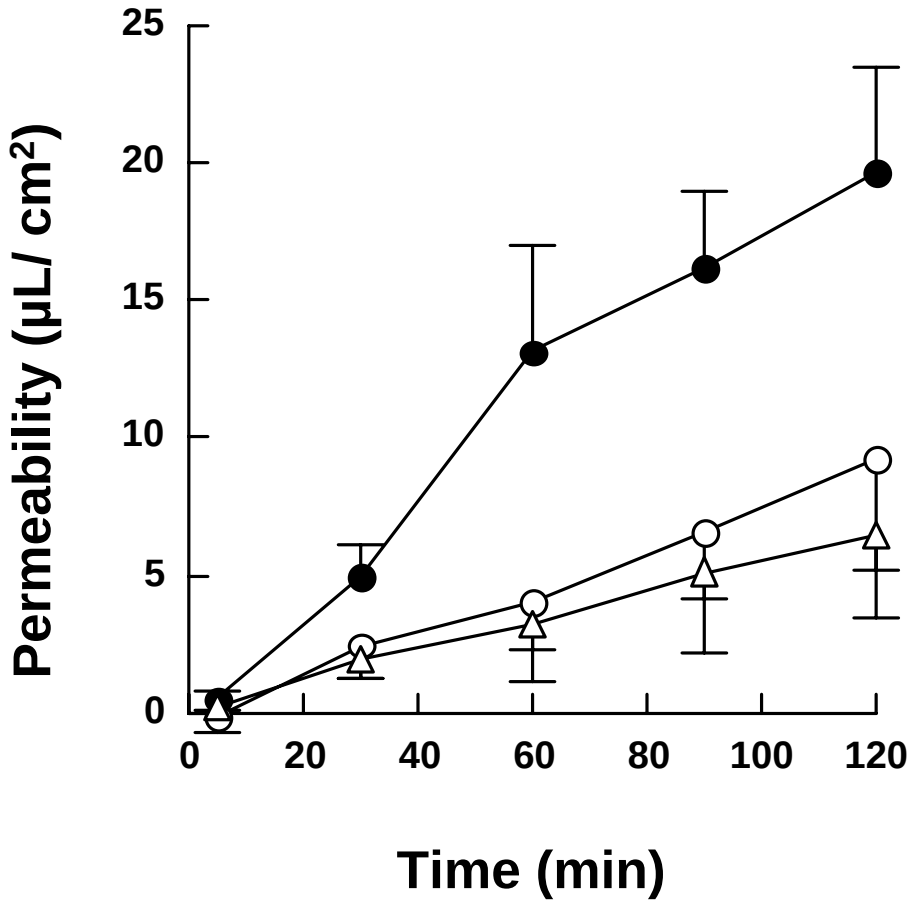


Figure ┌3

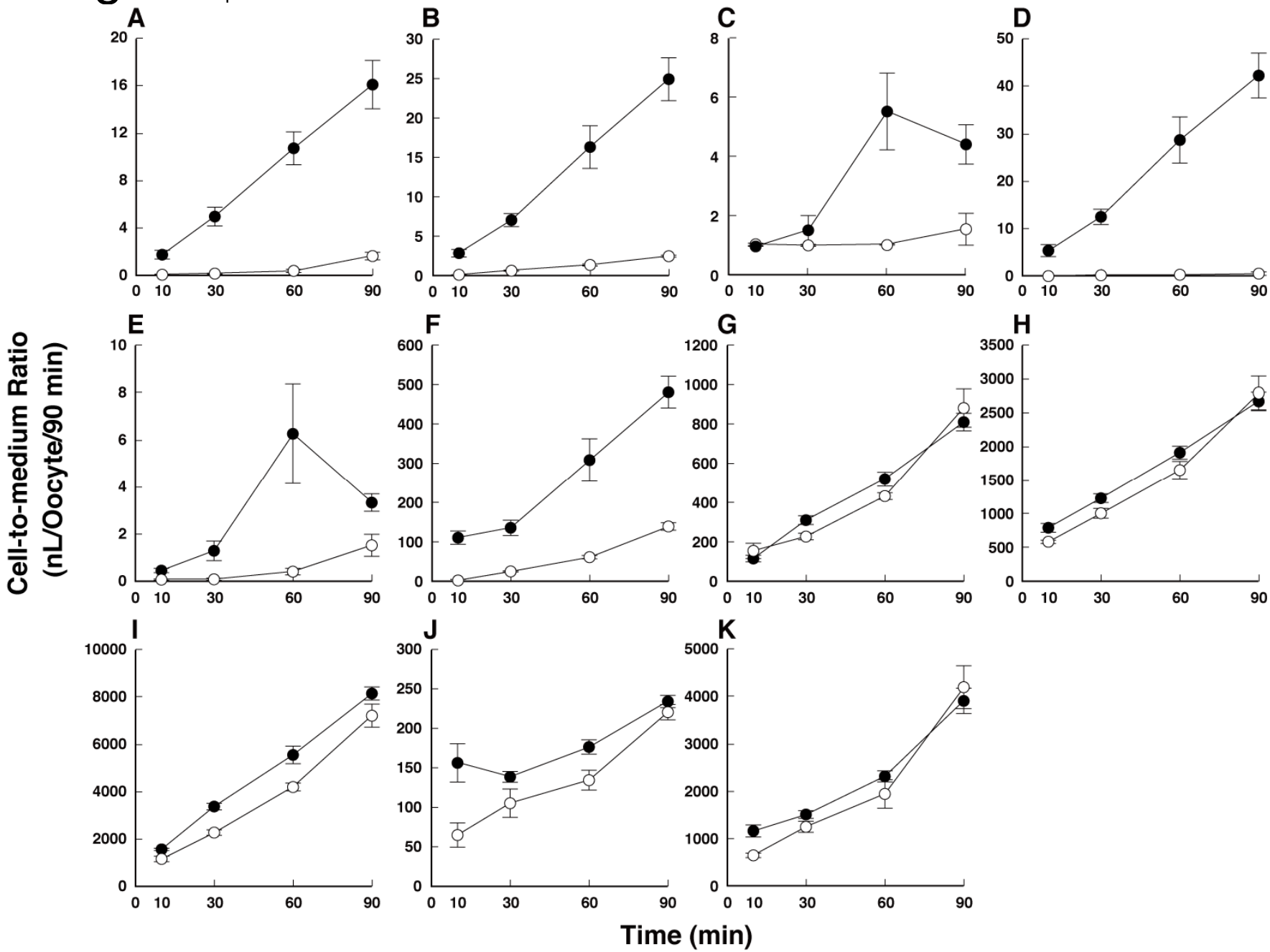
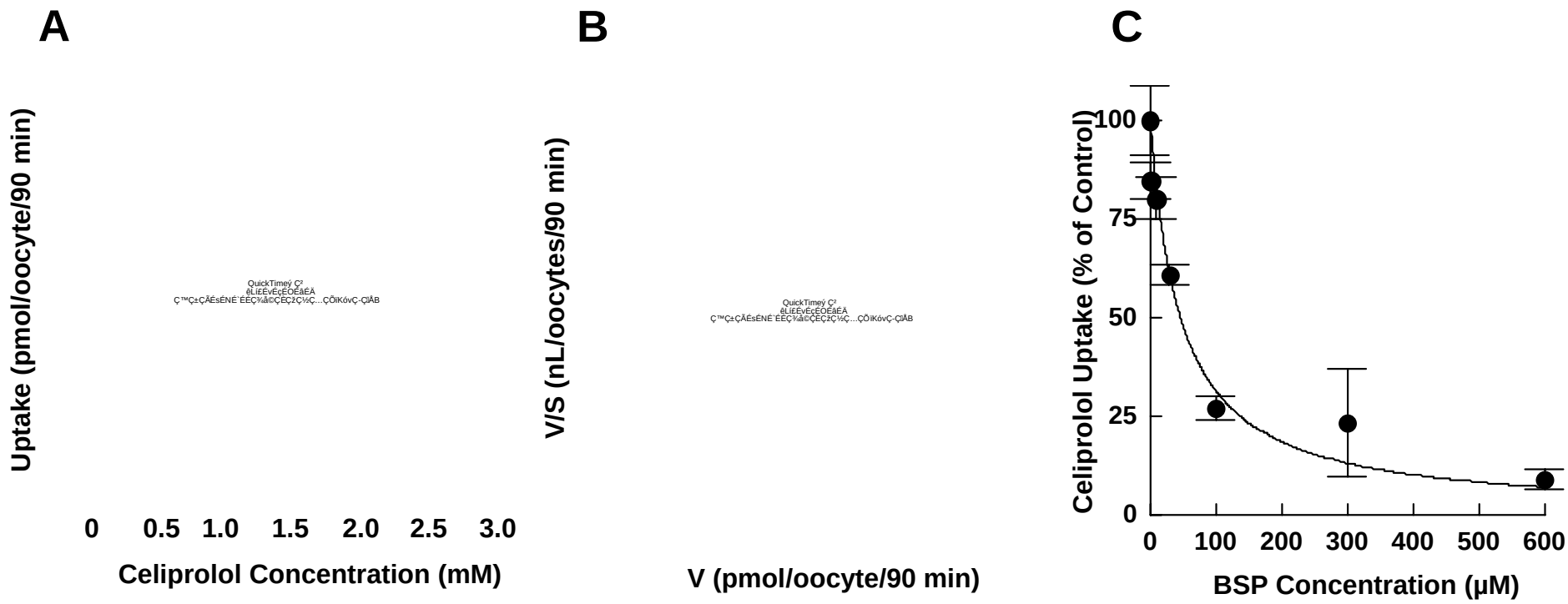


Figure 4


A
Uptake (pmol/oocyte/90 min)
0
0.5
1.0
1.5
2.0
2.5
3.0
Celiprolol Concentration (mM)
B
V/S (nL/oocytes/90 min)
V (pmol/oocyte/90 min)
C
Celiprolol Uptake (% of Control)
100
75
50
25
0
0
100
200
300
400
500
600
BSP Concentration (μM)