Edoxaban Transport via P-Glycoprotein Is a Key Factor for the Drug’s Disposition

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ABSTRACT

Edoxaban (the free base of DU-176b), an oral direct factor Xa inhibitor, is mainly excreted unchanged into urine and feces. Because active membrane transport processes such as active renal secretion, biliary excretion, and/or intestinal secretion, and the incomplete absorption of edoxaban after oral administration have been observed, the involvement of drug transporters in the disposition of edoxaban was investigated. Using a bidirectional transport assay in human colon adenocarcinoma Caco-2 cell monolayers, we observed the vectorial transport of [14C]edoxaban, which was completely inhibited by verapamil, a strong P-glycoprotein (P-gp) inhibitor. In an in vivo study, an increased distribution of edoxaban to the brain was observed in Mdr1a/1b knockout mice when compared with wild-type mice, indicating that edoxaban is a substrate for P-gp. However, there have been no observations of significant transport of edoxaban by renal or hepatic uptake transporters, organic anion transporter (OAT)1, OAT3, organic cation transporter (OCT)2, or organic anion transporting polypeptide (OATP)1B1. Edoxaban exhibited no remarkable inhibition of OAT1, OAT3, OCT1, OCT2, OATP1B1, OATP1B3, or P-gp up to 30 μM; therefore, the risk of clinical drug–drug interactions due to any edoxaban-related transporter inhibition seems to be negligible. Our results demonstrate that edoxaban is a substrate of P-gp but not of other major uptake transporters tested. Because metabolism is a minor contributor to the total clearance of edoxaban and strong P-gp inhibitors clearly impact edoxaban transport, the P-gp transport system is a key factor for edoxaban’s disposition.

Introduction

Drug transporters, along with cytochrome P450 enzymes, are key determinants governing drug disposition. As highlighted by the International Transporter Consortium, transporters are one of the major determinants of pharmacokinetics, and transporter-mediated drug–drug interactions (DDIs) are of clinical concern (Giacomini et al., 2010). Recently, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) published a draft guidance (FDA, 2012) and guidelines (EMA, 2012), respectively, on determining drug interactions for investigational drugs. Both the FDA draft guidance and the EMA guidelines list P-glycoprotein (P-gp), organic anion transporter (OAT), organic cation transporter (OCT), and organic anion transporting polypeptide (OATP) as key transporters that should be investigated during drug development.

P-gp is a relatively well understood example of a transport protein involved in transporter-mediated DDIs. A member of the ATP-binding cassette transporter superfamily, P-gp is an efflux transporter that pumps substrates out of cells and is highly expressed in the brush border membrane of the enterocytes. Therefore, it has an important role as a barrier in the absorption processes of xenobiotics. Because P-gp is located on the apical membrane of the renal tubular cells and the canalicular membrane of hepatocytes, its involvement in drug elimination is considered to be an important component of P-gp–mediated DDIs.

Edoxaban (the free base of DU-176b) is an oral direct factor Xa inhibitor approved in Japan for the prevention of venous thromboembolism after orthopedic surgery (Edoxaban tosilate hydrate (Furugohri et al., 2008; Lixiana, 2011). Ongoing phase 3 clinical trials are investigating the use of edoxaban for stroke prevention in patients with atrial fibrillation (Ruff et al., 2010) and for the treatment and prevention of recurrent thromboembolic events in patients with deep vein thrombosis and/or pulmonary embolism (Raskob et al., 2013).

A B B R E V I A T I O N S: AUC, area under the curve; BSA, bovine serum albumin; Caco-2, human colon adenocarcinoma; DDI, drug–drug interaction; DMEM, Dulbecco’s modified Eagle’s medium; D-PBS, Dulbecco’s modified phosphate-buffered saline; EG, estradiol 17β-d-glucuronide; EMA, European Medicines Agency; ES, estrone sulfate; FDA, US Food and Drug Administration; HBSS, Hank’s balanced salt solution; HEK, human embryonic kidney; HPLC, high-performance liquid chromatography; Kapp, apparent Michaelis-Menten constant; Kp, tissue-to-plasma partition coefficient; LSC, liquid scintillation counter; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; P-gp, P-glycoprotein; Papp, permeability coefficient; S2, second segment of proximal tubules cells derived from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen gene.
In humans, an involvement of transporters was speculated in both absorption and elimination of edoxaban. An incomplete absorption was observed with a bioavailability value of 61.8% (Matsushima et al., 2011). Clinical DDI studies with P-gp inhibitors found an elevated maximum plasma concentration and area under the curve (AUC) of edoxaban (Mendell et al., 2013). The maximum AUC increase was a 2-fold (Mendell et al., 2013). Although most P-gp inhibitors also inhibit CYP3A4, the inhibition of P-gp was considered to be the main cause of the DDIs, since CYP3A4 is a minor contributor to edoxaban’s elimination (Bathala et al., 2012). In addition, most P-gp inhibitors interact with P-gp in the gut, not in the kidney (Fenner et al., 2009); thus, intestinal P-gp may be the main site of the DDI. Further, the high inherent membrane permeability of edoxaban, which will be discussed later, also suggested the possibility of complete absorption in the absence of P-gp efflux activity. Therefore, the incomplete absorption of edoxaban can be attributed to P-gp efflux.

As for its elimination, half of edoxaban has been found to be excreted into urine unchanged (Matsushima et al., 2011). Renal clearance (222 ml/min) exceeded the elimination via glomerular filtration (unbound fraction = 0.41 – 0.60, glomerular filtration rate = 125 ml/min, unbound fraction x glomerular filtration rate = ca. 75 ml/min) (Ogata et al., 2010; Nutescu et al., 2011), indicating that edoxaban is eliminated via tubular secretion as well as filtration. In addition, biliary excretion and/or intestinal secretion are considered to be involved, based on the following clinical observation: after oral administration of [14C]edoxaban, a maximum of 10% of the dose was detected as metabolites, suggesting a minor contribution of metabolism (Bathala et al., 2012). Furthermore, after oral administration, 49.1% of the dose was recovered in feces, which exceeded the theoretical unabsorbed fraction (38.2%) calculated from the bioavailability value of 61.8% (38.2% = 100% – 61.8%) (Matsushima et al., 2011; Bathala et al., 2012). Data from a study using bile duct-cannulated rats, in which 8.3% of the edoxaban dose was excreted into bile, also support the involvement of biliary excretion in its clearance (Yoshigae et al., 2010). Thus, the involvement of the active membrane transport in the uriniferous and fecal excretion of edoxaban was surmised.

Collectively, drug transporters are important proteins to understand the mechanisms of absorption, renal tubular secretion, biliary excretion, and/or intestinal secretion of edoxaban. In this study, to elucidate the molecular mechanisms of edoxaban’s disposition in humans, we used uptake transporter–expressing cells and human hepatocytes for the evaluation of OAT1, OAT3, OCT2, and OATP1B1, while P-gp–expressing human colon adenocarcinoma (Caco-2 cell) monolayers and P-gp knockout mice were used for the evaluation of P-gp. The inhibitory potential of edoxaban on the drug transporters was also investigated in vitro.

Materials and Methods

[14C]Eadoxaban tosylate hydrate (DU-176b; 2.96 MBq/mg) and [14C]M-4 (4.1 MBq/mg), a metabolite of edoxaban, were synthesized by Sekisui Medical Co. Ltd. (Tokyo, Japan; Fig. 1). [3H]Digoxin (1.48 TBq/mmol), [3H]-aminohippuric acid ([3H]PAH; 93.6 GBq/mmol), [3H]estrone sulfate, ammonium salt ([3H]E; 1687 GBq/mmol), and [3H]estradiol 17β-d-glucuronide ([3H]EG; 1853.7 GBq/mmol) were purchased from PerkinElmer (Wellesley, MA). [14C]Meflofin hydrochloride (92.7 μCi/mmol) was purchased from Moravek (Brea, CA). [3H]C2O [53 μCi/mmol]) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Digoxin was purchased from Alfa Aesar (Ward Hill, MA). Ketocanozole, verapamil, amiodarone, erythromycin, quinidine, atorvastatin (acid form), cyclosporin A, probenecid, quinidine, estrone sulfate, rifampicin, Dulbecco’s modified phosphate-buffered saline (D-PBS), bovine serum albumin (BSA), epidermal growth factor, and insulin were purchased from Sigma-Aldrich (St. Louis, MO). RITC80-7 was purchased from the Research Institute for the Functional Peptides (Yamagata, Japan). 

Transferrin and butyric acid was purchased from Wako Pure Chemical Industries (Osaka, Japan). Penicillin-streptomycin liquid, antibiotic-antimycotic (100×) liquid, t-glutamine-200 mM liquid, Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and 10× Hanks’ balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA).

Cell Culture

Caco-2 Cells. Caco-2 cells were purchased from American Type Culture Collection (Manassas, VA). They were maintained in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in an atmosphere of 5% CO2. For the transcellular transport assay, Caco-2 cells were seeded on an HTS Transwell 24-well plate (polycarbonate membrane, 0.4 μm pore size, 0.33 cm2 culture surface area; Corning, Inc., Corning, NY) at 5 × 105 cells/well for 14 to 21 days. The culture medium was changed every 3 to 4 days. On the day of the experiment, the transepithelial electrical resistance was measured to check the integrity of the monolayer using an EVOM voltohmmeter (World Precision Instruments Ltd., FL) and the cell monolayers with transepithelial electrical resistance above 600 ohm-cm2 were used for the assay.

OAT1-, OAT3- and OCT2-Expressing S2 Cells. S2 cells, the second segment of proximal tubules cells derived from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen gene, transfected and stably expressing human OAT1, OAT3, or OCT2, were constructed and maintained by Sekisui Medical Co, Ltd. The OAT1-, OAT3-, and OCT2-expressing S2 cells and the control S2 cells were cultured in RITC80-7 medium supplemented with 5% FBS, 10 μg/ml transferrin, 0.08 U/ml insulin, and 10 ng/ml epidermal growth factor, and subjected to passage every 2 to 4 days. For these experiments, the OAT1-, OAT3-, and OCT2-expressing S2 cells and the control S2 cells were seeded in 24-well plates at a density of 2.4–4.0 × 105 cells/well and incubated at 33°C in an atmosphere of 5% CO2 for 2 days.

OCT1-, OCT2-, OATP1B1-, and OATP1B3-Expressing HEK293 Cells. Human embryonic kidney (HEK) 293 cells transfected and stably expressing human OCT1, OCT2, OATP1B1, or OATP1B3 were constructed and maintained by Sekisui Medical Co, Ltd. The OCT1-, OCT2-, OATP1B1-, and OATP1B3 cells and the control cells were cultured in DMEM supplemented with 10% FBS, 2 mM t-glutamine, and 1% antibiotic-antimycotic solution, and subjected to passage every 3 to 5 days. For these experiments, the OCT1-, OCT2-, OATP1B1-expressing cells, and the control cells were seeded in 24-well plates coated with collagen I at a density of 2.5 × 106 cells/well and incubated at 37°C in an atmosphere of 5% CO2 for 2 days. The OATP1B3-expressing cells and the control cells were seeded in 24-well plates coated with...
collagen 1 at a density of 3.0 × 10^5 cells/well and incubated at 37°C in an atmosphere of 5% CO_2 for 1 day; then the cells were incubated for an additional day with culture medium containing 10 mM butyric acid.

**Transport Assay for P-gp Using Caco-2 Cell Monolayers**

**Transcellular Transport of Edoxaban via P-gp Using Caco-2 Cell Monolayers and the Effect of P-gp Inhibitors on Edoxaban Transport.**

For the bidirectional transport assay of [^14C]edoxaban and a typical P-gp substrate, [^3H]digoxin, the culture medium on both the donor and receiver sides was replaced with HBSS buffer and preincubated for 15 minutes at 37°C. The HBSS buffer on the receiver side was then replaced with fresh HBSS buffer, while the HBSS buffer on the donor side was replaced with HBSS buffer containing 1, 3, 10, 30, and 100 µM of [^14C]edoxaban or 25 nM of [^3H]digoxin.

A typical P-gp inhibitor, verapamil (100 µM), was also added to both donor and receiver sides to confirm the effect on P-gp in the bidirectional transport assay. After incubation for 120 minutes at 37°C, aliquots of the solution from the receiver side were collected and mixed with 4 ml of Pico-Fluor 40 (PerkinElmer). Radioactivity was measured with a liquid scintillation counter (LSC) 2300TR (PerkinElmer) for 2 minutes. The radioactivity in the samples was calculated by subtracting the value of the background radioactivity from the observed values of the samples. The incubation period of 120 minutes was set by checking a linear time-dependent transport of edoxaban and digoxin over 120 minutes. Inhibitory effects of marketed drugs (amiodarone, atorvastatin, cyclosporin A, digoxin, erythromycin, ketoconazole, quinidine, and verapamil) on the vectorial transport of [^14C]edoxaban were also performed in the same manner.

**Inhibitory Effects of Edoxaban on P-gp.**

The bidirectional transport assay via P-gp using [^3H]digoxin was performed in the presence of various concentrations of edoxaban. For the transcellular transport assay from apical to basal and from basal to apical directions, the culture medium on the donor side and the receiver side was replaced with HBSS buffer and 4% BSA-HBSS buffer with or without edoxaban, respectively, and preincubated for 30 minutes at 37°C. Then the buffer on the donor side was replaced with HBSS buffer containing 1 µM [^3H]digoxin with or without edoxaban. After incubation at 37°C for 120 minutes, the radioactivity in the samples was determined as described in the transcellular transport assay for [^14C]edoxaban.

**Data Analysis.** The permeability coefficient (P_app) and P_app ratio of the test compounds were calculated using the following equations:

\[
P_{app} = \frac{dQ}{dt} \cdot \frac{C_0}{A}
\]

where dQ/dt is the steady-state appearance rate of the substrate on the receiver side (dpm/s), C_0 is concentration of the test compound on the donor side (dpm/µl), and A is the surface area of the monolayer (0.33 cm²);

\[
P_{app} \text{ ratio} = \frac{P_{app, A \rightarrow B}}{P_{app, B \rightarrow A}}
\]

where P_{app, A \rightarrow B} is the P_app value from the apical to basal direction and P_{app, B \rightarrow A} is the P_app value from the basal to apical direction.

The apparent Michaelis–Menten constant (K_m(app)) values of [^14C]edoxaban for P-gp-mediated transport was calculated by fitting a maximum effect model to the plots of P_app ratio–1 versus edoxaban concentration using Prism 4 (GraphPad Software, San Diego, CA). To calculate the concentration of half-maximal inhibition (IC_50), the remaining transport activity (% of control) in the presence of various concentrations of the inhibitor was calculated by dividing the P_app ratio–1 in the presence of the inhibitors by that in the absence of the inhibitors. When the maximal inhibition was greater than 50%, the IC_50 values against the P-gp-mediated vectorial transport were calculated by fitting a sigmoidal dose-response regression curve to the data using Prism 4.

**Transport Assay for OAT1, OAT3, OCT1, OCT2, OATP1B1, and OATP1B3 Using S2 or HEK293 Cells**

**Uptake Transport of Edoxaban via Renal Uptake Transporters, OAT1, OAT, and OCT2.** After the culture medium was removed from cell culture plates containing OAT1-, OAT3-, and OCT2-expressing S2 cells or control cells, the cells were preincubated with D-PBS at 37°C for 15 minutes. Next, they were incubated at 37°C for 1 and 5 minutes with prewarmed buffer containing [^14C]edoxaban or radiolabeled probe substrate as a positive control in the presence or absence of a typical inhibitor for each transporter. After incubation, the assay solution was removed and the cells were rinsed three times with 1 ml of ice-cold buffer. After adding 500 µl of 0.1 M NaOH to the wells, 300 µl of each cell lysate was collected into vials for LSC and mixed with 10 ml of Hionic-Fluor to measure the radioactivity with an LSC2500TR (PerkinElmer) for 2 minutes. Each 20 µl of cell lysate was used to determine the protein concentrations by a BCA protein assay kit (Pierce; Rockford, IL) standardized with BSA.

**Inhibitory Effects of Edoxaban on OAT1, OAT3, OCT1, OCT2, OATP1B1, and OATP1B3.** After the culture medium was removed from the cell culture plates containing OAT1- or OAT3-expressing S2 cells, OCT1-, OCT2-, OATP1B1-, or OATP1B3-expressing HEK293 cells and corresponding control cells, the cells were preincubated with D-PBS (for S2 cells) or HBSS buffer (for HEK293 cells) at 37°C for 15 minutes. The uptake assay was then initiated by adding a prewarmed mixture of probe substrate and various concentrations of edoxaban for the designated time. The conditions of the probe substrate and incubation time for each transporter-expressing cells were as follows:

OAT1, [^3H]PAH (1 µM), 2 minutes; OAT3, [^3H]HES (50 nM), 2 minutes; OCT1, [^3H]TEA (5 µM), 15 minutes; OCT2, [^3H]metformin (10 µM), 2 minutes; and OATP1B1 and OATP1B3, [^3H]Hég (50 nM), 2 minutes. After the incubation, the amount of radioactivity associated with the cells was determined as described in the uptake transport assay for [^14C]edoxaban.

**Data Analysis.** For the substrate assay, the uptake clearance of the test substrates via transporter (µl/min/mg protein) was given as the amount of radioactivity associated with the cells (dpm/min/mg protein) divided by its initial concentration in the medium (dpm/µl). For the inhibition assay, the transporter-mediated net uptakes were obtained by subtracting the uptake in control cells from that in transporter-expressing cells. The transport activities (% of control) in the presence of various concentrations of edoxaban were calculated from control experiments in the absence of inhibitors (100% uptake). When the maximal inhibition was greater than 50%, the corresponding IC_50 values, the concentration at which half of the transport activity was inhibited against the transporter-mediated probe substrate uptake, were calculated by fitting a sigmoidal dose-response regression curve to the data using Prism 4.

**Uptake Transport of Edoxaban Using Freshly Isolated Human Hepatocytes**

**Fresh Human Hepatocytes.** Human liver tissue was obtained from patients undergoing partial hepatectomy. The use of the samples for research purposes was reviewed and approved by an ethics committee and all patient donors provided written consent. Fresh female human hepatocytes were prepared by Biopredic International (Rennes, France) and used for the studies within 24 hours after the preparation.

**The Oil-Spin Assay and Data Analysis.** A total of 300 µl of hepatocyte suspension (2 × 10^6 cells/ml for probe substrates, 50 nM of [^3H]HES, or 4 × 10^6 cells/ml for 5 µM of [^14C]edoxaban and 5 µM of [^14C]M-4) was preincubated in carbogen (O_2:CO_2: 95:5)-saturated Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO_3, 4.8 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM MgSO_4, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM CaCl_2) adjusted to pH 7.4) at 37°C for 5 minutes. After preincubation, 300 µl of mixture of radiolabeled compound with or without an OATP inhibitor, cyclosporin A, or rifampicin, at a final concentration of 20 or 100 µM, respectively, was added. At 30, 60, and 90 seconds, 150 µl aliquots were taken and transferred into a tube containing a mixture of silicone and mineral oil layered over an alkaline layer (2 M NaOH). Samples were centrifuged for 30 seconds at 13,000 g, then snap frozen and stored at −70°C overnight. Afterward, the frozen tubes were cut and the upper and lower parts (medium and cells, respectively) were measured separately using an LSC. The incubations were performed in triplicate. The hepatic uptake was calculated as µl/10^6 cells for the uptake of radioactivity (Bq/10^6 cells) divided by the concentration of radioactivity in the incubation medium (Bq/µl).

**Uptake Transport of Edoxaban via Hepatic Uptake Transporter OATP1B1 Using OATP1B1-Expressing Oocytes**

**Preparation of OATP1B1-Expressing Oocytes.** *Xenopus laevis* was purchased from Kato-S-Science (Chiba, Japan) and then housed at 22°C
(acceptable range: 19°C to 25°C). The ovary was removed from X. laevis 4 days before the experiments, and oocytes were defolliculated with collagenase.

The next day, to prepare OATP1B1-expressing oocytes, the oocytes were injected with 25 ng of OATP1B1 cRNA, which was reverse-transcribed with T7 RNA polymerase using the OATP1B1 sequence containing pcDNA3.2 vector as a template. The OATP1B1-expressing oocytes and the control oocytes were incubated in modified Barth’s solution (88 mM NaCl, 1 mM KCl, 0.41 mM CaCl_2, 0.33 mM Ca(NO_3)_2, 0.82 mM MgSO_4, 2.4 mM NaHCO_3, 2.5 mM sodium pyruvate, and 7.5 mM Tris-HCl supplemented with 10 U/ml penicillin and 10 μg/ml streptomycin, adjusted to pH 7.4) for 3 days at 18°C. The culture medium was replaced twice a day.

**Uptake Transport of [14C]Edoxaban and Its Metabolite, [14C]M-4, Using OATP1B1-Expressing Oocytes.** OATP1B1-expressing oocytes and control oocytes were preincubated in polypropylene tubes (eight per tube) at room temperature for 30 minutes with the Na⁺ buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 10 mM HEPES, and 1 mM CaCl₂, adjusted to pH 7.4).

After removal of the buffer, 150 μl of Na⁺ buffer containing radiolabeled test compound, [14C]edoxaban (10 and 50 μM) or [14C]M-4 (10 and 50 μM), with or without a typical inhibitor, and 30 μM of ES were added and incubated at room temperature for 120 minutes. An OATP1B1 probe substrate, [3H]ES (50 nM), was also incubated for 60 minutes. After washing three times with 2 ml of ice-cold Na⁺ buffer, each oocyte was transferred to a glass vial and mixed with 1 ml of Soluene-350 (PerkinElmer) for 2 hours. Then, 10 ml of Hionic-Fluor was added to each vial and the radioactivity was measured using an LSC. Uptake amounts of the test substances and the typical substrate were determined from the obtained radioactivity. To measure the radioactivity used as the initial concentration, each assay solution (100 μl) was placed in vials (n = 2) and mixed with 10 ml of Hionic-Fluor before addition to the tube.

**Data Analysis and Statistical Analysis.** The uptake amounts into the OATP1B1-expressing oocytes and control oocytes were determined and the uptake clearance (nl/min/oocyte) = uptake amount into oocyte (dpm/oocyte)/initial concentration (dpm/μl)/incubation time (min) × 1000.

Uptake into OATP1B1-expressing oocytes in the absence of the inhibitor was compared with uptake in the control oocytes in the absence of the inhibitor or uptake in OATP1B1-expressing oocytes in the presence of the inhibitor by the unpaired Student’s t test using EXSUS version 7.7 (CAC Corporation, Tokyo, Japan).

**Tissue Distribution of Edoxaban in P-gp Knockout Mice.**

**Animals.** Male FVB mice (wild-type) and male P-gp knockout mice [Mdr1a/1b(−/−,−/−)] were purchased from Taconic Farm (Hudson, NY). The mice used in the study were 7 weeks old and weighed 19–23 g. All animals were maintained at a controlled temperature on a 12-hour light/dark cycle. Food and water were available ad libitum.

**In Vivo Animal Study with Wild-Type and Mdr1a/1b(−/−,−/−) Mice.** [14C]Edoxaban (0.1 mg/ml) (as the active moiety) in 5% w/v glucose solution was intravenously injected into wild-type and Mdr1a/1b(−/−,−/−) mice at a dose of 1 mg/kg (3.99 MBq/kg). After 0.5, 1-, and 2-hour administration (n = 3 per group), the mice were anesthetized with diethyl ether and a heparinized syringe was used to collect blood samples from the abdominal vena cava. A sodium fluoride aqueous solution at a final concentration of 2 mg/ml was added to the blood sample and centrifuged at 4°C for 5 minutes at 8000g to separate the plasma. Immediately after the blood sampling, the mice were sacrificed by exsanguination and the liver, kidney, and brain were collected. Each entire tissue was weighed and mixed with approximately 2-fold volume of ice-cold saline with sodium fluoride at a final concentration of 2 mg/ml. Each tissue sample was cut into small pieces in an ice bath and homogenized using a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland). To measure the radioactivity, a 50-μl aliquot of plasma, or approximately 0.1 g of each homogenate, was dissolved in 2 ml of Soluene-350 tissue solubilizer and mixed with 10 ml of Hionic-Fluor. Then the mixtures were allowed to stand at room temperature for more than half a day prior to analysis. The radioactivity in each sample was measured with an LSC 2500TR for 2 minutes. The samples collected from the animals that had not been administered with [14C]edoxaban were used for the background radioactivity assay in the plasma and tissues. Double the background radioactivity was determined as the detection limit of the assay. The remaining plasma and tissue homogenates were frozen in liquid nitrogen and stored at −80°C until use.

**Radio High-Performance Liquid Chromatography Method.** The plasma and tissue homogenates were extracted by mixing aliquots with 3-fold volume of ice-cold acetonitrile for 10 minutes. After centrifugation at 4°C for 10 minutes at 1800g, the supernatants were collected. The pellets were resuspended in the same solvent and volume, and the process was repeated twice. The supernatants were combined and weighed aliquots of each extract, together with final residual pellets, were radioassayed by LSC for the

![Fig. 2. Transcellular transport of [14C]edoxaban via P-gp using Caco-2 cell monolayers seeded on the Transwell membrane. (A) [3H]Digoxin (25 nM), a typical P-gp substrate, and (B) [14C]edoxaban (1–100 μM) in the presence or absence of the P-gp inhibitor verapamil (100 μM), were incubated with Caco-2 cell monolayers for 2 hours and bidirectional transport from apical to basal (P_{app,A→B}) and basal to apical (P_{app,B→A}) was evaluated (left axis). The diamond indicates the P_{app} ratio calculated as P_{app,B→A}/P_{app,A→B} (right axis). (C) The K_{m,app} for the concentration-dependent vectorial transport of [14C]edoxaban was calculated by fitting a maximum effect (E_{max}) model to the data of P_{app} ratio–1 and donor concentrations of edoxaban using Prism 4. A calculated fitting curve is overlaid. Data are shown as the mean of duplicate determinations.]
calculation of recovery after extraction before radio high-performance liquid chromatography (HPLC) analysis. The remaining supernatant was evaporated to dryness under reduced pressure using the rotary evaporator. The resulting residue of plasma was reconstituted in 100 mM of ammonium acetate adjusted to pH 2.5 with trifluoroacetic acid/ethanol (9:1 v/v), and the resulting residue of the tissue homogenates was reconstituted in 100 mM of ammonium acetate adjusted to pH 4.5 with acetic acid/ethanol (9:1 v/v). Each sample was centrifuged at 4°C for 5 minutes at 1000g, and a supernatant fraction was subjected to HPLC analysis. For plasma collected at 2 hours and brain homogenates, samples were pooled from three animals at each sampling time and assayed due to insufficient radioactivity.

Radio HPLC chromatograms were generated on the HPLC Shimadzu system (Shimadzu Corp., Kyoto, Japan) equipped with a system controller (SCL-10A VP; Shimadzu Corp.), a pump unit (LC-20AD; Shimadzu Corp.), a column oven (CTO-20AC; Shimadzu Corp.), and a UV-visible detector (SPD-20A; Shimadzu Corp.). The sample separation was performed on a CAPCELLPAK C18 ACR column (100 × 4.6 mm, 5.0 μm; Shiseido Co., Ltd., Tokyo, Japan) using an isocratic mobile phase composed of 20 mM (NH4)3PO4 and acetonitrile (8:3 v/v) with a flow rate of 1.0 ml/min at a column temperature of 40°C. The eluate and scintillator Flo-Scint II (PerkinElmer) at a flow rate of 3 ml/min were mixed, and radioactivity was measured in a radioactivity detector (625TR; PerkinElmer).

From the radio-chromatograms obtained, the peak area over the run time was determined using software attached to RAD (Flore-One; PerkinElmer). The recovery after extraction was multiplied by the peak area ratio of edoxaban to calculate the proportion of edoxaban in the sample. The concentrations of edoxaban in plasma, brain, liver, and kidney (ng/ml or ng/g tissue) were calculated by multiplying the total radioactivity in the samples by the proportion of edoxaban to total radioactivity in the corresponding samples. Under the analytical conditions, HPLC column recoveries were determined to be 99.3% by collecting the total HPLC column eluate (0–40 minutes) and assaying the radioactivity to assess recovery of injected radioactivity.

Data Analysis. The radioactivity concentration in the plasma or tissues (ng eq. of edoxaban/ml or g of tissue) was obtained by LSC and expressed as equivalents of edoxaban. Concentrations of edoxaban in the samples (ng/ml or g tissue) were calculated by multiplying the radioactivity concentration in the samples by the proportion of edoxaban in the samples to the total radioactivity, which was obtained by the radio-HPLC analysis. The tissue-to-plasma partition coefficient (Kp) value of edoxaban was calculated by dividing the concentrations of edoxaban in the brain, liver, or kidney by that in the plasma.

Results

Transcellular Transport of Edoxaban across Caco-2 Cell Monolayers. In the bidirectional transport assay systems, [3H]digoxin, a well-characterized P-gp probe, showed the vectorial transport (Papp,A to B < Papp,B to A) with a Papp ratio of 24.0, which was completely inhibited by the P-gp inhibitor verapamil (Fig. 2A). To assess the transport of edoxaban via P-gp, [14C]edoxaban was applied to the Caco-2 cell system as shown in Fig. 2B. 1 μM of [14C]edoxaban exhibited much lower permeability in the apical-to-basal direction compared with that in the basal-to-apical direction, and the vectorial transport of edoxaban with Papp ratio of 19.4 was completely inhibited by verapamil (Papp ratio of 1.62). These results suggest the involvement of P-gp in the vectorial transport of edoxaban in Caco-2 cell monolayers. Further, the high inherent membrane permeability of edoxaban was also suggested, since Papp, A to B in the presence of verapamil (10.3 × 10−6 cm/s) was comparable to that of labetalol (12.9 × 10−6 cm/s; data on file), a class I drug in the biopharmaceutics classification system.

As shown in Fig. 2, B and C, edoxaban exhibited a saturable vectorial transport up to 100 μM with the reduction of Papp ratio. An apparent Michaelis–Menten constant, K Inapp, defined by the donor concentration, was calculated to be 74.0 μM.

The effects of P-gp inhibitors on the vectorial transport of edoxaban in Caco-2 cells were further investigated. As shown in Table 1, the P-gp-mediated transport of edoxaban was inhibited by all compounds tested with a wide range of the IC50 values: the strongest inhibition (IC50 = 0.244 μM) by ketoconazole and the weakest inhibition (IC50 = 62.9 μM) by atorvastatin. The IC50 values against digoxin transport using the Caco-2 cell monolayers were also investigated. By comparing the IC50 values, the IC50 of P-gp inhibitors tested against the vectorial transport of edoxaban in Caco-2 cells were similar to those of

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 for Edoxaban Transport via P-gp</th>
<th>IC50 for Digoxin Transport via P-gp</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>Digoxin</td>
<td>51.3</td>
<td>NT</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.244</td>
<td>0.685</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.498</td>
<td>1.12</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>24.0</td>
<td>NT</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>49.8</td>
<td>54.8</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.898</td>
<td>0.929</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>62.9</td>
<td>43.5</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>0.290</td>
<td>NT</td>
</tr>
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</table>

NT, not tested.

Fig. 3. Tissue distribution of edoxaban in male wild-type and Mdr1a/1b (−/−,−/−) mice after intravenous administration of [14C]edoxaban at a dose of 1 mg/kg. Edoxaban plasma and tissue concentrations at 0.5, 1, and 2 hours were calculated by multiplying the radioactivity concentration in samples by the proportion of edoxaban to the total radioactivity in the sample. Each value represents the mean ± S.E.M. (n = 3) except for plasma at 2 hours and brain homogenates, which were pooled from three animals at each sampling time and assayed.
digoxin, indicating that the recognition of edoxaban by P-gp was
distinct from that of digoxin.

**Brain Penetration of Edoxaban in P-gp Knockout Mice.** P-gp
expressed in the blood-brain barrier can restrict the permeation of
substrate compounds into the brain. To clarify whether the
pharmacokinetics of edoxaban are regulated by P-gp, the brain
penetration of edoxaban in P-gp knockout mice, Mdr1a/1b(−/−,−/−),
was evaluated. After intravenous administration of 1 mg/kg of
[14C]edoxaban, plasma, liver, kidney, and brain were sampled and the
radioactivity in each sample was determined. The proportion of
edoxaban to total radioactivity in each sample determined by radio-
HPLC was less than 50% and varied among the sampling times and
tissues (3.7 to 46%). Thus, the total radioactivity in the samples was
corrected by the proportion of edoxaban in each sample to determine
the concentration of edoxaban.

As shown in Fig. 3 and Table 2, the concentration of edoxaban in
the brains of wild-type mice was much lower than that in plasma, with
the $K_p$ values of 0.0330 to 0.105. In contrast, the $K_p$ values in
the brains of P-gp knockout mice were determined to be 0.608 to 1.8,
suggesting a similar concentration level of edoxaban between brain and
plasma. No marked difference in $K_p$ values in either the liver or lung
was observed between wild-type and P-gp knockout mice. These
results demonstrate that the tissue distribution of edoxaban is regulated
by P-gp.

**Potential Uptake of Edoxaban by OAT1, OAT3, and OCT2-
Expressing Cells.** Since renal tubular secretion of edoxaban into urine
was observed during clinical studies, the uptake studies of edoxaban
via renal uptake transporters on the basolateral membrane, OAT1, OAT3,
and OCT2 were investigated. The probe substrate for OAT1,
[3H]PAH, was highly transported by OAT1-expressing cells, compared
and OCT2 were investigated. The probe substrate for OAT1,
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Expressing Cells.

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The maximal inhibitory effects were less than 50% for both OAT1, OAT3,
OCT1, and OCT2, with control cells, and their transport was inhibited by a typical inhibitor,
probenecid, indicating the high transport activity of the OAT1-
expressing cells (Fig. 4A, left panel). In this assay system, no
remarkable uptake transport of [14C]edoxaban was observed at either
1- or 5-minute incubations (Fig. 4A, right panel). Similarly, potential
transport of [14C]edoxaban via OAT3 and OCT2 was also determined
(Fig. 4, B and C). Although both OAT3 and OCT2 assay systems
showed sufficient transport activities of probe substrates, [1H]HES,
and [14C]metformin, no significant transport of [14C]edoxaban was
demonstrated by these assay systems. These results indicate that
[14C]edoxaban is either a poor substrate or not a substrate at all for
OAT1, OAT3, and OCT2.

**Potential Uptake of Edoxaban and M-4 by Freshly Isolated
Human Hepatocytes and OATP1B1-Expressing Oocytes.** To
clarify the involvement of the active uptake mechanisms in biliary
excretion, active hepatic uptake studies using the isolated human
hepatocytes and OATP1B1-expressing cell systems were conducted.
M-4, which is a major plasma metabolite of edoxaban and also has
pharmacological potential in vitro (Bathala et al., 2012), was also
investigated. [1H]HES, a marker substrate of active hepatic uptake and a
probe substrate of OATP1B1, was used as a positive control. As
shown in Fig. 5A, [1H]HES exhibited higher uptake into freshly isolated
human hepatocytes compared with that in the presence of typical
OATP inhibitors, cyclosporin A, or rifampicin, ensuring the OATP-
mediated uptake activities. Using the same hepatocytes, M-4 exhibited
the time-dependent OATP-mediated uptake; however, no active
uptake of edoxaban was observed (Fig. 5, B and C).

OATP1B1-expressing oocytes were also used to assess the potential
transport of edoxaban and M-4 via OATP1B1. As shown in Fig. 6,
[1H]HES and [14C]M-4 (10 and 50 μM) showed significant uptake, and
the transport disappeared in the presence of the OATP inhibitor,
30 μM ES. In contrast, no significant transport of [14C]edoxaban (10 and
50 μM) via OATP1B1 was found. These data indicated that, although
M-4 is taken up by hepatocytes mainly via OATP1B1, hepatic uptake of
edoxaban via OATP was negligible.

**Inhibitory Potential of Edoxaban on Drug Transporters,OAT1,
OAT3, OCT1, OCT2, OATP1B1, OATP1B3, and P-gp.** Using the
drug transporter-expressing cell systems, the inhibitory potential of
edoxaban on OAT1, OAT3, OCT1, OCT2, OATP1B1, OATP1B3,
and P-gp was investigated. Maximal inhibitory effects greater than
50% were observed at the highest concentration (100 μM) of edoxaban
against OATP1B1, OATP1B3, and P-gp, with IC50 values of 62.7,
50.8, and 53.7 μM, respectively (Table 3). As for OAT1, OAT3,
OCT1, and OCT2, the maximal inhibitory effects were less than 50%,
therefore IC50 values were deemed to be higher than 100 μM.

**Discussion**

Incomplete absorption of edoxaban following oral administration has been observed in human (bioavailability = 61.8%), along with active renal secretion, biliary excretion, and/or intestinal secretion (Matsushima et al., 2011; Bathala et al., 2012). These observations suggested that the drug transporters expressed in the kidney, liver, and gastrointestinal tract were likely to be involved in the drug disposition of edoxaban. In this study, to clarify the molecular mechanism of the disposition of edoxaban in humans, we evaluated whether edoxaban was a substrate for relevant drug transporters, and we were consequently able to demonstrate that edoxaban is a substrate of P-gp but not of other major drug transporters.

Using a bidirectional transport assay system in Caco-2 cell
monolayers, the vectorial transport of edoxaban was observed with
a $K_{m,app}$ value of 74.0 μM (Fig. 2). Although the Caco-2 cells express
multiple efflux transporters, such as breast cancer resistance protein
and multidrug resistance-associated protein 2, we conclude that P-gp
is a major determinant for the vectorial transport of edoxaban, because
verapamil, a typical P-gp inhibitor, completely inhibited the vectorial
transport of edoxaban. Furthermore, the IC50 values of other P-gp
inhibitors tested against the vectorial transport of edoxaban were

### Table 2

<table>
<thead>
<tr>
<th>Tissues (h)</th>
<th>Brain</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.033 g</td>
<td>0.105 g</td>
<td>10.5 ± 3.7</td>
<td>10.1 ± 3.3</td>
</tr>
<tr>
<td>0.105 g</td>
<td>ND</td>
<td>14.4 ± 3.4</td>
<td>12.0 ± 6.0</td>
</tr>
<tr>
<td>1</td>
<td>17.2 ± 5.2</td>
<td>20.1 ± 6.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11.5 ± 0.9</td>
<td>7.97 ± 1.40</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>12.7 ± 3.8</td>
<td>14.0 ± 6.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>26.2 ± 10.1</td>
<td>13.4 ± 6.4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>$K_p$ Values in Wild-Type Mice</th>
<th>$K_p$ Values in Mdr1a/1b(−/−,−/−) Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>0.033 g</td>
<td>0.105 g</td>
</tr>
</tbody>
</table>

ND, not detected.

*Data were determined from the pooled samples of three animals.
similar to those of digoxin, a typical P-gp substrate (Table 1). In addition, in the in vivo study, the distribution of edoxaban to the brain in P-gp knockout mice was dramatically increased compared with wild-type mice (Fig. 3). In contrast, no significant transport of edoxaban by uptake transporters expressed in the liver (OATP1B1) or kidney (OAT1, OAT3, and OCT2) was observed in transporter-expressing cells or freshly isolated human hepatocytes (Figs. 4–6).

In the gut, P-gp pumps drugs back into the intestinal lumen and limits oral absorption (Zhang and Benet, 2001). This efflux activity should explain the incomplete absorption of edoxaban observed in}

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**Fig. 4.** Uptake transport of [14C]edoxaban via renal uptake transporters, OAT1 (A), OAT3 (B), and OCT2 (C) using transfected S2 cells. (A) The left and right panels show the uptake CL of the probe substrate, [3H]PAH (1 μM for 2 minutes), and [14C]edoxaban (10 μM for 1 and 5 minutes), respectively, into OAT1-expressing or control S2 cells in the presence or absence of a typical OAT1 inhibitor, probenecid (100 μM). (B) The left and right panels show the uptake clearance (CL) of the probe substrate, [3H]ES (50 nM for 2 minutes), and [14C]edoxaban (10 μM for 1 and 5 minutes), respectively, into OAT3-expressing or control S2 cells in the presence or absence of a typical OAT3 inhibitor, probenecid (100 μM). (C) The left and right panels show the uptake CL of the probe substrate, [14C]metformin (10 μM for 5 minutes), and [14C]edoxaban (10 μM for 1 and 5 minutes), respectively, into OCT2-expressing or control S2 cells in the presence or absence of a typical OCT2 inhibitor, quinidine (300 μM). Each value represents the mean ± S.E.M. (n = 3).
that edoxaban possesses high inherent membrane permeability suggests a possibility of the complete absorption of edoxaban in the absence of P-gp efflux activity, which also supports the involvement of P-gp as an absorption barrier. Further, in a clinical pharmacokinetic study with a single dose of edoxaban, a dose-proportional increase in exposure was observed in the range of 10–150 mg, implying the nonsaturable kinetics of P-gp within the clinical dose.

Because edoxaban is partially eliminated via the renal secretion process (Bathala et al., 2012), the inhibition of P-gp in the kidney could be a possible explanation for the observed DDIs. However, the intestine was considered to be a primary tissue for the P-gp-mediated DDIs, because in general, intestinal concentration of P-gp inhibitors is projected to be much higher than that in plasma following oral administration. This explanation was exemplified by the DDI documented between digoxin and amiodarone, during which amiodarone increased the Cmax and AUC of digoxin without affecting its renal CL (Robinson et al., 1989). The total plasma concentration of amiodarone did not reach the IC50 value against P-gp. In addition, based on the Fenner et al. (2009) report, only a few compounds affect the renal excretion of digoxin. Thus, most P-gp inhibitors causing DDIs are supposed to inhibit P-gp in the gut, not in the kidney. Therefore, intestinal P-gp will act as an absorption barrier of edoxaban, and DDIs caused by the inhibitors can be mainly attributed to the inhibition of P-gp in the gut.

Although edoxaban is eliminated by active renal secretion (Bathala et al., 2012), it was not a substrate of OAT1, OAT3, or OCT2 transporters expressed on the basal membrane of renal epithelial cells. In general, many drugs secreted by the kidneys, such as acyclovir, furosemide, and metformin, are substrates of OAT or OCT (Odlind et al., 2010). The renal secretion process of furosemide is also considered to be mediated mainly by OAT3, with a minor contribution of OCT2-mediated transport, not passive diffusion, which serves as the main mechanism to move metformin across the basal membrane. This is the first step of renal secretion before being excreted into the urine by multidrug and toxin extrusion–1 or 2-K efflux transporters located on the apical membrane (Chen et al., 2009; Meyer zu Schwabedissen et al., 2010). The renal secretion process of furosemide is also considered to be mediated mainly by OAT3, with a minor contribution of passive diffusion due to a low permeability (Odlind and Beermann, 1980). On the other hand, passive diffusion of edoxaban is relatively high based on the membrane permeability across Caco-2 cells in the presence of verapamil. Therefore, we inferred the mechanism of renal secretion of edoxaban as follows: edoxaban crosses the basal membrane of renal epithelial cells via passive diffusion and/or other transporters not tested in this study, then it is actively secreted by apical P-gp into the urine.

No active uptake transport of edoxaban was observed in either OATP1B1-expressing oocytes or human hepatocytes. Therefore, we speculate that passive diffusion across the hepatic basal membrane is

<table>
<thead>
<tr>
<th>Transporter</th>
<th>OAT1</th>
<th>OAT3</th>
<th>OCT1</th>
<th>OCT2</th>
<th>OATP1B1</th>
<th>OATP1B3</th>
<th>P-gp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (μM)</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>62.7</td>
<td>50.8</td>
<td>53.7</td>
</tr>
</tbody>
</table>

Fig. 6. Uptake transport of [14C]edoxaban and [14C]M-4 via hepatic uptake transporter, OATP1B1. (A) [3H]ES (50 nM) used as a probe substrate, (B) [14C]edoxaban (10 and 50 μM), and (C) [14C]M-4 (10 and 50 μM) were evaluated using OAT1- and OAT3-expressing S2 cells, OCT1-, OCT2-, OATP1B1-, and OATP1B3-expressing HEK293 cells, and Caco-2 cells expressing P-gp.
the sole pathway for edoxaban to enter the liver. On the other hand, M-4, an active metabolite of edoxaban produced via carboxyl esterase 1, which possesses a carboxylic moiety, was actively transported into the hepatocytes via OATP1B1. Although anionic compounds, such as 3-hydroxy-3-methylglutaryl-CoA inhibitors, are well characterized as substrates of OATP1B1 governing the disposition of substrate (Kalliokoski and Niemi, 2009), cationic compounds, like edoxaban, are minimally transported by OATP; therefore, the results of the OATP1B1 uptake assay for edoxaban and M-4 seem to be reasonable based on the current knowledge of OATP substrate recognition.

In an effort to characterize edoxaban disposition in animals, the involvement of intestinal secretion in edoxaban's elimination has also been observed. In bile duct–cannulated rats, after i.v. administration of edoxaban, 8.6% of the dose was detected in feces and 9% was detected in the bile, indicating the existence of both intestinal and biliary excretion in the clearance pathway (data on file). Although previously published data clearly demonstrate the involvement of P-gp in the intestinal secretion in P-gp knockout mice models (Smit et al., 1998; van Asperen et al., 2000), in humans, the mechanism and significance of the intestinal secretion in drug elimination remains unclear. Intestinal P-gp could play a role as an absorption barrier as well as an elimination pathway.

FDA draft guidance (FDA, 2012) and EMA guidelines (EMA, 2012) state that the DDI risks involving uptake transporters are judged from the blood/plasma concentration and IC₅₀ values. Based on the results of our transporter inhibition assays (Table 3), we conclude that there would be minimal risk of clinical DDIs due to edoxaban coadministration with drugs transported by transporters tested in this study. The total plasma concentration of edoxaban at the highest dose (C_max = 332 nmol/ml at a dose of 60 mg) (Bathala et al., 2012) is much lower than the estimated IC₅₀ values observed here, suggesting that no interactions with the drug transporters tested in this study would occur in clinical use. The potential of edoxaban to inhibit P-gp in the gut, [I₅₀] (hypothetical gut concentrations, dose [μmol/l]25 [I]) at a dose of 30 mg divided by the IC₅₀ value is less than 10 (Giacomin et al., 2010), indicating a minimal risk for P-gp–related edoxaban DDIs. Indeed, no significant interaction with digoxin was observed in a clinical study (Mendell et al., 2012).

In conclusion, we have demonstrated that edoxaban is a substrate for P-gp, but not for the other major uptake transporters tested. Since the metabolism of edoxaban is a minor contributor to the total clearance, and strong P-gp inhibitors elevate edoxaban exposure, the membrane transport process of edoxaban via P-gp is a key factor in edoxaban’s disposition in humans.

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Authorship Contributions
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Conducted experiments: Mikkaichi, Yoshigae, Masumoto, Imaoka, Rozehnal, Fischer.
Performed data analysis: Mikkaichi, Yoshigae, Masumoto, Imaoka, Rozehnal, Fischer.

Wrote or contributed to the writing of the manuscript: Mikkaichi, Yoshigae, Okudaia, Izumi.

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