Special Section on Transporters in Drug Disposition and Pharmacokinetic Prediction

Physiologically Based Pharmacokinetic Modeling of Bosentan Identifies the Saturable Hepatic Uptake As a Major Contributor to Its Nonlinear Pharmacokinetics

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ABSTRACT

Bosentan is a substrate of hepatic uptake transporter organic anion–transporting polypeptides (OATPs), and undergoes extensive hepatic metabolism by cytochrome P450 (P450), namely, CYP3A4 and CYP2C9. Several clinical investigations have reported a nonlinear relationship between bosentan doses and its systemic exposure, which likely involves the saturation of OATP-mediated uptake, P450-mediated metabolism, or both in the liver. Yet, the underlying causes for the nonlinear bosentan pharmacokinetics are not fully delineated. To address this, we performed physiologically based pharmacokinetic (PBPK) modeling analyses for bosentan after its intravenous administration at different doses. As a bottom-up approach, PBPK modeling analyses were performed using in vitro kinetic parameters, other relevant parameters, and scaling factors. As top-down approaches, three different types of PBPK models that incorporate the saturation of hepatic uptake, metabolism, or both were compared. The prediction from the bottom-up approach (models 1 and 2) yielded blood bosentan concentration-time profiles and their systemic clearance values that were not in good agreement with the clinically observed data. From top-down approaches (models 3, 4, 5-1, and 5-2), the prediction accuracy was best only with the incorporation of the saturable hepatic uptake for bosentan. Taken together, the PBPK models for bosentan were successfully established, and the comparison of different PBPK models identified the saturation of the hepatic uptake process as a major contributing factor for the nonlinear pharmacokinetics of bosentan.

Introduction

Bosentan is a dual endothelin (ET) receptor antagonist and is indicated for the treatment of patients with pulmonary arterial hypertension (Rubin et al., 2002; Dingemanse and van Giersbergen, 2004; Galiè et al., 2008). Several clinical investigations have so far reported apparently opposing results in regard to a nonlinear relationship between intravenous and oral administration of bosentan and its systemic exposure in humans. When single intravenous bosentan doses ranging from 10 to 750 mg were administered to healthy volunteers, the systemic plasma clearance of bosentan decreased with increasing doses (10.8 and 5.7 l/h for the bosentan doses of 10 and 750 mg, respectively) (Weber et al., 1996). In the case of oral dosing, the systemic exposure of bosentan increased in a dose-proportional manner up to 600-mg doses of bosentan in healthy volunteers. However, with oral doses of bosentan greater than 600 mg, the fold increases in the systemic exposure [i.e., Cmax, and areas under the plasma concentration–time curve (AUCs)] were less than dose proportional (Weber et al., 1996). After repeated oral dosing of 500 mg of bosentan, the plasma clearance of bosentan was increased by approximately 2-fold, accompanied by an approximately 1.7-fold increase in

ABBREVIATIONS: AIC, Akaike information criterion; AUC, area under the plasma concentration–time curve; CLint/met, hepatic intrinsic metabolic clearance; CLmet, metabolic clearance; CLr, renal clearance; ET, endothelin; fb, unbound fraction in blood; fip, hepatic intracellular unbound fraction; Km, Michaelis constant; Km, Michaelis constant of metabolism; Km, value for the production of desmethyl bosentan; Km, value for the production of hydroxyl bosentan; Km, Michaelis constant of uptake; LC-MS/MS, liquid chromatography–tandem mass spectrometry; m/z, charge/mass ratio; OATP, organic anion–transporting polypeptide; P450, cytochrome P450; PBPK, physiologically based pharmacokinetic; PK, pharmacokinetic; PSact, transporter-mediated active uptake clearance; PSdiff, passive diffusion clearance; PSdiff, passive diffusion efflux clearance; PSdiff, passive diffusion influx clearance; TMDD, target-mediated drug disposition; v, initial uptake rate; Vmax, maximum metabolic rate; Vmax, maximum uptake rate; WSS, weighted squared residuals.
24-hour urinary excretion of β-glucuronidase, indicating autoinduction of bosentan metabolism mediated by CYP3A4 (Weber et al., 1999c).

Bosentan has also been associated with various cases of drug interactions when coadministered with drugs that inhibit/induce some cytochrome P450 (P450) enzymes and/or hepatocellular transporters (Kreisberg et al., 2002; Markert et al., 2014). After the second concomitant dosing of bosentan with cyclosporine, average trough concentrations of bosentan were 31-fold higher than those after the first dosing of bosentan (Binet et al., 2000). In the case of rifampin coadministration, the changes in the bosentan pharmacokinetics (PKs) depended on the number of rifampin dosing (van Giersbergen et al., 2007). The systemic exposure of bosentan markedly increased after the single rifampin dose coadministered, but significantly decreased after multiple rifampin doses. The cases of drug interactions are also reported for bosentan when coadministered with simvastatin (Dingemanse et al., 2003) and warfarin (Weber et al., 1999a). These complex drug interactions with bosentan likely involve the saturation of OATP-mediated uptake, P450-mediated metabolism, or both in the liver, yet a detailed mechanistic understanding has been lacking.

Several clinical and nonclinical studies provided evidence supporting the involvement of P450 enzymes and OATPs in the disposition of bosentan. A clinical study with 14C-labeled bosentan (Weber et al., 1999b) indicated extensive hepatic elimination of bosentan with minor renal and fecal excretion. The two major metabolites hydroxyl bosentan and desmethyl bosentan are reported to be produced mainly by CYP3A4 and CYP3A4, respectively (Dingemanse and van Giersbergen, 2004). Bosentan is also a substrate of OATP1B1, OATP1B3, and OATP2B1 (Treiber et al., 2007; Jones et al., 2012). In rats, PK interactions between bosentan and cyclosporine A were reported with the proposed mechanism involving the inhibition of hepatic uptake of bosentan by cyclosporine A (Treiber et al., 2004).

Physiologically based PK (PBPK) modeling has increasingly shown its utility in providing the kinetic and mechanistic insights into nonlinear PKs and complex drug interactions (Fan et al., 2010; Watanebe et al., 2010; Rowland et al., 2011). In the current study, we developed PBPK models for analyzing the systemic nonlinear PKs of bosentan after its intravenous administration at different doses by incorporating saturable processes of hepatic uptake, metabolism, or both via bottom-up and top-down approaches.

Materials and Methods

Materials. Bosentan was purchased from the Cayman Chemical Company (Ann Arbor, MI). Bosentan-d4, hydroxyl bosentan, and desmethyl bosentan were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Pooled cryopreserved human hepatocytes from 20 mixed-sex donors (Caucasian, 14 donors; Hispanic, 4 donors; and black, 2 donors) were purchased from Veritas (Tokyo, Japan). Pooled human liver microsomes from mixed-sex donors were purchased from Corning Japan (Tokyo, Japan). All other chemicals and reagents were readily available from commercial sources.

Kinetic Parameters for Bosentan Uptake (Human Cryopreserved Hepatocytes). Uptake studies using human cryopreserved hepatocytes were performed using a rapid separation method, as described previously (Shitara et al., 2003). Briefly, cryopreserved hepatocytes were thawed out, washed, and resuspended in Krebs Henseleit buffer (at a density of 2 × 10⁶ cells/ml). After preincubation at 37°C for 5 minutes, bosentan uptake was initiated by adding an equal volume of bosentan-containing buffer (the final concentrations of 0.6, 3, 6, 10, 30, or 100 μM) to the hepatocyte suspensions. After incubation at 37°C for 0.5, 1.5, or 3 minutes, the reaction was terminated by separating the cells from the bosentan solution. The separation was performed using tubes containing 50 μl of 2.5 M ammonium acetate under a layer of 100 μl of oil mixture (a mixture of silicone oil and mineral oil; density = 1.015). After centrifugation at 2000g for 30 seconds, tubes were snap frozen immediately and kept at −80°C until analysis. After thawing on ice, the centrifuge tube was cut below the oil layer and cells were resuspended in 40 μl of water. This suspension was transferred to another tube containing an internal standard and acetonitrile, and sonicated for 4.5 minutes using a Bioruptor sonication device (Cosmo Bio Co., Ltd., Tokyo, Japan). After centrifugation at 15,000g for 5 minutes, the resulting supernatant was diluted 2-fold with 0.1% formic and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Bosentan uptake into the hepatocytes was expressed as the uptake volume of bosentan [in microliters per 10⁶ cells (the bosentan amount detected divided by the bosentan concentration in the assay buffer)]. The initial uptake velocity of bosentan was calculated from the slope of the uptake volume obtained from 0.5 to 3 minutes and was expressed as the uptake clearance (in microliters per minute per 10⁶ cells).

The kinetic parameters for the bosentan uptake of bosentan were calculated using the following equation:

\[ v = \frac{V_{\text{max, uptake}} \times S}{K_{\text{m, uptake}} + S} + \frac{PS_{\text{dif}} \times S}{K_{\text{m, uptake}} + S} \]

where v is the initial uptake rate (in picomoles per minute per 10⁶ cells), S is the substrate concentration (micromolar), \( V_{\text{max, uptake}} \) is the maximum uptake rate (in picomoles per minute per 10⁶ cells), \( K_{\text{m, uptake}} \) is the Michaelis constant of uptake (micromolar), and \( PS_{\text{dif}} \) (microliters per minute per 10⁶ cells) is the passive diffusion clearance.

The hepatic intracellular bound fraction (fβ) was calculated as described previously (Yoshikado et al., 2016). Briefly, the hepatocyte suspensions (2.0 × 10⁶ cells/ml) were incubated with an equal volume of buffer containing bosentan (the final concentration, 1 μM) on ice for 0.5, 15, 30, or 60 minutes, and cells were separated and processed using the same method as described above. Bosentan levels in cell lysates and medium were quantified by LC-MS/MS. It was assumed that the active transport and membrane potential were abolished on ice and that the protein unbound fraction in the medium was 1. Using the values at 60 minutes (when the uptake was presumed to be at the steady state), fβ was calculated using the following equation:

\[ \frac{C_{\text{cell}}(\rightarrow)}{C_{\text{medium}}(\rightarrow)} = \frac{C_{\text{cell}}(\rightarrow)}{C_{\text{medium}}(\rightarrow)} \times \frac{1}{f_{\beta}} \]

where \( C_{\text{cell}}(\rightarrow) \) and \( C_{\text{medium}}(\rightarrow) \) are the total bosentan concentrations in the cell and medium measured on ice at 60 minutes, respectively; and \( C_{\text{cell}}(\rightarrow) \) and \( C_{\text{medium}}(\rightarrow) \) are the unbound bosentan concentrations in the cell and medium, respectively.

Kinetic Parameters for Bosentan Metabolism (Human Liver Microsomes). The kinetic parameters for bosentan metabolism were assessed by monitoring the generation of both hydroxyl bosentan and desmethyl bosentan. The reaction mixture was prepared with pooled human liver microsomes (final concentration, 2 mg/ml) and 100 mM phosphate buffer containing bosentan (final concentrations, 2, 4, 10, 25, 60, or 150 μM). After preincubation at 37°C for 5 minutes, the reaction was initiated by the addition of a NADPH-generating system (final concentrations: 0.3 μM NADPH, 5 μM glucose 6-phosphate, 1 μM glucose-6-phosphate dehydrogenase, and 3 mM MgCl₂). The reaction was terminated by the addition of two equivalent volumes of ice-cold, acetonitrile containing an internal standard, followed by brief vortexing. After centrifugation at 13,000g for 10 minutes, the resulting supernatant was diluted with 0.1% formic acid and subjected to LC-MS/MS analysis.

The Michaelis constant of metabolism \( K_{\text{m, met}} \) (micromolar), the maximum velocity of metabolism \( V_{\text{max, met}} \) (in picomoles per minute per milligram protein), and nonsaturable metabolic clearance \( CL_{\text{met, nonsaturation}} \) (microliters per minute per 10⁶ cells) were calculated using the following equation (fitting was performed using the nonlinear least-squares method):

\[ v = \frac{V_{\text{max, met}} \times S}{K_{\text{m, met}} + S} + CL_{\text{met, nonsaturation}} \times S \]

where v is the initial velocity (in picomoles per minute per milligram protein) and S is the substrate concentration (micromolars).

LC-MS/MS Analysis. To quantify bosentan, hydroxyl bosentan, and desmethyl bosentan, the LC-MS/MS analyses were performed using a Nexera X2 separating module (Shimadzu Co., Kyoto, Japan) equipped with an LCMS-8040 Mass Spectrometer (Shimadzu Co.) with an electron ion spray interface. The mass spectrometer was operated in the multiple reaction-monitoring mode using the respective MH⁺ ions: charge/mass ratio (m/z) 552 → 520 for bosentan,
$m/z$ 568 $\rightarrow$ $m/z$ 202 for hydroxyl bosentan, $m/z$ 538 $\rightarrow$ $m/z$ 494 for desmethyl bosentan, and $m/z$ 556 $\rightarrow$ $m/z$ 494 for bosentan-d$_4$. The mobile phase was 55% acetonitrile containing 0.1% formic acid, and the flow rate was 0.2 ml/min with the stationary phase, a C18 column (Kintex C18, 2.1 $\times$ 100 mm, 2.6 $\mu$m; Phenomenex Inc., Torrance, CA) at 40°C.

**Parameter Optimization by Nonlinear Least Squares Fitting.** All fitting and simulation analyses were performed using a multiple-purpose nonlinear least-squares fitting computer program, Napp (version 2.31; available from http://plaza.umin.ac.jp/~todaiyak/download.php) (Hisaka and Sugiyama, 1998). Differential equations were numerically solved using the Runge-Kutta-Fehlberg method. To evaluate the goodness of the fit, the sum of the weighted squared residuals (WSS) and Akaike information criterion (AIC) were calculated using the following equations:

$$WSS = \sum_{i=1}^{n} \left( \frac{y_i - y'_i}{\sigma_i} \right)^2$$

where $y_i$ is the $i$th observed value; and $y'_i$ is the $i$th predicted value.

$$AIC = n \ln(WSS) + 2m$$

where $n$ is the number of observations; and $m$ is the number of estimated parameters in the model.

**Structure of the PBPK Models for Bosentan.** Figure 1 shows the structure of the constructed PBPK model for bosentan after intravenous bolus dosing in humans. EH, Extrahepatic; HC, hepatocellular; $K_p,a$, $K_p,m$, and $K_p,s$, the partition coefficient between adipose, muscle, and skin; $Q_a$, $Q_m$, and $Q_s$, blood flow rate in adipose, muscle, and skin.

![Diagram of PBPK model](https://via.placeholder.com/150)

**Parameters and Equations.**

$$f_{H} \text{ is fixed at the value determined by in vitro study on ice, which is approximately } 0.74.$$
shown to be consistent with that estimated at 37°C using human liver homogenates (Yoshikado et al., 2017). In all analyses conducted in this study, 

\[
PS_{\text{dif,eff}} = \frac{PS_{\text{dif,inf}}}{\gamma}
\]

The \( \gamma \) value was calculated to be 0.243 at 37°C with consideration of the following: 1) the ratio of the membrane permeability by passive diffusion of an ionized form of the drug to that of its unionized form (obtained from in vitro experiments that examine pH-dependent membrane permeability); 2) the concentration ratio of an ionized form of the drug to its unionized form, derived from the Henderson-Hasselbalch equation (intracellular pH 7.2; extracellular pH 7.4); and 3) the membrane potential estimated from the Nernst equation (Yoshikado et al., 2016).

Both bottom-up and top-down approaches were used for the current PBPK modeling analyses (summarized in Table 2). As bottom-up approaches, simulation analyses were performed using the kinetic parameters extrapolated from in vitro to in vivo using biologic scaling factors (model 1) or those obtained by fitting (model 2). Detailed description on the handling of various parameters is included in the Supplemental Material. As top-down approaches (models 3, 4, and 5), we performed simultaneous fitting analyses of the PBPK models that incorporate saturation processes for \( PS_{\text{act}} \), \( CL_{\text{int,met}} \), or both to blood bosentan.
In vivo $V_{\text{max, uptake}}$ and $K_{\text{m, uptake}}$ were calculated from this linear part of the time-uptake curves using proportional to time at least up to 3 minutes after the onset of incubation.

Simulated using the kinetic parameters of hepatic uptake and metabolism (Kato et al., 2003; Ito et al., 2017; Toshimoto et al., 2017). The initial uptake velocity of bosentan was calculated using the uptake $V_{\text{max, uptake}}$ and uptake clearance of bosentan, respectively. The initial uptake velocity of bosentan was calculated using the uptake

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
<th>Model 5</th>
</tr>
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<tbody>
<tr>
<td>$V_{\text{max, uptake}}$</td>
<td>µmol/h per 78 kg</td>
<td>642</td>
<td>642</td>
<td>1610 ± 159</td>
<td>1750 ± 314</td>
<td>642 (64.2–6420)</td>
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<tr>
<td>$K_{\text{m, uptake}}$</td>
<td>µM</td>
<td>1.33</td>
<td>1.33</td>
<td>0.534 ± 0.0845</td>
<td>0.667 ± 0.132</td>
<td>1.33 (&gt;0.001)</td>
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<tr>
<td>$PS_{\text{act}}$</td>
<td>l/h per 78 kg</td>
<td>2360 ± 629</td>
<td>2360 ± 629</td>
<td>5.05 ± 0.729</td>
<td>5.35 ± 2.11</td>
<td>4.22 ± 1.04</td>
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<tr>
<td>$PS_{\text{dif, inf}}$</td>
<td>l/h per 78 kg</td>
<td>161</td>
<td>161</td>
<td>20.8</td>
<td>22.0</td>
<td>17.4</td>
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<tr>
<td>$V_{\text{max, met, OH}}$</td>
<td>µmol/h per 78 kg</td>
<td>97.1</td>
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<td>24.8%</td>
<td>24.8%</td>
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<tr>
<td>$K_{\text{m, met, OH}}$</td>
<td>µM</td>
<td>6.4</td>
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<tr>
<td>$CL_{\text{met, OH,nonsaturable}}$</td>
<td>l/h per 78 kg</td>
<td>0.936</td>
<td>0.936</td>
<td>0.936</td>
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<tr>
<td>$V_{\text{max, met, DES}}$</td>
<td>µmol/h per 78 kg</td>
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<tr>
<td>$K_{\text{m, met, DES}}$</td>
<td>µM</td>
<td>4.8</td>
<td>4.8</td>
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<tr>
<td>$CL_{\text{met,nonsaturable}}$</td>
<td>l/h per 78 kg</td>
<td>1.62</td>
<td>1.62</td>
<td>1.62</td>
<td>1.62</td>
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<tr>
<td>$V_{\text{max, met}}$</td>
<td>µmol/h per 78 kg</td>
<td>868 ± 438</td>
<td>1140 ± 713</td>
<td>135 (13.5–1350)</td>
<td>27 (2.7–270)</td>
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<tr>
<td>$K_{\text{m, met}}$</td>
<td>µM</td>
<td>108 ± 59.4</td>
<td>163 ± 106</td>
<td>5 (&gt;0.001)</td>
<td></td>
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<tr>
<td>$SF_{\text{transport}}$</td>
<td>10.0 ± 47</td>
<td>10.0 ± 47</td>
<td>10.0 ± 47</td>
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<tr>
<td>$SF_{\text{act}}$</td>
<td>1.04 ± 39.1</td>
<td>1.06 ± 6.3</td>
<td>1.04 ± 39.1</td>
<td>1.06 ± 6.3</td>
<td>1.04 ± 39.1</td>
<td>1.06 ± 6.3</td>
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<tr>
<td>$V_e$</td>
<td>l/78 kg</td>
<td>6.3 ± 10.2 ± 289</td>
<td>7.43 ± 0.953</td>
<td>6.93 ± 2.54</td>
<td>6.94 ± 1.06</td>
<td>6.3 (5.25–10.5)</td>
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<td>6.84816</td>
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<tr>
<td>AIC</td>
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<td>201.076</td>
<td>6.7290</td>
<td>125.439</td>
<td>86.8143</td>
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</table>

$PS_{\text{act}}$ is the unbound fraction in blood, $C_{\text{HIC}}$ is the concentration in ith extrahepatic compartment, and $C_{\text{HIC}}$ is the concentration in ith hepatocellular compartment $CL_{\text{met,nonsaturable}}$ is the saturation model (models 4 and 5):

$$CL_{\text{met,nonsaturable}} = \frac{V_{\text{max, met}}}{K_{\text{m, met}} + f_H C_{\text{HIC}}}$$

Tables 2 and 3 summarize the characteristics of the PBPK models used and the initial value as well as the lower and upper limits (range) of each parameter for optimizing kinetic parameters, respectively.

**Monte Carlo Simulation of Bosentan Blood Concentration Profiles.** One set of blood bosentan concentration-time profiles for six virtual subjects (same as those in the previous report Weber et al., 1996) were generated from Monte Carlo simulation based on the constructed PBPK model (model 3), and the same process was repeated 40 times to generate additional sets. The CV values for in vivo $V_{\text{max, uptake}}$, in vivo $K_{\text{m, uptake}}$, and in vivo $PS_{\text{act}}$ (those displaying interindividual variability) were set as 25.8%, 25.8%, and 10% as per the previously reported modeling methodologies (Kato et al., 2003; Ito et al., 2017; Toshimoto et al., 2017), and that for in vivo metabolic clearance ($CL_{\text{met}}$) was set as 33%, as reported previously (Kato et al., 2010). For parameters displaying intra-individual variability, proportional CV values were set at 24.8% (Volz et al., 2017). The in vivo $V_{\text{max, uptake}}$, in vivo $K_{\text{m, uptake}}$, in vivo $PS_{\text{act}}$, and in vivo $CL_{\text{met}}$ parameters were assumed to follow a log-normal distribution.

**Results**

**Kinetic Parameters of Bosentan Uptake and Metabolism from In Vitro Studies.** The uptake of bosentan by human hepatocytes was proportional to time at least up to 3 minutes after the onset of incubation in all bosentan concentrations (data not shown). Thus, the uptake rates were calculated from this linear part of the time-uptake curves using differing bosentan concentrations and used to prepare the Eadie-Hofstee plot (Fig. 2) and to obtain the kinetic parameters (Table 1). $PS_{\text{act}}$ (calculated from $V_{\text{max}}/K_{\text{m}}$ under the unsaturated conditions) was 35.6 µl/min per 10⁶ cells, approximately 12 times higher than $PS_{\text{dif, inf}}$. The $f_H$ was obtained from the steady-state uptake study under ice-cold conditions and determined to be 0.0696 ± 0.0068 (Table 1). Similarly, the Eadie-Hofstee plots and the kinetic parameters for the production of hydroxyl bosentan and desmethyl bosentan by human liver microsomes were obtained (Fig. 3; Table 1). Under the unsaturated condition, the in vitro intrinsic metabolic clearance for the production of hydroxyl bosentan and desmethyl bosentan (calculated from $V_{\text{max}}/K_{\text{m}}$) were 2.56 and 1.57 µl/min per milligram microsomal protein, respectively.

**PBPK Modeling Via Bottom-Up Approaches (Models 1 and 2).** For model 1, the blood concentration-time profiles of bosentan were simulated using the kinetic parameters of hepatic uptake and metabolism.
obtained from in vitro studies and extrapolated using biologic scaling factors. The predicted blood concentrations of bosentan were consistently higher than the reported values at nearly all time points for every dose level (Fig. 4A), resulting in the underprediction of the total body clearances compared with the observed in vivo values.

Instead of biologic scaling factors, model 2 used scaling factors that were optimized by fitting. With this modification, the predicted values of dose-normalized AUCs became closer to the reported values. However, the blood bosentan concentration-time profiles simulated by model 2 were not in good agreement with the clinically observed data (Fig. 4B).

**PBPK Modeling Via Top-Down Approaches (Models 3, 4, and 5).** The next three PBPK models incorporated the saturable processes for hepatic uptake only (model 3), metabolism only (model 4), or both (models 5). Model 3 yielded the predicted profiles that were in good agreement with the observed values as well as the smallest AIC values among the tested models (Fig. 4C; Table 3). Model 4, which incorporated the saturable process for hepatic metabolism only, yielded the profiles that substantially deviated from the clinically observed data, especially at early times at high doses of bosentan (Fig. 4D). In model 5, which incorporated the saturable process for both hepatic uptake and metabolism, the simulated blood concentration-time profiles of bosentan were in much better agreement with the observed data than those predicted from model 4 (Fig. 4E). The AIC value also substantially improved from 125 (model 4) to 86.8 (model 5).

**Monte Carlo Simulation of PBPK Modeling.** Taking interindividual and intraindividual variability of the parameters of model 3 into consideration, Monte Carlo simulations were carried out. The simulated dose-normalized AUCs of every dose level were similar to the reported values, in terms of the average and S.E. (Fig. 5). These results suggest that the variation in AUCs after intravenous bosentan dosing may be explained mostly by the variation in the kinetic processes of hepatic uptake and metabolism.

**Discussion**

In our current study, the PBPK models for bosentan were developed to enhance our kinetic and mechanistic understanding of nonlinear PKs associated with bosentan therapy. Based on the results comparing different PBPK models (models 1–5), the saturable hepatic uptake of bosentan is a most likely contributor to the nonlinear PKs of intravenously administered bosentan.

To obtain the kinetic parameters necessary for our PBPK modeling analyses, we assessed the processes of both hepatic uptake and metabolism of bosentan in the current study. The in vitro $K_{m,uptake}$ value for bosentan was determined to be 1.33 µM using suspended human cryopreserved hepatocytes and was considered to be in a comparable range with the previously reported values using OATP1B1-expressing cells or sandwich-cultured hepatocytes (4.27–44 µM) (Jones et al., 2012; Ménochet et al., 2012; Izumi et al., 2015). And the in vitro $K_{m,met}$ values for the production of hydroxyl bosentan and desmethyl bosentan were determined to be 6.40 and 4.80 µM, respectively, using pooled human liver microsomes. The $K_{m}$ values for bosentan metabolism were 12.3–232 µM using recombinant CYP2C9 microsomes (Chen et al., 2014) or 13 µM using human liver microsome (Ubeaud et al., 1995). The $K_{m}$ values in our experiment appear comparable with those of previous reports. By using the method reported previously (Hallifax and Houston, 2006), the lipophilicity of bosentan and the experimental conditions used in our in vitro study, the unbound fraction of bosentan was predicted to be 0.867 in the presence of microsomal protein 2 mg/ml. This prediction result suggested that the microsomal protein binding of bosentan may not be so extensive in our experimental conditions.

These $K_{m}$ values for bosentan metabolism and uptake were comparable with the estimated unbound maximum bosentan blood concentration (over 5 µM) in healthy volunteers after receiving the 750-mg intravenous bosentan dose. The in vitro $V_{max}/K_{m}$ value for bosentan uptake (using pooled human cryopreserved hepatocytes) was 35.6 µl/min per 10⁶ cells, approximately 12 times higher than the in vivo $PS_{ui}$ value (2.89 µl/min per 10⁶ cells) (Table 1). These results indicate that bosentan is actively taken up into the liver from the blood in humans and the unbound bosentan concentrations are likely to be higher in the hepatocytes than in human blood. These considerations provide justifications for further interrogating the saturation of hepatic uptake and/or metabolism of bosentan as possible underlying mechanisms for nonlinear bosentan PKs.

For model 1 (a bottom-up approach with the use of biologic scaling factors), simulated bosentan blood concentration-time profiles and dose-normalized AUCs of bosentan substantially differed from the clinically observed data (Fig. 4A). When the kinetic parameters were scaled up to fit the clinically observed data (model 2), the prediction accuracy...
improved for dose-normalized AUC values, yet there were substantial deviations in terms of bosentan blood concentration-time profiles (Fig. 4B). These findings may suggest that the scaling factors for in vitro $V_{\text{max,uptake}}/K_{\text{m,uptake}}$, and in vitro $PS_{\text{dif}}$ need to be individually optimized instead of using a single scaling factor for both parameters. These findings are in line with previous reports, which proposed that the scaling factor for OATP-mediated uptake clearance should be greater than 1 and be determined independently from in vitro $PS_{\text{dif}}$ (Kusuhara and Sugiyama, 2009; Jones et al., 2012; Varma et al., 2014).

Among the PBPK models of top-down approaches, model 3 was deemed to yield the best fit to the clinically observed data based on the AIC values. The scaling factors for bosentan uptake were calculated by calculating the ratio of the in vivo $V_{\text{max,uptake}}/K_{\text{m,uptake}}$ value to the biologically scaled in vitro $V_{\text{max,uptake}}/K_{\text{m,uptake}}$ value (483 l/h per 78 kg), yielding 6.24, 4.89, and 5.43 for models 3, 4, and 5, respectively. The reported scaling factors of OATP substrates, calculated using the same method, displayed considerable variability: 12–161 (Jones et al., 2012) or 1.0–101.8 (Varma et al., 2014). The scaling factors in our models appear to be less variable than those reported in the literature.

When the in vivo $K_{\text{m,uptake}}$ value of 0.534 or 0.667 $\mu$M was obtained by fitting in model 3 or 5, respectively, which were similar to the experimentally obtained in vitro $K_{\text{m,uptake}}$ value of 1.33 $\mu$M, simulated bosentan blood concentration-time profiles were in good agreement with the clinically observed data (Fig. 4). The similarity between in vivo and in vitro $K_{\text{m,uptake}}$ values may further support the saturation of hepatic transporters in the presence of high concentrations of substrates. The similarity between in vivo and in vitro $K_{\text{m,uptake}}$ values may further support the saturation of hepatic transporters in the presence of high concentrations of substrates.

Fig. 4. Simulation results from the PBPK models (models 1, 2, 3, 4, and 5) of bosentan. Panels A, B, C, D, and E are the analysis results of Model 1, 2, 3, 4 and 5, respectively. Solid lines represent the simulation results. The open and closed circles, open and closed squares, and open triangles indicate the reported bosentan blood concentration-time profiles with intravenous doses of 10, 50, 250, 500, and 750 mg, respectively.

Fig. 5. Monte Carlo simulation of bosentan blood concentration profiles. The results of the Monte Carlo simulations that considered interindividual variability in $V_{\text{max,uptake}}$, $K_{\text{m,uptake}}$, $PS_{\text{dif}}$, and $CL_{\text{met}}$ and intraindividual variability in model 3. Observed mean and S.E. values of each dose are shown as closed circles and lines, and mean values of dose-normalized AUCs of each virtual study estimated from Monte Carlo simulation using model 3 are indicated as closed rectangles.
uptake as the most likely contributor to the nonlinear PKs of bosentan after intravenous dosing.

PBPK modeling analyses incorporating saturable hepatic metabolism yielded the $K_{\text{m,met}}$ values of 108 and 163 $\mu$M for models 4 and 5, respectively. The maximum unbound concentrations of bosentan in the liver were predicted to be approximately 65 and 45 $\mu$M based on the simulation results using models 4 and 5, respectively (Supplemental Fig. 1), and on the unbound fraction in hepatocytes (0.0696) obtained by our in vitro study. Therefore, we reasoned that the saturation of bosentan metabolism in the liver is unlikely to occur at clinically relevant concentrations.

The $K_{\text{m,met}}$ values derived from models 4 (108 $\mu$M) and 5 (163 $\mu$M) differed from our in vitro experiment results using human liver microsomes ($K_{\text{m,met}}$ value for the production of hydroxyl bosentan ($K_{\text{m,met,OH}}$) was 6.40 ± 1.20 $\mu$M; $K_{\text{m,met}}$ value for the production of desmethyl bosentan ($K_{\text{m,met,DES}}$) was 4.80 ± 2.61 $\mu$M). These discrepancies may be related to the effects of CYP2C9 polymorphism on bosentan metabolism. Chen et al. (2014) reported that the $K_{\text{m,met,OH}}$ values mediated by CYP2C9 vary widely from 12.3 to 232 $\mu$M depending on the CYP2C9 polymorphism. We were not able to further investigate this possibility due to the limited information on CYP2C9 polymorphisms in the study participants.

The results of the Monte Carlo simulation also demonstrated that the variation in the systemic exposure (AUCs) after bosentan intravenous dosing can be explained mostly by the variations in $V_{\text{max,uptake}}$, $K_{\text{uptake}}$, $P_{\text{S,diff}}$, and $CL_{\text{uni,met}}$ (Fig. 5).

We initially attempted the PBPK analyses of the nonlinear PKs of bosentan after intravenous and oral dosing at the same time. Different from the intravenous data, the dose-normalized AUC values (AUC/dose) decreased with escalating oral doses of bosentan (Weber et al., 1996). To describe nonlinear PKs after oral administration, PBPK models included the components for solubility-limited absorption and saturable intestinal absorption mediated by OATP2B1 (detailed information is provided in Supplemental Figs. 2–7; Supplemental Material; and Supplemental Tables). Currently, we have limited confidence in our PBPK models for oral bosentan data, mainly due to the lack of information on excipients used for making bosentan suspensions. Further investigations are warranted to establish reliable PBPK models for PO bosentan data. Very recently, PBPK models, which described intravenous and oral data of bosentan, have been reported (Li et al., 2018). The results from our current study provide new information that saturation of hepatic uptake, but not of hepatic metabolism, likely contributes to nonlinear PKs after bosentan intravenous dosing.

Nonlinear bosentan PKs was recently described by a two-compartment, target-mediated drug disposition (TMDD) model (Volz et al., 2017). This model showed that bosentan binds to ET receptors with high affinity (dissociation constant, $\approx$ 1.9 nM), comparable to the measured binding constant (0.79–1.1 nM). In addition, the study reported that the receptor binding of bosentan is saturated with escalating doses (>50 mg, i.v.). However, such findings differ from the reported clinical data where the systemic plasma clearance of bosentan decreased with escalating intravenous doses (11.5, 7.9, 6.4, and 4.8 l/h for intravenous bosentan doses of 50, 250, 500, and 750 mg, respectively) (Weber et al., 1996). Currently, the reasons for these apparent discrepancies are unknown. We are not aware of solid experimental evidence showing the internalization of the bosentan-ET receptor complex. For other ET receptor antagonists such as ambrisentan and macitentan, there is no report that they undergo TMDD. We thus believe that further efforts may be needed to determine the necessity of including TMDD in the bosentan PBPK model.

On the other hand, the $K_{\text{in}}$ value (0.534 $\mu$M) for hepatic uptake derived from our current PBPK model was comparable to that (1.33 $\mu$M) obtained from in vitro experiments. After 250-mg bosentan intravenous dosing, a maximum unbound bosentan concentration in blood was calculated as $\approx$ 1.4 $\mu$M. Thus, it is reasonable to interpret that hepatic uptake of bosentan may be saturated with intravenous doses greater than 250 mg, affecting bosentan PKs. Further investigation is warranted to examine the contribution of TMDD to nonlinear PKs of bosentan, but saturation of hepatic uptake appears to be a plausible mechanism for nonlinear PKs of bosentan with high intravenous bosentan doses.

In conclusion, we established a PBPK model that can account for the nonlinear PKs of intravenously administered bosentan by incorporating the saturable process of transporter-mediated hepatic uptake. The PBPK model established in this study may prove useful in explaining and predicting complex PK behaviors of bosentan and drug-drug interactions.

Acknowledgments

We thank Dr. Sibylle Neuhoff, Dr. Shriram Pathak, and Dr. Matthew Harwood of Certara, the maker of Simcyp Simulator, and Dr. Amin Rostami of the University of Manchester for providing advice about performing analyses of oral administration data using the Simcyp Simulator.

Authorship Contributions

Participated in research design: Sato, Toshimoto, Tanaka, Hisaka, and Sugiyama.

Conducted experiments: Tomaru.

Performed data analysis: Sato, Tomaru, and Tanaka.

Wrote or contributed to the writing of the manuscript: Sato, Toshimoto, Tomaru, Yoshikado, Lee, and Sugiyama.

References


**Supplementary File**

Article’s Title;
Physiologically Based Pharmacokinetic Modeling of Bosentan Identifies the Saturable Hepatic Uptake as A Major Contributor to Its Nonlinear Pharmacokinetics

Authors;
Masanobu Sato, Kota Toshimoto, Atsuko Tomaru, Takashi Yoshikado, Yuta Tanaka, Akihiro Hisaka, Wooin Lee and Yuichi Sugiyama

Journal Title;
Drug Metabolism and Disposition
In our constructed PBPK model, the concentration profile of bosentan in each compartment can be expressed by the following differential equations.

Central:

\[ V_c \frac{dC_c}{dt} = Q_h(C_{EH5} - C_c) + Q_a \left( \frac{C_a}{K_{p,a}} - C_c \right) + Q_m \left( \frac{C_m}{K_{p,m}} - C_c \right) + Q_s \left( \frac{C_s}{K_{p,s}} - C_c \right) - CL_r C_c \]

(V, volume; C, concentration; Q, blood flow rate of each organ; subscript c, h, a, m, and s, the compartment of central, hepatic, adipose, muscle and skin, respectively; EH5, the fifth extrahepatic compartment)

Hepatocyte compartments 1 to 5:

\[ \frac{1}{5} V_{HCi} \left( \frac{dC_{HCi}}{dt} \right) = \frac{1}{5} \left\{ (PS_{act} + PS_{dif,inf}) f_B C_{HEi} - (PS_{dif,eff} + CL_{int,met}) f_H C_{HCi} \right\} \]

Hepatic extracellular compartment 1:

\[ \frac{1}{5} V_{HE1} \left( \frac{dC_{HE1}}{dt} \right) = Q_h(C_c - C_{HE1}) + \frac{1}{5} \left\{ PS_{dif,eff} f_H C_{HC1} - (PS_{act} + PS_{dif,inf}) f_B C_{HE1} \right\} \]

(HC1, the first hepatocellular compartment; f_B, the unbound fraction in blood)

Hepatic extracellular compartments 2 to 5:
\[ \frac{1}{5} V_{HEi} \left( \frac{dC_{HEi}}{dt} \right) \]

\[ = Q_h (C_{HE(i-1)} - C_{HEi}) \]

\[ + \frac{1}{5} \left\{ P_{S_{diff,eff}} f_H C_{HCl} - (P_{S_{act}} + P_{S_{diff,inf}}) f_B C_{HEi} \right\} \]

(subscript i, the number of the compartment)

Non-elimination organ (adipose, muscle and skin):

\[ V_a \frac{dC_a}{dt} = Q_a (C_c - \frac{1}{K_{p,a}} C_a) \]

\[ V_m \frac{dC_m}{dt} = Q_m \left( C_c - \frac{1}{K_{p,m}} C_m \right) \]

\[ V_s \frac{dC_s}{dt} = Q_s (C_c - \frac{1}{K_{p,s}} C_s) \]

For bottom-up approaches (Models 1 and 2), the metabolism of bosentan into both hydroxyl bosentan and desmethyl bosentan was taken into consideration. \( P_{S_{act}}, P_{S_{diff,inf}} \) and \( CL_{int,met} \) were expressed by the following equations.

\[ P_{S_{act}} = \frac{\text{In vitro } V_{max,uptake} \times SF_{uptake}}{\text{In vitro } K_{m,uptake} + f_B C_{HEi}} \]

\[ P_{S_{diff,inf}} = \text{In vitro } P_{S_{diff,inf}} \times SF_{uptake} \]

\[ CL_{int,met} = \left( \frac{\text{In vitro } V_{max,met,OH}}{\text{In vitro } K_{m,met,OH} + f_H C_{HCl}} + \text{In vitro } CL_{met,OH,non saturable} \right) \]

\[ + \left( \frac{\text{In vitro } V_{max,met,DES}}{\text{In vitro } K_{m,met,DES} + f_H C_{HCl}} + \text{In vitro } CL_{met,DES,non saturable} \right) \times SF_{met} \]
where $SF_{\text{uptake}}$ and $SF_{\text{met}}$ were calculated based on $1.2 \times 10^8$ hepatocytes/g of liver, 24.1 g of liver/kg body, and 52.5 mg microsomal protein/g of liver (Davies and Morris, 1993; Iwatsubo et al., 1997). In the simulation analysis, the central compartment volume ($V_c$) was assumed to be 6.3 L/78 kg. In Model 2, the $SF_{\text{uptake}}$, $SF_{\text{met}}$, and $V_c$ were optimized to fit the bosentan blood concentration profiles of the intravenous bosentan doses from 10 to 750 mg. In these analyses (Models 1 and 2), the values of $V_{\text{max,uptake}}$, $K_{m,\text{uptake}}$, $PS_{\text{dif,inf}}$, kinetic parameters representing the production of hydroxyl bosentan including $V_{\text{max,met,OH}}$, $K_{m,\text{met,OH}}$ and $CL_{\text{met,OH,nonsaturable}}$, and kinetic parameters representing the production of desmethyl bosentan including $V_{\text{max,met,DES}}$, $K_{m,\text{met,DES}}$ and $CL_{\text{met,DES,nonsaturable}}$ were fixed to the values from the in vitro kinetic parameters.

For top-down approaches (Models 3, 4 and 5), we performed simultaneous fitting analyses of the PBPK models that incorporate saturable processes for $PS_{\text{act}}$, $CL_{\text{int,met}}$ or both to bosentan blood concentration profiles. In Models 3, 4 and 5, the following parameters were optimized:

(1) Model 3

$In\ vivo\ V_{\text{max,uptake}}, in\ vivo\ K_{m,\text{uptake}}, in\ vivo\ PS_{\text{dif,inf}}, in\ vivo\ CL_{\text{int,met}},$ and $V_c$

(2) Model 4

$in\ vivo\ PS_{\text{act}}, in\ vivo\ PS_{\text{dif,inf}}, in\ vivo\ V_{\text{max,met}}, in\ vivo\ K_{m,\text{met}}$ and $V_c$

(3) Model 5

$In\ vivo\ V_{\text{max,uptake}}, in\ vivo\ K_{m,\text{uptake}}, in\ vivo\ PS_{\text{dif,inf}}, in\ vivo\ V_{\text{max,met}}, in\ vivo\ K_{m,\text{met}}$ and $V_c$
The initial values of these fitting parameters were determined using the following methods: *in vivo* $V_{\text{max,uptake}}$ and *in vivo* $PS_{\text{diff}}$ were extrapolated biologically from *in vitro* parameters described above; *in vivo* $K_{\text{m,uptake}}$ was set at the equivalent with *in vitro* $K_{\text{m,uptake}}$ values; *in vivo* $PS_{\text{act}}$ was determined by calculating $in vivo \ V_{\text{max,uptake}}/in vivo \ K_{\text{m,uptake}}$ assuming a linear condition in which $f_B C_{\text{HEI}}$ was much lower than *in vivo* $K_{\text{m,uptake}}$; *in vivo* $CL_{\text{int,met}}$ was calculated by the sum of $in vivo \ V_{\text{max,met,OH}}/K_{\text{m,met,OH}}$, $CL_{\text{met,OH,nonsaturable}}$, $V_{\text{max,met,DES}}/K_{\text{m,met,DES}}$, and $CL_{\text{met,DES,nonsaturable}}$; *in vivo* $K_{\text{m,met}}$ was set at 5 μM according to *in vitro* $K_{\text{m,met,OH}}$ and $K_{\text{m,met,DES}}$; *in vivo* $V_{\text{max,met}}$ was determined by the calculation of *in vivo* $CL_{\text{met}}$ and *in vivo* $K_{\text{m,met}}$; and $V_c$ were set at 6.3 L/78 kg, respectively.
Supplementary text 2

Efforts to apply the PBPK model to reproduce pharmacokinetic profiles following oral bosentan dosing

The reported pharmacokinetic profiles of orally administered bosentan display deviations from dose-proportional behaviors, but in the opposite direction to the results of intravenously administered bosentan (Supplementary Figure 2). The dose-normalized AUCs of orally administered (as 100 ml aqueous suspension) showed a decreasing trend as the bosentan doses increased from 3 to 2,400 mg (Clin Pharmacol Ther 60: 124-37). This decreasing trend with oral bosentan dosing may arise from multiple mechanisms including the saturation in plasma protein binding, solubility, and/or uptake transporters (e.g. OATP2B1) expressed in the luminal side of enterocytes. To obtain mechanistic insights, we attempted to establish a PBPK model that can capture non-linear pharmacokinetic behavior of orally administered bosentan.

To develop a PBPK model for orally administered bosentan, several modifications were incorporated. First, the ADAM (Advanced Dissolution, Absorption and Metabolism) model was incorporated into the systemic PBPK model using SimCYP (version 16.0, SimCYP Ltd, Sheffield, UK, Supplementary Figure 3). The kinetic parameters are described in Supplementary Table 1 ($V_{\text{max}}$ for CYP2C9 or CYP3A4, $J_{\text{max}}$ for OATP1B1, and $K_p$ scalar were re-optimized to reproduce the bosentan concentration-time profiles following intravenous bolus dosing). The following saturable components were also
incorporated into the PBPK model: i) hepatic efflux (PS_{dif,eff}) ii) intestinal solubility-pH profiles (reported in the interview form, Supplementary Figure 4), iii) intestinal efflux (by P-glycoprotein, obtained from Caco-2 permeability experiments with and without 100 µM verapamil), iv) intestinal uptake (by OATP2B1, obtained from the uptake study using HEK293 cells expressing OATP2B1). The possibility of supersaturation of bosentan in oral dosing solution (100 mL aqueous suspension) was also considered (the solubility of bosentan was described to be 0.01 mg/mL in the interview). Given each oral bosentan dose was given as 100 mL aqueous suspension, the fraction of API dissolved for each dose was set as described in Supplementary Table 2. Two cases of simulations were performed with the critical supersaturation ratio of 1 and 10, respectively, using SimCYP. For each case, the simulation was performed with J_{max} of OATP2B1 = 0, 5, 15, and 45 pmol/min, respectively in 100 virtual subjects generated from Sim-Healthy Volunteers.

The constructed PBPK model reasonably reproduced the non-linear pharmacokinetic profiles of intravenously administered bosentan (Supplementary Figure 5). However, it was not the case for orally administered bosentan, regardless of the consideration of the supersaturation of bosentan (Supplementary Figures 6 and 7). With low bosentan doses (3 – 100 mg), the simulation results were in a relatively good agreement with the reported profiles when J_{max} of OATP2B1 was set as 0 (i.e. no consideration of OATP2B1 uptake). However, the simulation results showed substantial deviations from
the reported profiles for high bosentan doses (600 - 2,400 mg), regardless of the consideration of apical uptake for OATP2B1.
Legends for Supplementary Figures

Supplementary Figure 1 Simulated Bosentan Concentration in Hepatocyte Compartment 1 by Model 3, 4 and 5

Supplementary Figure 2 Relationship between bosentan doses and the dose-normalized AUCplasma following intravenous (A) or oral (B) dosing. Error bars show the standard deviation.

Supplementary Figure 3 Structure of a PBPK model for orally administered bosentan. The ADAM (Advanced dissolution, absorption and metabolism) model was used for intestinal absorption of bosentan using SimCYP version 16.0.

Supplementary Figure 4 Solubility-pH profiles of bosentan. Square symbols represent the observed data in the interview form. Three colored curves represent simulated solubility-pH profiles; red, using in silico estimated pKa (= 4.0) and the intrinsic solubility (Sunionized, 0.0038mg/mL); green, using pKa (= 5.1) and Sunionized, (= 0.001mg/mL) described in the interview form; blue, using optimized pKa (= 5.4) and Sunionized, (= 0.0011mg/mL).

Supplementary Figure 5 (A) Mean plasma bosentan concentration-time profiles following intravenous dosing of various bosentan doses: 10 mg (black), 68 mg (purple), 308 mg (green), 500 mg (blue), and 904 mg (red), respectively. Solid symbols represent the observed mean values while the solid lines represent simulation results in 100 virtual subjects. (B) Comparison of dose-normalized AUCs between the observed and predicted values. Red and blue symbols represent the simulated and observed values, respectively. Error bars show the standard deviation.

Supplementary Figure 6 Plasma concentration-time profiles of orally administered bosentan without consideration of supersaturation (critical supersaturation ratio = 1). The Jmax values of OATP2B1 are set as 0 (A), 5 (B), 15 (C) and 45 (D) pmol/min, respectively. Solid symbols represent the observed mean values while the lines represent simulation results. Black, 3 mg; purple, 10 mg; blue, 30mg; red, 100 mg; black, 300 mg; purple, 600 mg; blue, 1,200 mg; red, 2,400 mg.
Supplementary Figure 7 Plasma concentration-time profiles of orally administered bosentan considering supersaturation (critical supersaturation ratio = 10)
The Jmax values of OATP2B1 are set as 0 (A), 5 (B), 15 (C) and 45 (D) pmol/min, respectively. Solid symbols represent the observed mean values while the lines represent simulation results. Black, 3 mg; purple, 10 mg; blue, 30 mg; red, 100 mg; black, 300 mg; purple, 600 mg; blue, 1,200 mg; red, 2,400 mg.
### Supplementary table 1

Input parameters for bosentan in SimCYP

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<td>µM</td>
<td>1.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transport</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OATP1B1 J&lt;sub&gt;max&lt;/sub&gt;</td>
<td>pmol/min/million</td>
<td>179</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sinusoidal Efflux CL&lt;sub&gt;int,T&lt;/sub&gt;</td>
<td>µL/min/million</td>
<td>7.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Intestine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-gp K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>µM</td>
<td>4.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-gp J&lt;sub&gt;max&lt;/sub&gt;</td>
<td>pmol/min</td>
<td>124</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Set K<sub>m,met,DES</sub> value in Table 1

Estimated using bonsentan i.v. concentration time profile

Renal clearance described in Table 1 (0.144 L/h) *

B/P (0.6) = 0.0864

Set PS<sub>dif,inf</sub> value in Table 1

Set f<sub>H</sub> value in Table 1

Set K<sub>m,uptake</sub> value in Table 1

estimated using bonsentan i.v. concentration time profile

The difference between PS<sub>dif,eff</sub> and PS<sub>dif,inf</sub> (PS<sub>dif,inf</sub> * (γ - 1))

in-house study
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp A</td>
<td>cm²</td>
<td>1</td>
<td>in-house study</td>
</tr>
<tr>
<td>P-gp System</td>
<td></td>
<td></td>
<td>Caco-2</td>
</tr>
<tr>
<td>Apical uptake Kₘ</td>
<td>µM</td>
<td>0.421</td>
<td>as an apical uptake transporter.</td>
</tr>
<tr>
<td>Apical uptake Jₘₐₓ</td>
<td>pmol/min</td>
<td>0, 5, 15, 45</td>
<td></td>
</tr>
<tr>
<td>Apical uptake A</td>
<td>cm²</td>
<td>1</td>
<td>User</td>
</tr>
</tbody>
</table>

OATP2B1 was considered as an apical uptake transporter.
**Supplementary Table 2**

Fraction of API dissolved value for each dose of bosentan

<table>
<thead>
<tr>
<th>Bosentan dose (mg)</th>
<th>Fraction of API dissolved (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>33</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td>3.3</td>
</tr>
<tr>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>300</td>
<td>0.33</td>
</tr>
<tr>
<td>600</td>
<td>0.17</td>
</tr>
<tr>
<td>1200</td>
<td>0.083</td>
</tr>
<tr>
<td>2400</td>
<td>0.042</td>
</tr>
</tbody>
</table>
Supplementary Table 3

Solubility-pH profile of bosentan described in the interview form

<table>
<thead>
<tr>
<th>pH</th>
<th>Solubility (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.001</td>
</tr>
<tr>
<td>5</td>
<td>0.002</td>
</tr>
<tr>
<td>7.5</td>
<td>0.43</td>
</tr>
<tr>
<td>8</td>
<td>0.53</td>
</tr>
<tr>
<td>8.5</td>
<td>0.93</td>
</tr>
</tbody>
</table>
Supplementary Fig. 2

A) IV

B) Oral

AUC_{plasma}/Dose vs. Dose (mg)

- AUC increases with IV administration.
- AUC decreases with oral administration.

Dose range: 0 to 2400 mg.
Supplementary Fig. 3

Systemic PBPK model

Venous Blood

- Lung
- Adipose
- Bone
- Brain
- Heart
- Kidney
  - $CL_r$
  - Muscle
  - Skin
  - Liver
    - $PS_{\text{diff,eff}}$
    - $PS_{\text{act}} + PS_{\text{diff,inf}}$
    - $CL_{\text{met}}$

Arterial Blood

- Portal vein
- Spleen
- Pancreas
- Gut

ADAM* model

PO Dose

- Stomach
- Duodenum
- Jejunum I
- Jejunum II
- Ileum I
- Ileum II
- Ileum III
- Ileum IV
- Colon

IV Dose

- Liver

Systemic PBPK model

- Lung
- Adipose
- Bone
- Brain
- Heart
- Kidney
  - $CL_r$
  - Muscle
  - Skin
  - Liver
    - $PS_{\text{diff,eff}}$
    - $PS_{\text{act}} + PS_{\text{diff,inf}}$
    - $CL_{\text{met}}$

Arterial Blood

- Portal vein
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- Jejunum I
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- Ileum I
- Ileum II
- Ileum III
- Ileum IV
- Colon

IV Dose

- Liver
Supplementary Fig. 4

The graph shows the relationship between pH and solubility (mg/mL). The x-axis represents pH values ranging from 1 to 9, while the y-axis shows solubility values ranging from 0.00001 to 100. The fitted curves indicate trends for solubility at different pH levels, with observed data points marked as squares.

- The fitted curve for in silico calculation is shown as a green line.
- The observed data points are marked with red squares.

The graph illustrates how solubility changes with pH, highlighting the importance of pH in drug solubility studies.
Supplementary Fig. 6
Supplementary Fig. 7