Pharmacokinetics of primary oxidative metabolites of thalidomide in rats and in chimeric mice humanized with different human hepatocytes

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ABSTRACT — The approved drug thalidomide is teratogenic in humans, nonhuman primates, and rabbits but not in rodents. The extensive biotransformation of 5′-hydroxythalidomide after oral administration of thalidomide (250 mg/kg) in rats was investigated in detail using liquid chromatography–tandem mass spectrometry. Probable metabolites 5′-hydroxythalidomide sulfate and glucuronide were extensively formed, with approximately tenfold and onefold peak areas, respectively, to the primary 5′-hydroxythalidomide measured using authentic standards. As a minor metabolite, 5-hydroxythalidomide was also detected. The output of simplified physiologically based pharmacokinetic rat models was consistent with the observed in vivo data under a metabolic ratio of 0.05 for the hepatic intrinsic clearance of thalidomide to unconjugated 5′-hydroxythalidomide. The aggregate of unconjugated and sulfate/glucuronide conjugated 5′-hydroxythalidomide forms appear to be the predominant metabolites in rats. Two hours after oral administration of thalidomide (100 mg/kg) to chimeric mice humanized with four different batches of genotyped human hepatocytes, the plasma concentration ratios of 5-hydroxythalidomide to 5′-hydroxythalidomide were correlated with replacement indexes of human liver cells previously transplanted in immunodeficient mice. These results indicate that rodent livers mediate thalidomide primary oxidation, leading to extensive deactivation in vivo to unconjugated/conjugated 5′-hydroxythalidomide and suggest that thalidomide activation might be dependent on the humanized livers in mice transplanted with human hepatocytes.

Key words: 5′-Hydroxythalidomide, 5-Hydroxythalidomide, Rodent, PBPK modeling, Human albumin

INTRODUCTION

The sedative drug thalidomide [α-(N-phthalimido) glutarimide] was originally introduced in the 1950s but was withdrawn in the 1960s because of its now well-known teratogenic effects (Speirs, 1962). Among experimental animals, thalidomide is teratogenic in nonhuman primates and rabbits (Calabrese and Resztak, 1998) but not in rodents (Kim and Scialli, 2011). Its teratogenic effects notwithstanding, thalidomide was subsequently approved for the clinical treatment of multiple myeloma (Palumbo et al., 2008; Nakamura et al., 2013). The pharmacokinetics of thalidomide have been reported for mice, rabbits, and multiple myeloma patients (Chung et al., 2004a). The mechanism of action and teratogenicity of thalidomide remains unclear, but it has been shown that the metabolism of thalidomide is important for both teratogenicity and cancer treatment outcomes, especially as its pharmacokinetics are affected when coadministered with other anticancer drugs (Chung et al., 2004b). There is renewed interest in both the toxicity and pharmacological mechanisms of thalidomide, with attention focused on its primary oxidative metabolic pathways mediated by liver cytochromes P450 (P450 or
CYP) to form 5′- or 5-hydroxythalidomide (Chowdhury et al., 2010, 2014). In a system of human liver microsomal P450 enzymes in vitro and in humanized-liver mice in vivo, further oxidation of 5-hydroxythalidomide [leading to conjugations with glutathione (Yamazaki et al., 2012) and nonspecific proteins (Yamazaki et al., 2016)] was evident and may be relevant to toxicological activation. However, the rodent metabolite 5′-hydroxythalidomide seemed to lack virtually all the modulating effects on human pregnane X or constitutive androstane receptors evident for 5′-hydroxythalidomide (Murayama et al., 2014). Nevertheless, as yet, the metabolic profiles of thalidomide have not been determined in rats in terms of the concentrations of primary metabolites 5′- and 5-hydroxythalidomide, the major and minor products, respectively, formed in rodents. Further detailed investigation of the primary oxidative metabolism of thalidomide in rodents is therefore of interest.

Immunodeficient mice with transplanted human hepatocytes (humanized-liver mice, Hasegawa et al., 2011) have proven useful for investigation of the metabolism of thalidomide (Yamazaki et al., 2012; Nishiyama et al., 2015) and pomalidomide (Shimizu et al., 2017). The purpose of the current study was to investigate in detail the oxidative metabolism of thalidomide in rodents in vivo to better understand the deactivation of thalidomide. Using in vivo pharmacokinetic data obtained in the rat, a simplified physiologically based pharmacokinetic (PBPK) model consisting of gut, liver, kidney, and central compartments was created. Metabolic ratios of these activated/deactivated metabolites of thalidomide could be evaluated using the simplified PBPK models for 5′- or 5-hydroxythalidomide. In this study, to investigate species differences in thalidomide metabolism, different batches of genotyped human hepatocytes were transplanted into immunodeficient mice, and the plasma concentrations of 5′- and 5-hydroxythalidomide in rats and humanized-liver mice were measured. We report herein extensive 5′-hydroxythalidomide formation in rats compared with that in the humanized-liver mice.

**MATERIALS AND METHODS**

**In vivo metabolic studies**

Thalidomide-related metabolites were obtained from the same sources as those reported previously (Murayama et al., 2014; Chowdhury et al., 2014). Three male Sprague-Dawley rats (7 weeks old, Charles River Laboratory, Tokyo, Japan) and 20 male humanized-liver immunodeficient TK-NOG mice (20-30 g body weight) (Hasegawa et al., 2011) were used in this study. Four different sources of human hepatocytes were used: lot BPI-HEP187273, genotyped as CYP3A5*3/*3, was obtained from Biopredic, St Gregoire, France, whereas lots TRL-HUM4122B, genotyped as CYP3A5*1/*3; TRL-HUM4119F, genotyped as CYP3A5*1/*7; and TRL-HUM4129, genotyped as CYP3A5*3/*3 were obtained from Lonza TRL, Morrisville, NC, USA (Miura et al., 2019b, 2020, 2021). After liver-specific damage was induced in the TK-NOG mice, these human hepatocytes were transplanted to reconstitute the liver. In the resulting humanized mice, the percentage of liver cells replaced by the different batches of human hepatocytes was estimated by measurements of choline esterase activities (Suemizu et al., 2018) and human albumin concentrations (Hasegawa et al., 2011) in plasma. Blood samples (~ 30-120 µL) were collected 2 hr after oral doses of 100 mg/kg thalidomide in mice and between 0.08 and 24 hr after single oral doses of 250 mg/kg thalidomide (Carbosynth, Compton, UK) in rats. After treatment of the plasma with an equal volume of acetonitrile, the aqueous supernatant was centrifuged at 2 × 10³ g for 10 min at 4°C and analyzed using liquid chromatography (LC)-tandem mass spectrometry (MS). The use of animals for this study was approved by the Ethics Committees of the Central Institute for Experimental Animals and Showa Pharmaceutical University.

Quantitative LC-MS/MS analyses of thalidomide, 5′-hydroxythalidomide, and 5-hydroxythalidomide were performed according to previously reported methods (Yamazaki et al., 2011, 2012; Nishiyama et al., 2015; Shimizu et al., 2017; Murayama et al., 2018) with minor modifications. A Xevo TQ-XS tandem mass analyzer (Waters, Milford, MA, USA) was used in electrospray positive or negative ionization mode and was directly coupled to a Shimadzu LC-30AD system equipped with an octadecylsilane (C₁₈) column (YMC-Triart, 3 µm, 2.0 mm × 150 mm, YMC, Kyoto, Japan). The LC conditions were as follows: solvent A was methanol and solvent B was 10 mM ammonium acetate in water. The following gradient program was used, with a flow rate of 0.30 mL min⁻¹: 0-10 min, linear gradient from 5% A to 55% A (v/v); 10.1-12 min, hold at 100% A; and 12.1-15 min, hold at 5% A. The temperature of the column was maintained at 40°C. Prepared samples (1.0 µL) were injected with an auto-sampler. The retention times (tᵣ) of thalidomide, 5′-hydroxythalidomide, and 5-hydroxythalidomide were 8.1, 6.7, and 7.2 min, respectively. Thalidomide, 5′-hydroxythalidomide, and 5-hydroxythalidomide were quantified using the m/z 259→186 transition of thalidomide in positive ion mode, the m/z 273→146 transition of 5′-hydroxythalidomide in negative
ion mode, and the m/z 273→161 transition of 5-hydroxythalidomide in negative ion mode, as described previously (Yamazaki et al., 2012). Qualitative LC-MS/MS analyses of thalidomide metabolites were also performed in this study, and the results are shown in Fig. 1. Under the present conditions, thalidomide, 5′-hydroxythalidomide, and 5-hydroxythalidomide levels in plasma were measurable (≥ 1.0 ng/mL) or detectable (≥ 0.10 ng/mL).

Estimation of rat plasma concentrations using physiologically based pharmacokinetic model

A simplified rat PBPK model consisting of chemical receptor (gut), metabolizing (liver), excreting (kidney), and central compartments was set up as described elsewhere (Kamiya et al., 2020b, 2021). The molecular weights (258, 274, and 274), octanol-water partition coefficients (clogP; 0.528, -0.138, and 0.402), plasma unbound fractions (fu,p; 0.588, 0.615, and 0.237), and blood-plasma concentration ratios (Rb; 0.893, 0.885, and 0.904) of thalidomide, 5′-hydroxythalidomide, and 5-hydroxythalidomide, respectively, were used as described previously (Nishiyama et al., 2015; Yamazaki et al., 2012). The values used for the hepatic and renal blood flow rates (Qh and Qr) in rats were 0.853 L/hr, and the hepatic and renal volumes (Vh and Vr) of 8.5 mL and 3.7 mL, respectively, as described previously (Miura et al., 2019a). The initial values for the fraction absorbed × intestinal availability (Fa·Fg), the hepatic clearance (CLh), and the renal clearance (CLR) for PBPK modeling were derived from the elimination constants obtained using one-compartment models (Miura et al., 2019a).

The model input parameters, i.e., the absorption rate constant (k0), the volume of the systemic circulation (V), and the hepatic intrinsic clearance (CLint) (Table 1), were computed using simplex and modified Marquardt methods so that the model results were consistent with the measured plasma substrate and metabolite concentrations obtained in this study. The general ratio of CLh to CLR was set at 9:1 (Kamiya et al., 2019, 2020a). In the present rat PBPK models, the metabolic ratios to unconjugated 5′-hydroxythalidomide and 5-hydroxythalidomide were estimated to be 0.05 and 0.0025, respectively, by fitting. The following system of differential equations was solved to model the concentrations of the substrate and its primary metabolites (indicated with subscript m):

\[
\frac{dX_g}{dt} = -k_a \cdot X_g \quad \text{when } t = 0, X_g(0) = F_a \cdot \text{Fig. dose}
\]

\[
\frac{dX_h}{dt} = -Q_h \cdot F_a \cdot \text{gut} - CL_{int} \cdot X_h \quad \text{to} \quad X_h = \text{CL}_{int} \cdot \text{gut} + \text{gut} + \text{gut}
\]

\[
\frac{dC_h}{dt} = -Q_h \cdot F_a \cdot \text{gut} - CL_{int} \cdot C_h \quad \text{to} \quad C_h = \text{CL}_{int} \cdot \text{gut} + \text{gut} + \text{gut}
\]

\[
\frac{dC_r}{dt} = -Q_r \cdot F_a \cdot \text{gut} - CL_{int} \cdot C_r \quad \text{to} \quad C_r = \text{CL}_{int} \cdot \text{gut} + \text{gut} + \text{gut}
\]

\[
\frac{dC_b}{dt} = -Q_b \cdot C_b \quad \text{to} \quad C_b = \text{CR}_{int} \cdot \text{gut} + \text{gut} + \text{gut}
\]

where Xg, Vh, Vr, Cg, Ch, and Cr are the amounts of compound in the gut compartment, the liver and kidney volumes, and the hepatic, renal, and blood substrate concentrations, respectively.

RESULTS AND DISCUSSION

In the present study, the oral dose of 250 mg/kg thalidomide for rats was taken from its classical embryopathy dose of 250 mg/kg in rabbits (McBride, 1976) within the range of 150-500 mg/kg for thalidomide-sensitive rabbits (Hui et al., 2014; Teo et al., 2004). After oral administration of thalidomide to rats, the pharmacokinetics of thalidomide and its metabolites were quantitatively and qualitatively investigated using LC-MS/MS analyses with authentic standard metabolites of 5′-hydroxythalidomide and 5-hydroxythalidomide. As indicated in Fig. 1, estimated possible metabolite ion peaks corresponding to 5′-hydroxythalidomide sulfate (tR 5.2 min, m/z 353→273 transition in negative ion mode) and 5′-hydroxythalidomide glucuronide (tR 3.3 min, m/z 491→273 transition in negative ion mode) were extensively formed, with area ratios of approximately tenfold and onefold, respectively, to the measured ion peak area of 5′-hydroxythalidomide.
an approximate area of one-tenth that of 5-hydroxythalidomide quantitatively detected in rat plasma. Furthermore, small ion peaks corresponding to possible metabolites formed by oxidative reactions at the glutarimide ring of thalidomide, i.e., phthaloylglutamine (t<sub>R</sub> 3.5 min, m/z 275→146 transition in positive ion mode) and phthaloylisoglutamine (t<sub>R</sub> 3.3 min, m/z 275→146 transition in positive ion mode), were detected but were not confirmed with authentic standards.

After single oral doses of 250 mg/kg thalidomide in rats, plasma concentrations of thalidomide and unconjugated 5′-hydroxythalidomide and 5-hydroxythalidomide were quantitatively determined using LC-MS/MS analyses (Fig. 2). Thalidomide was detected 5 min after oral administration. Plasma concentrations of 5′-hydroxythalidomide were higher than those of 5-hydroxythalidomide in rats. From these data, the values of k<sub>a</sub>, V<sub>1</sub>, and CL<sub>h,int</sub> for use in rat PBPK models were determined by fitting procedures and are shown in Table 1; the coefficients of variation for k<sub>a</sub>, V<sub>1</sub>, and CL<sub>h,int</sub> were within 30%. The metabolic ratios of thalidomide to unconjugated 5′-hydroxythalidomide and 5-hydroxythalidomide, respectively, were set at 0.05 and 0.0025 for the rat PBPK models, in accordance with the plasma concentration curves. By solving the equations that make up the PBPK models, virtual plasma concentration curves were generated for rats, and the resulting in silico concentration curves are shown in Fig. 2. These PBPK-generated data were consistent with the measured in vivo data points. The measured maximum

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<th>Abbreviation (unit)</th>
<th>Rat</th>
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<tr>
<td>Fraction absorbed × intestinal availability</td>
<td>F&lt;sub&gt;a&lt;/sub&gt;·F&lt;sub&gt;g&lt;/sub&gt;</td>
<td>1</td>
</tr>
<tr>
<td>Absorption rate constant</td>
<td>k&lt;sub&gt;a&lt;/sub&gt; (1/hr)</td>
<td>0.279 ± 0.026&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Volume of systemic circulation for thalidomide</td>
<td>V&lt;sub&gt;1&lt;/sub&gt;_substrate (L)</td>
<td>0.413 ± 0.028&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Hepatic intrinsic clearance for thalidomide</td>
<td>CL&lt;sub&gt;h,int&lt;/sub&gt;_substrate (L/hr)</td>
<td>0.176 ± 0.026&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Hepatic clearance for thalidomide</td>
<td>CL&lt;sub&gt;h&lt;/sub&gt;_substrate (L/hr)</td>
<td>0.0925</td>
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<tr>
<td>Renal clearance for thalidomide</td>
<td>CL&lt;sub&gt;r&lt;/sub&gt;_substrate (L/hr)</td>
<td>0.0097</td>
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<tr>
<td>Volume of systemic circulation for 5′-hydroxythalidomide</td>
<td>V&lt;sub&gt;1&lt;/sub&gt;_5′-hydroxythalidomide (L)</td>
<td>2.59 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Volume of systemic circulation for 5-hydroxythalidomide</td>
<td>V&lt;sub&gt;1&lt;/sub&gt;_5-hydroxythalidomide (L)</td>
<td>2.00 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Hepatic intrinsic clearance for 5′-hydroxythalidomide</td>
<td>CL&lt;sub&gt;h,int&lt;/sub&gt;_5′-hydroxythalidomide (L/hr)</td>
<td>0.514 ± 0.160&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Hepatic intrinsic clearance for 5-hydroxythalidomide</td>
<td>CL&lt;sub&gt;h,int&lt;/sub&gt;_5-hydroxythalidomide (L/hr)</td>
<td>1.91 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Hepatic clearance for 5′-hydroxythalidomide</td>
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<td>Hepatic clearance for 5-hydroxythalidomide</td>
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<td>Renal clearance for 5-hydroxythalidomide</td>
<td>CL&lt;sub&gt;r&lt;/sub&gt;_5-hydroxythalidomide (L/hr)</td>
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<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; in plasma for thalidomide</td>
<td>ng/mL</td>
<td>56100 (1.28)</td>
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<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; in plasma for 5′-hydroxythalidomide</td>
<td>ng/mL</td>
<td>338 (0.71)</td>
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<td>C&lt;sub&gt;max&lt;/sub&gt; in plasma for 5-hydroxythalidomide</td>
<td>ng/mL</td>
<td>17.2 (0.87)</td>
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<td>AUC in plasma for thalidomide</td>
<td>ng·hr/mL</td>
<td>562000 (0.90)</td>
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<tr>
<td>AUC in plasma for 5′-hydroxythalidomide</td>
<td>ng·hr/mL</td>
<td>5100 (0.76)</td>
</tr>
<tr>
<td>AUC in plasma for 5-hydroxythalidomide</td>
<td>ng·hr/mL</td>
<td>246 (0.81)</td>
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<td>ng/mL</td>
<td>43700</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; in plasma for 5′-hydroxythalidomide</td>
<td>ng/mL</td>
<td>474</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; in plasma for 5-hydroxythalidomide</td>
<td>ng/mL</td>
<td>19.7</td>
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<tr>
<td>AUC in plasma for thalidomide</td>
<td>ng·hr/mL</td>
<td>627000</td>
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<tr>
<td>AUC in plasma for 5′-hydroxythalidomide</td>
<td>ng·hr/mL</td>
<td>6700</td>
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<tr>
<td>AUC in plasma for 5-hydroxythalidomide</td>
<td>ng·hr/mL</td>
<td>305</td>
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<sup>a</sup> Data are means ± standard deviations.  
<sup>b</sup> Values in parentheses are ratios to the estimated/observed values.

The metabolic ratios to 5′-hydroxythalidomide and 5-hydroxythalidomide in rats were 0.05 and 0.0025, respectively.  
C<sub>max</sub> and AUC data are for a single oral dose of 250 mg/kg thalidomide.
plasma concentrations ($C_{\text{max}}$) and areas under the curve (AUCs) and the PBPK-modeled concentration profiles of single oral doses were consistent and are also shown in Table 1.

In our preliminary study, five control mice had no apparent no-observed adverse effects after the oral dose of 270 mg/kg (~1 mmol) thalidomide/kg. On the other hand, because the oral dose of 270 mg/kg was approximately half-lethal for humanized-liver mice (2 dead out of five animals in two days), the lower dose of 100 mg/kg was for humanized-liver mice selected according to our previous reports (Yamazaki et al., 2011, 2012; Nishiyama et al., 2015). Thalidomide and its primary metabolites, 5′- and 5-hydroxythalidomide, were also determined by LC-MS/MS analyses in plasma samples obtained from humanized-liver mice (different mice were humanized with different batches of human hepatocytes) following oral administration of thalidomide (100 mg/kg). Given that under the present conditions, the secondary oxidative or conjugated metabolites did not account for all the primary 5′- and 5-hydroxylation of thalidomide, the ratios of 5-hydroxythalidomide to 5′-hydroxythalidomide at 2 hr after oral administration were calculated. As shown in Fig. 3, both indexes of the extent of replacement of the mouse livers with transplanted human hepatocytes in mice were roughly but significantly correlated with the ratios of 5-hydroxythalidomide concentrations to 5′-hydroxythalidomide concentrations in plasma from humanized-liver mice in vivo ($r = -0.5$, $p < 0.05$, $n = 20$). A significant correlation among plasma choline esterase activities and human albumin concentrations in plasma and the degree of replacement of mouse hepatocytes by human hepatocytes as assessed by the morphometric analysis of liver sections from humanized-liver mice was previously established (Suemizu et al., 2018).

Because drug oxidations may be increased by thalidomide via the heterotropic and homotropic cooperativi-

Fig. 2. Measured and estimated concentrations of thalidomide and its primary oxidative metabolites in plasma from rats after oral administration. The observed values for thalidomide (circles), 5′-hydroxythalidomide (triangles), and 5-hydroxythalidomide (squares) are shown after single oral doses of 250 mg/kg in rats. Lines show the rat PBPK model results for thalidomide (solid lines), 5′-hydroxythalidomide (dashed lines), and 5-hydroxythalidomide (dotted lines) after virtual single oral doses of 250 mg/kg.

Fig. 3. Relationship between the concentration ratios of 5-hydroxythalidomide to 5′-hydroxythalidomide determined in plasma 2 hr after oral administrations of thalidomide (100 mg/kg) to 20 humanized-liver mice (that had received different batches of genotyped human hepatocytes) and their individual humanized-liver index (percentage of hepatocyte replacement) determined using choline esterase activity (A) or human albumin levels (B). The four different sources of human hepatocytes were lot BPI-HEP187273, genotyped as CYP3A5*3/*3, closed circles; TRL-HUM4122B, CYP3A5*1/*3, open circles; TRL-HUM4119F, CYP3A5*1/*7, light gray circles; and TRL-HUM4129, CYP3A5*3/*3, dark gray circles.
ty of human P450 3A4/5 enzymes in vitro (Okada et al., 2009; Chowdhury et al., 2010) and in vivo (Yamazaki et al., 2013). It would appear that CYP3A5.1 (from the CYP3A5*1 allele) does not contribute greatly to enhancing the plasma concentration ratios of 5-hydroxythalidomide to 5′-hydroxythalidomide in humanized-liver mice under the present conditions. These results suggest that the humanized liver could be one of the factors that determine the formation of 5-hydroxythalidomide in humanized-liver mice in vivo. The livers of humanized mice mediated thalidomide oxidation at the aromatic ring, thereby leading to thalidomide activation (Chowdhury et al., 2010; Yamazaki et al., 2012; Nishiyama et al., 2015).

It has been reported that metabolites of thalidomide that are 5-hydroxylated at the aromatic ring have been recovered in the urine of rabbits but not from rats (Schumacher et al., 1965). Differences in species susceptibility to thalidomide teratogenicity may result from differences in the biotransformation of the compound by drug metabolizing enzymes. In the current study, a metabolic ratio of 0.05 for the hepatic intrinsic clearance of thalidomide to unconjugated 5′-hydroxythalidomide in rat PBPK modeling (Fig. 2) gave results consistent with the observed data (Table 1); however, unconjugated 5′-hydroxythalidomide plus its sulfate and glucuronide conjugated forms appear to be predominant and are estimated to make up more than half the metabolic pathway in rats (Fig. 1). Different batches of genotyped human hepatocytes transplanted into immunodeficient mice in this study were used to investigate the species differences in terms of the concentrations of 5′-hydroxythalidomide and 5-hydroxythalidomide in rodent plasma (Fig. 3). Although the ubiquitously expressed E3 ligase protein cereblon is reportedly one of the common direct protein targets for the antiproliferative activities of thalidomide, lenalidomide, and pomalidomide (Ito et al., 2010; Lopez-Girona et al., 2012), this target could also exist in rodents, which show no thalidomide teratogenicity (Kim and Scialli, 2011). Further investigations with thalidomide-sensitive nonhuman primates or rabbits and PBPK modeling would be of interest in terms of the toxicokinetics of thalidomide. Such approaches will support further characterization of the metabolic clearance rates (mediated by P450 enzymes) and ratios of these activated/deactivated metabolites of thalidomide and will foster further improvement of the current PBPK models.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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Thalidomide PK in rats and humanized-liver mice