Prediction of Species Differences (Rats, Dogs, Humans) in the In Vivo Metabolic Clearance of YM796 by the Liver from In Vitro Data

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ABSTRACT

The bioavailability after oral administration of (S)-(−)-2,8-dimethyl-3-methylene-1-oxa-8-azaspiro[4,5]decane-L-tartarate monohydrate (YM796), which is being developed as an antidepressant drug, at a dose of 1 mg/kg was very low (3.4%) in rats, but considerably higher (16.1%) in dogs. The oral clearances (CLoral) Dose/AUCoral in rats and dogs were, respectively, 300 and 18 times more than that already reported in humans. We have previously reported successful attempts to predict the in vivo hepatic metabolic clearance of YM796 from in vitro data in humans. In our study, the in vitro metabolism of YM796 was determined using liver microsomes prepared from both rats and dogs and we also investigated if the species difference observed in vivo could be quantitatively reproduced in vitro. In rats, total metabolite formation could be described by single component kinetics with a Km of 13.4 μM and a Vmax of 520 nmol/min/g liver. However, in dogs, total metabolite formation could be described by three components, as also reported for humans. The Km and Vmax values for the high-affinity, low-capacity component (Km1 and Vmax1) in dogs and humans were, respectively, 8.1 and 1.7 μM, and 19.9 and 1.2 nmol/min/g liver. The overall intrinsic metabolic clearances estimated from the in vitro studies (CLint,in vitro) for rats and dogs were 38.8 and 2.6 ml/min/g liver, respectively, being approximately 40 and 3 times more than that previously reported for humans (0.94 ml/min/g liver). The overall intrinsic hepatic clearances (CLint,in vivo) calculated from in vivo CLoral were 30.4, 3.4 and 0.73 ml/min/g liver for rats, dogs and humans, respectively, indicating that the in vivo hepatic clearance of YM796 can be predicted from in vitro metabolism data in each species. Thus, the pronounced species difference in the metabolic clearance observed in vivo can be quantitatively predicted from in vitro metabolic data using liver microsomes, and was predominantly due to the large difference in the Vmax values.

Because most of drugs are eliminated from the body predominantly by hepatic metabolism and/or renal excretion, it is important to be able to predict the CLh, and CLv in humans. Application of the method for animal-scaling has been successful in predicting CLv in humans for many drugs using information obtained from animal experiments (Dedrick, 1974; Boxenbaum, 1982; Sawada et al., 1984). The application of animal-scaling to the prediction of CLv, however, is limited because of large interspecies differences in the CLint (Boxenbaum, 1980; Lin, 1995).

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ABBREVIATIONS: AUCoral area under the plasma concentration-time curve after oral administration; CLint, hepatic clearance; CLoral, oral clearance; CLint,in vitro, overall intrinsic metabolic clearance (intrinsic hepatic clearance); CLint,in vivo, overall intrinsic metabolic clearance estimated from the in vivo study; CLoral, oral clearance (= Dose/AUCoral); CLr, renal clearance; CLtot, total body clearance; CYP, cytochrome P-450; DN, dispersion number; Fu, the fraction absorbed from the intestinal tract; Fs, hepatic availability; fb, unbound fraction in blood; fp, unbound fraction in plasma; GC, gas chromatography; Km,i, Michaelis-Menten constant for the i-th component of the metabolic reaction; MS, microsomal; MS-MS, tandem mass spectrometry; Qh, hepatic blood flow rate; Rh, blood-to-plasma concentration ratio; TLC, thin-layer chromatography; Vmax,i, maximum metabolic rate for the i-th component of the metabolic reaction.
factors which should be taken into account to improve the predictability (Iwatsubo et al., 1997a, Suzuki et al., 1995).

In our study, we examined the interspecies difference in the metabolic clearance of a model drug, YM796 (fig. 1), which is being developed for the treatment of dementia, both in vitro and in vivo. We calculated CL_{int,in vitro} and CL_{int,in vivo} using in vitro and in vivo metabolism data in rats and dogs. We compared the parameters obtained with those previously reported for humans (Iwatsubo et al., 1997b), and also examined the possibility of predicting species differences in the in vivo metabolic clearances from in vitro metabolism data.

Materials and Methods

Chemicals and reagents. YM796 and [14C]-YM796 were synthesized by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan) and by Amersham International (Buckinghamshire, UK), respectively. Acetonitrile, methanol and other reagents of analytical grade were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim (Mannheim, Germany). Anti-sera for rat CYP3A2 were purchased from Daiichi Pure Chemical Co. Ltd. (Tokyo, Japan).

Preparation of rat and dog liver microsomes. Liver specimens from male F344 rats and/or male Beagle dogs were rinsed with ice-cold 1.15% KCl and homogenized in 100 mM potassium phosphate buffer (pH 7.4). Microsomes were prepared by differential centrifugation, and a 105,000-g pellet was rinsed and resuspended in 100 mM potassium phosphate buffer (pH 7.4). The suspension was divided into aliquots, frozen and stored at -80°C until used.

YM796 metabolism in rat and dog liver microsomes. YM796 and [14C]-YM796 (1 μM; specific activity, 40 mCi/mmol) were incubated with a reaction mixture (0.25 ml) consisting of 25 μg rat or dog liver MS protein and NADPH-generating system (0.33 mM NADP, 8 mM glucose-6-phosphate, 0.1 U/ml glucose-6-phosphate dehydrogenase) in the presence of 100 mM potassium phosphate buffer (pH 7.4). Enzyme reactions were initiated by adding 25 μl of NADPH-generating system. After incubation at 37°C in a shaking water-bath for 2 min, the reaction was terminated by adding 250 μl methanol.

Experiments were performed in triplicate. YM796 concentrations used to estimate the kinetic parameters were 1 to 1000 μM. After stopping the metabolic reaction, the reaction mixture was centrifuged at 10,000 × g for 5 min then an aliquot of supernatant was spotted on to silica-gel plates (E. Merck, Darmstadt, Germany) to separate metabolites from the parent drug by TLC using chloroform/methanol/27% ammonia (100:10:1) as mobile phase. Quantitation of metabolites was performed using BAS-2000 equipment (Fuji-film, Tokyo, Japan).

Immunoinhibition of YM796 metabolism. Rat and dog liver microsomes were used at a final concentration of 0.1 mg MS protein/ml and were preincubated for 30 min at room temperature with increasing volumes of anti-sera (from 10 to 80 μl/mg MS protein) for rat CYP3A2 or rat control sera. The final YM796 concentration was 1 μM.

Protein binding of YM796 in rat and dog plasma. To 2-ml aliquots of rat or dog plasma, 20-μl aliquots of phosphate buffered isotonic solution containing [14C]-YM796 were added to give concentrations of 0.5, 50 and 2500 μM. After incubation for 30 min at 37°C, a 50-μl aliquot was taken from each plasma sample to measure the total plasma concentration and the remainder was transferred to an ultrafiltration tube (Ultraflee CL, Millipore Corp., Bedford, MA). These tubes were centrifuged for 15 min (1000 × g, 37°C), and then a 50-μl aliquot of filtrate was removed and used to measure the unbound plasma concentration. Aliquots of plasma and filtrate samples underwent liquid scintillation counting with 10-ml liquid scintillator.

Blood-to-plasma concentration ratio (R_p) of YM796 in rats and dogs. The R_p of YM796 was determined using heparinized whole blood (Lin et al., 1982). To 1-ml aliquots of rat and dog blood preincubated at 37°C, 20-μl aliquots of phosphate-buffered isotonic solution containing [14C]-YM796 were added to give concentrations of 0.5, 50 and 2500 μM. After incubation for 5 min at 37°C, the blood samples were centrifuged for 5 min at 1500 × g, and then aliquots of plasma underwent liquid scintillation counting with 10 ml liquid scintillator.

Calculation of CL_{int,in vitro}. The kinetic data for YM796 metabolism obtained with liver microsomes were fitted to equations (1) and (2), (3) and (4) in dogs using MULTI (Yamaoka et al., 1981) to estimate V_max, V_max and CL_{int}.

\[
V = V_{\text{max}} \cdot S/(K_m + S) + \text{CL}_{\text{int}} \cdot S
\]

\[
V = V_{\text{max}} \cdot S/(K_m + S) + \text{CL}_{\text{int}} \cdot S
\]

\[
V = V_{\text{max}} \cdot S/(K_m + S) + \text{V}_{\text{max}} \cdot S/(K_n + S)
\]

\[
V = V_{\text{max}} \cdot S/(K_m + S) + \text{V}_{\text{max}} \cdot S/(K_n + S) + \text{CL}_{\text{int}} \cdot S
\]

The CL_{int,in vitro} values expressed per mg MS protein calculated from the in vitro metabolism study were expressed per gram liver by taking the MS protein content per gram liver shown in table 1 into consideration. For all parameters in humans, the reported values were used (Iwatsubo et al., 1997b).

Pharmacokinetics of YM796 in rats and dogs. Male F344 rats weighing 150 to 200 g were given YM796 i.v. (0.3 mg/kg) or p.o. (1.0 mg/kg). At defined time points after dosing, blood was collected from the inferior vena cava using a heparinized syringe under ether anesthesia. Male Beagle dogs weighing 15.0 to 17.5 kg were also given YM796 i.v. (0.1 mg/kg) or p.o. (1.0 mg/kg). Blood was collected from the cephalic vein using a heparinized syringe at defined time points after dosing. After centrifugation, plasma was separated and stored at -20°C until analysis. An aliquot of plasma (2.5 ml) was buffered with 0.5 ml saturated sodium bicarbonate solution after addition of 0.1 ml aqueous internal standard solution, and the resulting mixture was stirred and applied to a disposable column (Chem Elute, Analytichem International, Harbor City, CA) for liquid-liquid extraction. YM796 was extracted by passing 4 ml dichloroethane through the column twice. The extract was evaporated to dryness under reduced pressure, the residue dissolved in 0.5 ml 0.1N hydrochloric acid and washed with 8 ml diethylether. After stirring and centrifugation, the upper layer (ether) was discarded. To the aqueous layer, 1 ml saturated sodium bicarbonate solution was added and YM796 was extracted from the resulting mixture using 7 ml dichloroethane. After stirring and centrifugation, the aqueous layer was discarded and the organic layer evaporated to dryness. The residue was dissolved in chloroform and a small aliquot (25 μl) was
TABLE 1

Basic values used for the conversion of intrinsic clearance per mg microsomal protein into that per g liver in rats, dogs and humans

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44.8 mg MS protein/g liver (n = 3)</td>
<td>77.9 mg MS protein/g liver (n = 4)</td>
<td>48.8 mg MS protein/g liver (n = 60)</td>
</tr>
<tr>
<td>Reference</td>
<td>Knaak et al., 1993</td>
<td>Grant et al., 1987; Seddon et al., 1989; Bayliss et al., 1990; Shimada et al., 1994</td>
<td></td>
</tr>
</tbody>
</table>

*P-450 content was measured in both liver microsomes (0.563 nmol/mg MS protein) and homogenates corresponding to 1 g of liver (25.2 nmol/g liver) in our study. From these data, 1 g of rat liver will contain 44.8 mg of MS protein.

Animal scaling. The $CL_{int,in vivo}$ values in rats and dogs calculated by the aforementioned method were plotted against the body weight on a log-log scale and the following allometric equation was used to predict $CL_{int,in vivo}$ (ml/min/kg) in humans based on a body weight of 70 kg.

$$CL_{int,in vivo} = a \times (BW)^b$$

where the BW is the body weight in kg, $a$ (a’) and $b$ (b’) are the coefficient and exponent of the allometric equations, respectively, and MLP represents the maximum lifespan potential (4.68, 19.7 and 95.4 year in rats, dogs, and humans, respectively, Boxenbaum, 1982).

**YM796 metabolism in rat small intestinal microsomes.** Microsomes were prepared from rat jejunal mucosa as previously described (Stohs et al., 1976). Under similar conditions to those used for rat and dog liver microsomes except the incubation time (5 and 20 min), YM796 metabolism in rat intestinal microsomes were examined. The final YM796 concentration used was 1 $\mu$M.

Estimation of the fraction of unchanged YM796 absorbed from the small intestine. Male Wistar rats weighing 270 to 280 g were used. After 16 hr fasting, under light ether anesthesia, the abdomen of each rat was opened by a midline incision. The first cannula filled with heparinized normal saline was implanted in the upper part on the portal system through the pyloric vein. The free end of the cannula was drawn out through the midline incision. Simultaneously, the femoral artery of each rat was cannulated. After preparing the proximal jejunal loop (around 5 cm), YM796 solution in potassium phosphate buffer (pH 7.4) was administered to the loop at a dose of 1 mg/kg. Blood samples were collected simultaneously from the portal vein and femoral artery 5, 10, 20, 30, 40 and 60 min after administration and centrifuged at 10,000 x $g$ for 5 min to separate plasma. Unchanged YM796 concentrations in plasma were determined by TLC and a BAS-2000 system in a similar way de...
scribed above. The $F_a$ of unchanged YM796 into the portal system was calculated from the following equation (14) (Fujieda et al., 1996).

$$F_a = Q_{pv} \cdot R_p \cdot \frac{AUC_p - AUC_a}{Dose}$$  \hspace{1cm} (14)

where $Q_{pv}$ and $R_p$ are blood flow rates in the portal vein and the blood-to-plasma concentration ratio of YM796, respectively. $AUC_p$ and $AUC_a$ are the area under plasma concentration-time curves of YM796 for the portal vein and the systemic artery, respectively. A literature value of 14.7 ml/min was used for $Q_{pv}$.

**Results**

**Plasma concentration of YM796 in rats and dogs.** All parameters obtained from the *in vivo* studies in rats and dogs.
are summarized in Table 2. The previously reported parameters for humans (Iwatsubo et al., 1997b) are also shown in Table 2 for comparison. The plasma concentration-time profiles of YM796 after i.v. administration (dose: 0.3 and 0.1 mg/kg in rats and dogs, respectively) showed a biexponential behavior in both animals (Fig. 2). The CL_{tot} was 3.6 times more in rats compared with dogs. The plasma concentration of YM796 after oral dosing (dose: 1.0 mg/kg in rats and dogs) reached C_{max} at approximately 0.5 hr in both animals (Fig. 2), and C_{max}, AUC_{oral} and the bioavailability in dogs were 4.2, 18 and 4.7 times more than in rats, respectively. A species difference was also observed in the CL_{oral}, which were 300 and 18 times more in rats and dogs, respectively, than in humans (Iwatsubo et al., 1997b).

**YM796 metabolism in rat and dog liver microsomes.** As shown in Figure 3, at least four different metabolites of YM796 could be detected by TLC for both of rat and dog microsomes. The Rf values of unchanged YM796 and each metabolite (M1, M2, M3 and M4) were 0.52, 0.12, 0.19, and 0.45, respectively, for both animals, the same as the previously reported values for the metabolites formed by human liver microsomes (Iwatsubo et al., 1997b). Eadie-Hofstee plots for total metabolite formation in rat and dog liver microsomes are shown in Figure 4. For rats, the formation of YM796 metabolites could be described by a single component with a K_{m} of 13.4 μM and a V_{max} of 520 nmol/min/g liver (Fig. 4a; Table 3). Because Eadie-Hofstee plots for the formation of YM796 metabolites derived from dog liver microsomes showed that multiple metabolic components were involved in YM796 metabolism (Fig. 4b), the following three models were tried for data fitting: 1) one saturable and one nonsaturable components (equation (2), 2) two saturable components (equation (3) and 3) two saturable components and one nonsaturable component (equation (4)). The kinetic parameters obtained based on each model are shown in Table 4. The AIC value was smallest for model 3, indicating that equation (4) gave the statistically best fit of the data.

**Immunoinhibition of YM796 metabolism.** Because we have already found that CYP3A4 was predominantly responsible for the metabolism of YM796 in humans (Iwatsubo et al., 1997b), the effects of anti-rat CYP3A2 sera on YM796 metabolism in rat and dog liver microsomes were examined. Anti-rat CYP3A2 sera inhibited the formation of total metabolites of YM796 by up to approximately 70 and 50% in rats and dogs, respectively (Fig. 5).

### Table 3: Comparison of kinetic parameters among rats, dogs and humans

<table>
<thead>
<tr>
<th>Species</th>
<th>V_{max1} (nmol/min/g liver)</th>
<th>K_{m1} (μM)</th>
<th>V_{max2} (nmol/min/g liver)</th>
<th>K_{m2} (μM)</th>
<th>CL_{int, in vitro} (ml/min/g liver)</th>
<th>V_{max1}</th>
<th>K_{m1}</th>
<th>V_{max2}</th>
<th>K_{m2}</th>
<th>CL_{int, in vitro}</th>
<th>V_{max1}</th>
<th>K_{m1}</th>
<th>V_{max2}</th>
<th>K_{m2}</th>
<th>CL_{int, in vitro}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>13.4 (5.1)</td>
<td>38.8 (6.4)</td>
<td>520 (14.2)</td>
<td>264 (3.9)</td>
<td>0.040 (0.031)</td>
<td>1.00</td>
<td>1.00</td>
<td>0.040</td>
<td>1.00</td>
<td>0.040 (0.031)</td>
<td>1.00</td>
<td>1.00</td>
<td>0.040</td>
<td>1.00</td>
<td>0.040 (0.031)</td>
</tr>
<tr>
<td>Dog</td>
<td>8.10 (3.2)</td>
<td>38.8 (6.4)</td>
<td>10.9 (4.1)</td>
<td>264 (3.9)</td>
<td>0.040 (0.031)</td>
<td>1.00</td>
<td>1.00</td>
<td>0.040</td>
<td>1.00</td>
<td>0.040 (0.031)</td>
<td>1.00</td>
<td>1.00</td>
<td>0.040</td>
<td>1.00</td>
<td>0.040 (0.031)</td>
</tr>
<tr>
<td>Human</td>
<td>1.67 (0.82)</td>
<td>38.8 (6.4)</td>
<td>1.365 (0.63)</td>
<td>264 (3.9)</td>
<td>0.040 (0.031)</td>
<td>1.00</td>
<td>1.00</td>
<td>0.040</td>
<td>1.00</td>
<td>0.040 (0.031)</td>
<td>1.00</td>
<td>1.00</td>
<td>0.040</td>
<td>1.00</td>
<td>0.040 (0.031)</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the standard deviations of the mean values.
YM796 metabolism in the small intestine. No metabolites of YM796 could be detected in rat microsomal samples prepared from the upper intestine and incubated with YM796 for 5 and 20 min. The plasma concentration-time profiles of unchanged YM796 in the portal vein and systemic artery after administering drug to the small intestinal loop in rats are shown in figure 6. The plasma concentration of YM796 in the portal vein was constantly higher than in the systemic artery, and this concentration difference in unchanged YM796 was considered to reflect the absorption of YM796 into the portal vein through the small intestine. The AUC values of unchanged YM796 for the portal vein and systemic artery were 15.1 and 0.193 μg·min/ml, respectively, resulting in a figure for the estimated fraction of YM796 absorbed through the small intestine of 88% based on equation 14.

Comparison of $\text{CL}_{\text{int,in vitro}}$ and $\text{CL}_{\text{int,in vivo}}$ among rats, dogs and humans. To calculate $\text{CL}_{\text{int,in vivo}}$, the $f_p$ and $R_B$ of YM796 were measured for rats and dogs. The $f_p$ values were approximately 0.7 for both animals, similar to the value reported for humans, and almost constant irrespective of the YM796 concentration, ranging from 0.5 to 2500 μM (table 2). The $R_B$ values were 1.10, 1.07 and 1.11 for rats, dogs and humans, respectively, showing no marked species difference or concentration-dependence. The calculated $\text{CL}_{\text{int,in vitro}}$, under linear conditions was 38.8 and 2.6 ml/min/g liver for rats and dogs, respectively, being approximately 40 and 3 times more than for humans (tables 3 and 5). The $\text{CL}_{\text{int,in vivo}}$ calculated from the in vivo pharmacokinetic data in rats and dogs was 30.4 and 3.4 ml/min/g liver, respectively, comparable with the $\text{CL}_{\text{int,in vitro}}$ in each species, being 40 and 3 times more than the human value (table 5). Although only two animal species were available, the $\text{CL}_{\text{int,in vivo}}$ in humans was also predicted by animal scaling based on equation (12). The coefficient (a) and exponent (b) of the allometric equation were 645 ml/min/kg and 0.475, and the $\text{CL}_{\text{int,in vivo}}$ in humans was estimated to be 69.3 ml/min/kg (i.e., 2.85 ml/min/g liver) indicating significant overestimation, when compared with the $\text{CL}_{\text{int,in vivo}}$ (17.7 ml/min/kg, i.e., 0.73 ml/min/g liver) calculated from the observed in vivo data in humans. When corrected by MLP based on equation (13), the $\text{CL}_{\text{int,in vivo}}$ predicted for humans became comparable (25.0 ml/min/kg, i.e., 1.03 ml/min/g liver at $a' = 25.8 \times 10^5$ l/MLP/kg, $b' = 0.825$) with that obtained from the in vivo human data as well as that predicted from the in vitro metabolism data in humans (22.8 ml/min/kg, i.e., 0.94 ml/min/g liver).
Discussion

Eadie-Hofstee plots for the formation of total YM796 metabolites in rat liver microsomes were linear, while they appeared curved in dog liver microsomes, suggesting that a single component and multiple components contributed to the metabolic reactions of YM796 in the liver of rats and dogs, respectively (fig. 4). Thus, the metabolism data derived from dog liver microsomes were fitted to equations based on each of the following models to obtain the kinetic parameters: 1) one saturable and one nonsaturable components (equation 2), 2) two saturable components (equation 3), 3) two saturable and one nonsaturable components (equation 4). The calculated AIC value for each model was -27.6, -31.7 and -35.5, indicating that equation 4 gave the best fit to the data (table 4). We have already reported that the metabolism data in human liver microsomes were also described by multiple component kinetics and that model 3 was the most appropriate for data fitting as was the case with dogs (Iwatsubo et al., 1997b). For both dogs and humans, the formation of YM796 metabolites could be described by three components: 1) high-affinity with low-capacity, 2) low-affinity with high-capacity and 3) nonsaturable. The contribution of the high-affinity component under linear conditions was 100% (single component) in rats and approximately 80 and 51% in humans and dogs, respectively (table 3). Furthermore, anti-rat CYP3A2 sera inhibited YM796 metabolite formation in rat liver microsomes by up to more than 70% as also reported for human liver microsomes (Iwatsubo et al., 1997b), although up to 50% inhibition was observed in dog liver microsomes (fig. 5). Thus, CYP3A seemed to make the predominant contribution to YM796 metabolism in all species examined. As described previously, the contribution of the high-affinity component to CLint,al was predominant for both rats and humans, although it was as low as 50% for dogs. These facts suggest that it is possible that the high-affinity component for YM796 metabolism was primarily inhibited by anti-rat CYP3A2 sera in both dog and human liver microsomes.

The CLoral after oral administration of YM796 to rats and dogs at a dose of 1 mg/kg was 3330 and 205 ml/min/kg, respectively, indicating that there is a pronounced interspecies difference in the in vivo metabolic clearance of YM796. The CLoral for humans after an oral dose (5 mg/body) was 11.1 ml/min/kg (Iwatsubo et al., 1997b), the lowest among the species used, which corresponded to approximately 1/300 and 1/350, indicating that equation 4 gave the best fit to the data (table 4). To reproduce the CLoral of YM796, considering the metabolism of YM796 only in the liver, was comparable with the CLint, in vitro.

In conclusion, although the kinetics of YM796 metabolism differed markedly among species (rats, dogs and humans), the large species difference in the metabolic clearance observed in vivo could be reproduced in the in vitro experiments using liver microsomes prepared from each animal, and it was found that such differences may be ascertained predominantly to the large difference in Vmax. In addition, the absolute values of CLint, in vitro and CLint, in vivo were comparable for each animal examined, suggesting the usefulness of predicting the in vivo metabolic clearance of a drug from in vitro metabolism data obtained using liver microsomes. It should be noted, however, that the application of this method is restricted to drugs that are eliminated from the body mainly by hepatic microsomal metabolism, with no significant intestinal metabolism and insignificant or predictable renal elimination.
Acknowledgments

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