Species Difference in the Mechanism of Nonlinear Pharmacokinetics of E2074, a Novel Sodium Channel Inhibitor, in Rats, Dogs, and Monkeys

Yoko Nagaya, Osamu Takenaka, Kazutomi Kusano, and Tsutomu Yoshimura

Global Drug Metabolism and Pharmacokinetics, Eisai Product Creation Systems, Eisai Co., Ltd., Ibaraki, Japan

Received November 15, 2012; accepted February 11, 2013

ABSTRACT

New chemical entities often exhibit nonlinear pharmacokinetics (PK) profiles in experimental animals. However, the number of studies that have focused on species differences in nonlinear PK is very limited; thus, the aim of this study was to clarify the mechanism of the nonlinear PK of E2074 (2-[(2R)-2-fluoro-3-[(3R)-[(3-fluorobenzyl)oxy]-8-azabicyclo[3.2.1]oct-8-yl]propyl]-4,5-dimethyl-2,4-dihydro-3H-1,2,4-triazol-3-one), a novel sodium channel inhibitor, in rats, dogs, and monkeys. Nonlinear PK profiles with more than dose-proportional increases of Cmax and area under the plasma concentration curve were observed in all species after oral administration. The Michaelis–Menten constant (Km) values of hepatic microsomal metabolism were 7.23 and 0.41 μM in rats and dogs in vitro, respectively, which were lower than the unbound maximum plasma concentrations after oral administration in vivo, indicating that the nonlinear PK in rats and dogs was attributable to the saturation of hepatic metabolism. However, we do not believe that the saturation of hepatic metabolism was the mechanism of nonlinearity in monkeys because of the high Km value (42.44 μM) observed in liver microsomes. Intestinal metabolism was observed in monkey intestinal microsomes but not in rats and dogs, and the nonlinear PK in monkeys was diminished by inhibition of intestinal metabolism with a concomitant oral dose of ketoconazole. These results suggest that saturation of the intestinal metabolism is the potential mechanism of nonlinearity in monkeys. P-glycoprotein was not involved in the nonlinear PK profiles in any species. In conclusion, the mechanism of the nonlinear PK of E2074 is species dependent, with the saturation of hepatic metabolism in rats and dogs and that of intestinal metabolism in monkeys being the primary cause.

Introduction

In the drug discovery process, the prediction of the human pharmacokinetics (PK) profiles of new chemical entities is routinely performed at the preclinical stage. The predicted PK profile provides useful information about the clinical dosing regimen, the PK/pharmacodynamic analysis, and the estimation of safety margins. Assessment of the nonlinear PK profiles of new chemical entities and the prediction of PK profiles are important because nonlinearity occasionally results in unexpected adverse effects. For example, as phenytoin exhibits a nonlinear PK profile in the therapeutic range, a slight increase of the dose leads to remarkable escalation in plasma phenytoin levels, resulting in unexpected adverse effects (Richens, 1975; Pitner et al., 1998). Nonlinear PK profiles are often observed in preclinical animal studies, particularly in toxicologic studies, because a wide range of doses and relatively high doses are administered. This nonlinearity makes assessment of dose–toxicity and dose–efficacy relationships difficult.

Nonlinearity is caused by several different mechanisms (Ludden, 1991; Lin, 1994). Less than dose-proportional increases in exposure with oral doses could be caused by decreased drug absorption. Most drugs are lipophilic and are absorbed by passive diffusion; thus, less than dose-proportional increases in exposure could be caused by limited solubility (Chapelsky et al., 1998). Decreased absorption could also be due to the saturation of carrier-mediated intestinal transport. For example, gabapentin exhibits less than dose-proportional increases in exposure at high doses due to saturation of absorptive capacity from the intestinal lumen (L-type amino acid transporter) (Su et al., 2005). For carbapenem antibiotic (MK-826), the saturation of plasma protein binding also contributes to its nonlinear PK profile by increasing clearance, resulting in less than dose-proportional increases in exposure (Wong et al., 1999). A greater than dose-proportional increase in exposure is most likely due to decreased clearance. Phenytoin, salicylate, and theophylline are the classic examples of drugs that exhibit decreased clearance due to saturated hepatic metabolism (Ludden, 1991; Lin, 1994).

This work was previously partly presented as a poster presentation at the following conference: Nagaya Y, Takenaka O, Kusano K, and Yoshimura T (2009) Clarification of species difference in the cause of nonlinear PK profile. 16th North American Meeting of the International Society for the Study of Xenobiotics; 2009 Oct 18–22; Baltimore, MD. dx.doi.org/10.1124/dmd.112.050062.

ABBREVIATIONS: AUC, area under the plasma concentration curve; BA, bioavailability; CL, clearance; E2074, 2-[(2R)-2-fluoro-3-[(3R)-[(3-fluorobenzyl)oxy]-8-azabicyclo[3.2.1]oct-8-yl]propyl]-4,5-dimethyl-2,4-dihydro-3H-1,2,4-triazol-3-one; fp, unbound fraction in plasma; HBSS, Hank’s balanced salts solution; IS, internal standard; iv, intravenous administration; KCZ, ketoconazole; Km, Michaelis–Menten constant; KO, knockout; LC/MS/MS, high-performance liquid chromatography with tandem mass spectrometry; MK-826, carbapenem antibiotic; MR2P, multidrug resistance-associated protein 2; P450, cytochrome P450; PAR, peak area ratio; P-gp, P-glycoprotein; PK, pharmacokinetics; r, Hill coefficient; Rb, blood to plasma partition ratio; rP450, recombinant human cytochrome P450; tf/2, half-life; tmax, time to reach Cmax; Vmax, maximum velocity; Vss, steady-state volume of distribution; WT, wild-type.
Decreased biliary clearance is reported to be another cause of nonlinear PK profiles in rats. Mefloquine is mainly excreted in bile, and reductions in its biliary clearance result in nonlinear PK profiles with disproportional increases in exposure (Bremnes, et al., 1989). The decrease in biliary excretion is caused by saturation of a transporter on the biliary canalicular membrane. This transporter was confirmed to be multidrug resistance-associated protein 2 (MRP2) using Eisai hyperbilirubinemice, which are MRP2-deficient (Masuda, et al., 1997). In addition, P-glycoprotein (P-gp) expressed on the brush border membrane of enterocytes can act as an efflux pump that limits intestinal absorption. Talinolol, a good substrate for P-gp, displays a nonlinear PK profile due to the saturation of P-gp transport activity in therapeutic dose ranges in healthy subjects (Bolger, et al., 2009).

As mentioned previously, many reports have attempted to elucidate the mechanisms responsible for nonlinear PK profiles, but these studies describe the nonlinearity of specific drugs in a specific species. Reports about species differences in the mechanisms of nonlinearity are very limited (Sugimoto, et al., 1999; Roller, et al., 2009). The purpose of this study was to clarify the determinant factor of the mechanism of nonlinearity in different species. E2074, 2-[((2R,3S)-4-methyl-2,4-dihydro-3H-1,2,4-triazol-3-yl)oxy]-2,4,6-trifluorobenzyl]-3-(3-methyl-2-fluorophenyl)oxazoline, was used as a model compound in this study because nonlinear PK profiles with more than dose-proportional increases in exposure were observed in preclinical species. Because one of the presumed causes of nonlinearity is decreased metabolic clearance, kinetic parameters—the Michaelis–Menten constant (Km) and maximum velocity (Vmax)—were estimated using liver microsomes to assess the possibility of saturated hepatic metabolism. In addition to hepatic metabolism, the impact of intestinal metabolism was also verified using intestinal microsomes in vitro in addition to hepatic metabolism, the impact of intestinal metabolism was also verified using intestinal microsomes in vitro to assess the possibility of saturated hepatic metabolism. In the case of concomitant administration of E2074 and KCZ in monkeys, KCZ (5 mg/4 ml/kg) was administered orally immediately before E2074 administration. The dog and monkey studies were conducted using a crossover design. Blood samples (0.25–0.5 ml) were collected using heparinized syringes via the jugular (rats) or cephalic vein (dogs and monkeys) at 30 minutes after i.v. dosing, 15, 30, and 60 minutes, and 1, 2, 4, 6, and 8 hours after dosing. For double-cannulated rats, blood samples were collected using heparinized syringes through the jugular and portal vein canulae at 5, 20, and 40 minutes, and 1, 1.5, 2, and 4 hours after dosing. For mice, blood samples (approximately 20 µl) were collected using heparinized capillaries through the tail vein at 15 and 30 minutes, and 1, 2, 4, 6, 8 hours and 12 hours after dosing. Plasma was obtained by centrifugation at 12,000g for 5 minutes at 4°C (Kubota 1910; Kubota Co., Ltd., Tokyo, Japan). Aliquots of plasma (rat, dog, and monkey) were transferred to polypropylene tubes and stored at −15°C until analysis. To estimate brain penetration, mice were decapitated at 30 minutes after administration, and brain tissue was obtained. The brain tissue was stored at −15°C and homogenized with distilled water to prepare 20% (w/v) homogenates before analysis.

Methods and Materials

Chemicals. E2074 was synthesized by Eisai Co. Ltd. (Ibaraki, Japan). Ketoconazole (KCZ), loperamide hydrochloride, and propranolol hydrochloride were purchased from Sigma Aldrich (St. Louis, MO). Pool rat liver microsomes (Sprague-Dawley (SD) rats, male, 8 weeks old), dog liver microsomes (beagle, male, 0.5–3 years old), monkey liver microsomes (cytomolgus, male, 4–10 years old), rat intestinal microsomes (SD, male, 8 weeks old), dog intestinal microsomes (beagle, male, more than 6 months old), and monkey intestinal microsomes (cytomolgus, male, 4–8 years old) were purchased from XenoTech (Lenexa, KS). Recombinant human cytochrome P450 (rP450) was obtained from BD Gentest Products and Service (Woburn, MA). All other reagents and solvents were of analytical grade.

Animals. Male SD rats were supplied by Charles River Laboratories Japan (Kanagawa, Japan). SD rats with jugular and portal vein double cannulation were provided by Charles River Laboratories Japan. Male cynomolgus monkeys were obtained from Guangxi Grandforest Scientific Primate Company, Ltd. (Guangxi, China). Male beagles were supplied by Narc Corporation (Chiba, Japan). Mdr1a/1b KO and wild-type (WT) mice were purchased from Taconic (Germantown, NY). All experimental protocols were approved by the Institutional Animal Care and Use Committee of Eisai.

In Vivo PK Studies. For the PK of E2074 in rats (i.v.: 1 mg/1 ml/kg; oral: 3, 10, and 30 mg/5 ml/kg), dogs (i.v.: 1 mg/0.5 ml/kg; oral: 3 and 10 mg/3 ml/kg), monkeys (i.v.: 1 mg/0.5 ml/kg; oral: 3 and 10 mg/3 ml/kg), and mice (oral: 3 mg/10 ml/kg), the dosing solution was 5% glucose/1 M HCl (97:3, v/v). The oral dosing solution of KCZ was 5% glucose/1 M HCl (95:5, v/v). In the case of concomitant administration of E2074 and KCZ in monkeys, KCZ (5 mg/4 ml/kg) was administrated orally immediately before E2074 administration.

In Vitro Metabolic Studies. To estimate the enzyme kinetic parameters in liver and intestinal microsomes, the reaction solution comprising 0.1 mM EDTA, 100 mM phosphate buffer (pH 7.4), 25 µl of NADPH-generating system, E2074 (0.025, 0.074, 0.25, 0.74, 2.5, 7.4, 24.6, 49.2, and 73.8 µM), and liver or intestinal microsomes for each animal (0.5, 0.2, and 1 mg/ml for rat/monkey liver, dog liver, and monkey intestine, respectively) in a final volume of 250 µl was used. The NADPH-generating system was prepared as a mixture containing 3.3 mM β-NADP+, 80 mM glucose 6-phosphate, 60 mM MgCl2, and 1 unit/ml glucose 6-phosphate dehydrogenase. After the reaction solution lacking the NADPH-generating system was preincubated at 37°C for 5 minutes, the reaction was initiated by adding the NADPH-generating system or 60 mM MgCl2, and the mixture was incubated at 37°C for 7, 30, and 15 minutes for rat, dog, and monkey microsomes, respectively. Each sample was prepared in duplicate.

Protein Binding and Blood-to-Plasma Partition Ratio. The in vitro unbound fraction in plasma (fp) and liver microsomes (fmic) was estimated using equilibrium dialysis. E2074 was added to plasma and liver microsomes (0.5 and 0.2 mg/ml for rat/mouse and dog microsomes, respectively) at a concentration of 0.25 µM. We added 1 ml of the matrix to the donor side of a dialysis chamber, and 1 ml of phosphate-buffered saline (pH 7.4) was added to the receiver side chamber. The dialysis chamber was maintained at 37°C (plasma) or 4°C (liver microsomes) for 24 hours. The incubated samples were collected from both sides of the chamber and stored at −15°C until analysis.

We calculated fp and fmic as the receiver side to donor side concentration ratio. To estimate the blood to plasma partition ratio (Rp), E2074 was added to blood at a concentration of 0.25 µM in a final volume of 0.5 ml. After the blood was incubated at 37°C for 10 minutes, an aliquot of the blood sample was collected, and the remaining sample was centrifuged to obtain plasma. The blood and plasma samples were stored at −15°C until analysis. Rp was calculated from the blood and plasma concentrations.

Transport Assay Using Mouse mdr1a-Overexpressing Cell Line. We purchased mdr1a-expressing (porcine kidney epithelial LLC-PK1 cells transformed with mouse mdr1a cDNA) and control cells (LLC-PK1 cells) from the
Cells were seeded at a density of $4 \times 10^5$ cells/cm$^2$ on a filter well in cell culture insert plates (Millicell-24 Cell Culture Plate PET, pore size 1 $\mu$m, membrane area 0.7 cm$^2$; EMD Millipore, Billerica, MA) and incubated in a CO$₂$ incubator (37°C, 5% CO$₂$) for 4 days. Medium 199 containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 $\mu$g/ml streptomycin was used for cell culture. On the day of the experiment, cells were washed with 10 mM HEPES/Hanks’ balanced salt solution (HBSS) (pH 7.4) before the transport assay. In the plates seeded with cells, both the apical and basal sides were preincubated with 10 mM HEPES/HBSS for 2 hours at 37°C. Then, the solution on the apical or basal side was replaced with 10 mM HEPES/HBSS containing E2074 or loperamide as a positive control (1 $\mu$M). After the cells were incubated for 2 hours at 37°C, 100 $\mu$l of the medium were collected from the compartment opposite of the one spiked with E2074.

**Extraction Procedure.** Plasma, blood, microsomal samples, and medium samples were deproteinized with acetonitrile containing 0.3 $\mu$M propranolol as an internal standard (IS) followed by centrifugation. The obtained supernatant was filtrated, and the filtrate was analyzed by high-performance liquid chromatography with tandem mass spectrometry (LC/MS/MS).

**Analytical Procedure.** The concentration of E2074 was measured using a Waters LC/MS/MS system (Milford, MA). The ionization mode was positive electrospray ionization. Chromatography was performed using an Acquity UPLC BEH C18 column (1.7 $\mu$m, 2.1 mm i.d. $\times$ 50 mm; Waters). The mobile phase consisted of water (containing 0.1% formic acid) and acetonitrile (containing 0.1% formic acid) at a flow rate of 0.5 ml/min. The elution time was 10 minutes. The UV detector was set at 210 nm. The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting standards at various concentrations and were found to be 0.01 $\mu$g/ml and 0.02 $\mu$g/ml, respectively.

**TABLE 1**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Rat</th>
<th>i.v.</th>
<th>Oral</th>
<th>Oral</th>
<th>Oral</th>
<th>Dog</th>
<th>i.v.</th>
<th>Oral</th>
<th>Oral</th>
<th>Oral</th>
<th>Monkey</th>
<th>i.v.</th>
<th>Oral</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NA</td>
<td>0.25–0.5 &amp;superscript;</td>
<td>0.25</td>
<td>0.25–0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>NA</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>NA</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>NA</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$t_{max}$ (h) = time to reach $C_{max}$; $t_{1/2}$, half-life; $V_{ss}$, steady-state volume of distribution.

**Fig. 2.** Plasma concentration–time profiles of E2074 in rats (A), dogs (B), and monkeys (C) (●, i.v. 1 mg/kg; ○, oral 3 mg/kg; □, oral 10 mg/kg; △, oral 30 mg/kg). Each point represents the mean ± S.E. of five rats for the rat oral 3 mg/kg dose; the monkey oral 3 mg/kg dose represents the mean of two animals, and the other represents the mean of three animals.

**TABLE 1**

Pharmacokinetic parameters of E2074 in rats, dogs, and monkeys

The pharmacokinetic parameters represent the mean ± S.E. of three animals or five rats, or the mean of two monkeys.

AUC, area under the plasma concentration curve; BA, bioavailability; CL, clearance; E2074, 2-[(2R)-2-fluoro-3-{(3R)-[(3-fluorobenzyl)oxy]-8-azabicyclo[3.2.1]oct-8-yl}propyl]-4,5-dimethyl-2,4-dihydro-3H-1,2,4-triazol-3-one; NA, not applicable;

* Mean ± S.E. of three animals or five rats.

$^{a}$ Mean ± S.E. of three animals or five rats.

$^{b}$ Mean of two monkeys.
phases were water containing 0.1% formic acid (A) and acetonitrile containing
0.1% formic acid (B). The initial condition was 100% (A)/0% (B), and (B) was
increased linearly to 50% over 3 minutes. The flow rate was 0.3 ml/min. The
injection volume of the extracted sample was 5 μl. The selected ion monitoring
conditions were as follows:

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Precursor Ion</th>
<th>Cone Voltage</th>
<th>Product Ion</th>
<th>Collision Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2074</td>
<td>407.1</td>
<td>30</td>
<td>172.0</td>
<td>30</td>
</tr>
<tr>
<td>Propranolol (IS)</td>
<td>260.3</td>
<td>30</td>
<td>183.1</td>
<td>20</td>
</tr>
</tbody>
</table>

**PK Analysis.** The PK parameters of E2074, excluding the maximum
congestion in plasma (Cmax) and time to reach Cmax (tmax), were determined
by model-independent analysis in WinNonlin (ver 6.1; Pharsight Corporation,
St. Louis, MO). Cmax and tmax were taken from the observed values. Bioavailability
(BA) was calculated according to the following equation:

\[
BA(\%) = \left( \frac{\text{AUC}_{\text{p.o.}}}{\text{Dose}_{\text{p.o.}}} \right) \times 100
\]

**Data Analysis.** The peaks of E2074 and IS in samples were integrated using
MassLynx (Waters). The peak area ratio (PAR) of E2074 to IS was calculated,
and the residual ratio was calculated using the following equation:

\[
\text{Residual ratio (\%)} = \frac{\text{PAR}_{t}}{\text{PAR}_{0}} \times 100
\]

where PARt represents the mean PAR after incubation and PAR0 represents the
mean PAR at 0 minutes.

The enzyme kinetic parameters in rat and monkey microsomes were
calculated from the Michaelis–Menten equation with a Hill coefficient for rats
and monkeys or biphasic components for dogs as follows:

\[
v = \frac{V_{\text{max1}}C_{\text{m1}}}{K_{\text{m1}}C_{\text{m1}} + C} + \frac{V_{\text{max2}}C_{\text{m2}}}{K_{\text{m2}}C_{\text{m2}}}
\]

where v is the metabolic velocity of E2074, C is the E2074 concentration, and
Km, Vmax, and r are the Michaelis–Menten constant, maximum velocity, and
Hill coefficient, respectively. The v value was directly calculated from the following equation:

\[
v = \frac{V_{\text{max}}C}{K_{\text{m}} + C}
\]

The predicted hepatic blood clearance (CLh, vitr) was calculated from CLint,u by
the dispersion model with a dispersion number of 0.17 (Roberts and Rowland,
2017).
metabolism of E2074 in rats and monkeys was typified by a sigmoidal Michaelis–Menten reaction with a Hill coefficient. The $K_m$, $V_{\text{max}}$, and $r$ values were 7.23 $\mu$M, 1.42 nmol/min per mg protein, and 1.10, respectively, for rats and 42.44 $\mu$M, 3.58 nmol/min/mg protein, and 1.04, respectively, for monkeys. The metabolism in dogs was represented by a Michaelis–Menten reaction with biphasic components. The $K_m$ and $V_{\text{max}}$ values were 0.41 $\mu$M and 0.07 nmol/min/mg protein, respectively, for high-affinity component and 21.5 $\mu$M and 0.72 nmol/min/mg protein, respectively, for the low-affinity component. In microsomes, fmic was in unity for all species tested. The fp values were 0.675, 0.601, and 0.676 in rats, dogs and monkeys, respectively, and the corresponding Rb values were 0.89, 0.86, and 1.01, respectively (Table 3). The $\text{CL}_{\text{int},\text{vivo}}$ values calculated from $K_m$ and $V_{\text{max}}$ were 0.196, 0.208, and 0.084 ml/min per mg protein in rats, dogs, and monkeys, respectively, and the corresponding $\text{CL}_{h,vitro}$ values were 3.19, 1.83, and 2.00 l/h/kg, respectively (Table 3). The intestinal metabolism in monkeys displayed a classic Michaelis–Menten pattern ($r = 1$) with a $K_m$ of 34.60 $\mu$M and a $V_{\text{max}}$ of 0.66 nmol/min per mg protein. The P450 enzyme involved in E2074 metabolism was evaluated by rP450s, and CYP3A4 was revealed to play a major role (Fig. 4).

**Results**

**Pharmacokinetics of E2074 in Rats, Dogs, and Monkeys.** The plasma concentration–time profiles and PK parameters are shown in Fig. 2 and Table 1, respectively. After i.v. dosing at 1 mg/kg, E2074 was characterized in all species by moderate to high plasma clearance (3.21 l/h/kg in rats, 1.70 l/h/kg in dogs, and 1.35 l/h/kg in monkeys) and a moderate volume of distribution (2.6 to 15.1%). In addition, in dogs and monkeys after oral administration, the PK profile of E2074 was not proportional, and the resultant BA increased from 1.2 to 43.7%.

**In Vitro Metabolism.** E2074 was metabolized by liver microsomes in all species tested. Monkey intestinal microsomes, but not rat or dog intestinal microsomes, displayed E2074 metabolic activity. The enzyme kinetic parameters of E2074 for hepatic and intestinal metabolism were estimated in rat, dog, and monkey liver microsomes and monkey intestinal microsomes (Fig. 3; Table 2). The hepatic membrane permeability (P$_{\text{app}}$) of E2074 across the cell monolayer was calculated using the following equation:

$$P_{\text{app}}(\text{cm/s}) = \frac{\text{Permeated amount of E2074}}{\text{Incubation time} \times \text{Initial concentration of E2074} \times \text{Membrane area}}$$

where the incubation time was 2 hours and the membrane area was 0.3 $\times$ 10$^6$ cm$^2$.

**Table 3**

Estimation of hepatic clearance of E2074 by using the dispersion model

<table>
<thead>
<tr>
<th>Species</th>
<th>fp</th>
<th>Rb</th>
<th>fmic</th>
<th>$\text{CL}_{h,vitro}$</th>
<th>$\text{CL}_{h,vitro}$</th>
<th>$\text{CL}_{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>0.675</td>
<td>0.89</td>
<td>1</td>
<td>0.196</td>
<td>3.19</td>
<td>3.60 ± 0.14</td>
</tr>
<tr>
<td>Dog</td>
<td>0.601</td>
<td>0.86</td>
<td>1</td>
<td>0.208</td>
<td>1.83</td>
<td>1.97 ± 0.30</td>
</tr>
<tr>
<td>Monkey</td>
<td>0.676</td>
<td>1.01</td>
<td>1</td>
<td>0.084</td>
<td>2.00</td>
<td>1.34 ± 0.14</td>
</tr>
</tbody>
</table>

$\text{CL}$, clearance; $\text{CL}_{h,vitro}$, hepatic blood clearance; $\text{CL}_{b}$, intrinsic clearance; E2074, 2-[(2R)-2-fluoro-3-[(3-[6-fluorobenzyl]oxy]-8-azabicyclo[3.2.1]oct-8-yl[3-propyl]-4,5-dimethyl-2,4-dihydro-3H-1,2,4-triazol-3-one; fp, unbound fraction in plasma; Rb, blood to plasma partition ratio.

**Fig. 4.** Comparison of E2074 metabolic activity in recombinant human P450s. The protein concentration of each P450 was 25 pmol/ml, and the E2074 concentration was 0.25 $\mu$M. The data are presented as the mean of two experiments.
oral PK profile of E2074 (Fig. 6B). At a dose of 3 mg/kg, the C\text{max} and AUC with similar dose normalized values were found in the same dose range (Table 4). Conversely, KCZ coadministration significantly affected the PK profile in dogs; in preclinical animals, nonlinear PK profiles with greater than dose-proportional increases in AUC and C\text{max} were observed after oral administration of E2074, and BA increased as the dose level increased (Fig. 2 and Table 1). An evident prolongation of t\text{1/2} at higher oral dose was observed only in dogs, suggesting that the major cause of the nonlinear PK profile of E2074 was saturation of the first-pass effect in rats and monkeys, and saturation of the elimination of E2074 after entering the systemic circulation also contributed to the nonlinear PK profile in dogs in the dose range studied. E2074 was metabolized by liver microsomes in rats, dogs, and monkeys, and the estimated CL\text{h,\text{vitr}} from CL\text{int,u} was comparable with in vivo blood clearance (CL\text{b}) after i.v. administration (Table 3).

The accomplishment of in vitro–in vivo extrapolation using liver microsomes indicated that the major elimination route of E2074 after i.v. dosing in all species tested was hepatic metabolism; thus, the major reason for the nonlinear PK profile of E2074 after oral administration was initially believed to be saturation of hepatic metabolism. The K\text{m} estimated from enzyme kinetic studies using liver microsomes is one of the key parameters for evaluating the saturation of hepatic metabolism, and the estimated K\text{m} values were 7.23 μM for rats, 0.41 μM for dogs, and 42.44 μM for monkeys (Table 2).

The other parameter for assessing the saturation of hepatic metabolism is the concentration at the site of metabolism in the liver after oral administration of E2074. Estimation of the concentration at the metabolic site is difficult, but unbound C\text{max} in the portal vein (C\text{max,pv,u}) could be used as a surrogate concentration. In double-cannulated rats, C\text{max,pv,u} values were 10-fold higher than unbound systemic C\text{max} values (Fig. 5), thus, the C\text{max,pv,u} values at E2074 doses of 3, 10, and 30 mg/kg in intact rats were assumed to be 0.4, 3.3, and 22.6 μM, respectively (Tables 1 and 3). The ratios of C\text{max,pv,u} to K\text{m} (7.23 μM) at the three aforementioned E2074 doses in rat liver microsomes were 0.06, 0.46, and 3.1, respectively, suggesting that saturation of first-pass hepatic metabolism at 10 and 30 mg/kg was a key cause of the nonlinear PK profile in rats. In dogs, there were no data for C\text{max,pv,u}, and the unbound systemic plasma C\text{max} values (0.02 and 1.57 μM at 3 and 10 mg/kg oral doses, respectively) were used for preclinical animals.

Discussion

In preclinical animals, nonlinear PK profiles with greater than dose-proportional increases in AUC and C\text{max} were observed after oral administration of E2074, and BA increased as the dose level increased (Fig. 2 and Table 1). An evident prolongation of t\text{1/2} at higher oral dose was observed only in dogs, suggesting that the major cause of the nonlinear PK profile of E2074 was saturation of the first-pass effect in rats and monkeys, and saturation of the elimination of E2074 after entering the systemic circulation also contributed to the nonlinear PK profile in dogs in the dose range studied. E2074 was metabolized by liver microsomes in rats, dogs, and monkeys, and the estimated CL\text{h,\text{vitr}} from CL\text{int,u} was comparable with in vivo blood clearance (CL\text{b}) after i.v. administration (Table 3).

The accomplishment of in vitro–in vivo extrapolation using liver microsomes indicated that the major elimination route of E2074 after i.v. dosing in all species tested was hepatic metabolism; thus, the major reason for the nonlinear PK profile of E2074 after oral administration was initially believed to be saturation of hepatic metabolism. The K\text{m} estimated from enzyme kinetic studies using liver microsomes is one of the key parameters for evaluating the saturation of hepatic metabolism, and the estimated K\text{m} values were 7.23 μM for rats, 0.41 μM for dogs, and 42.44 μM for monkeys (Table 2).

The other parameter for assessing the saturation of hepatic metabolism is the concentration at the site of metabolism in the liver after oral administration of E2074. Estimation of the concentration at the metabolic site is difficult, but unbound C\text{max} in the portal vein (C\text{max,pv,u}) could be used as a surrogate concentration. In double-cannulated rats, C\text{max,pv,u} values were 10-fold higher than unbound systemic C\text{max} values (Fig. 5), thus, the C\text{max,pv,u} values at E2074 doses of 3, 10, and 30 mg/kg in intact rats were assumed to be 0.4, 3.3, and 22.6 μM, respectively (Tables 1 and 3). The ratios of C\text{max,pv,u} to K\text{m} (7.23 μM) at the three aforementioned E2074 doses in rat liver microsomes were 0.06, 0.46, and 3.1, respectively, suggesting that saturation of first-pass hepatic metabolism at 10 and 30 mg/kg was a key cause of the nonlinear PK profile in rats. In dogs, there were no data for C\text{max,pv,u}, and the unbound systemic plasma C\text{max} values (0.02 and 1.57 μM at 3 and 10 mg/kg oral doses, respectively) were used for preclinical animals.
The monkeys were different individuals than those found in Table 1. The KCZ dose was 5 mg/kg orally. The pharmacokinetic parameters represent the mean ± S.E. of three monkeys or mean (two monkeys). The figures in parentheses are the ratio without KCZ in the same dosing route.

### Table 4

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>i.v.</th>
<th>Oral</th>
<th>i.v.</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>t&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
<td>1.93 ± 0.20</td>
<td>5.49</td>
<td>1.96 ± 0.05</td>
<td>2.20 ± 0.32</td>
</tr>
<tr>
<td>AUC (µg·h/ml)</td>
<td>0.70 ± 0.09</td>
<td>0.34</td>
<td>0.82 ± 0.03</td>
<td>1.55 ± 0.27</td>
</tr>
<tr>
<td>CL (l/h/kg)</td>
<td>3.07 ± 0.44</td>
<td>NA</td>
<td>1.22 ± 0.04</td>
<td>NA</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (l/kg)</td>
<td>2.92 ± 0.56</td>
<td>NA</td>
<td>3.29 ± 0.29</td>
<td>NA</td>
</tr>
<tr>
<td>Dose normalized C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>NA</td>
<td>0.03 (1.0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NA</td>
<td>0.13 ± 0.03 (3.9)</td>
</tr>
<tr>
<td>Dose normalized AUC</td>
<td>0.70 ± 0.09 (1.0)</td>
<td>0.11 (1.0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82 ± 0.03 (1.2)</td>
<td>0.52 ± 0.09 (4.6)</td>
</tr>
</tbody>
</table>

AUC, area under the plasma concentration curve; CL, clearance; E2074, 2-[(2R)-2-fluoro-3-[[3-fluorobenzyl]oxy]-8-azabicyclo[3.2.1]oct-8-yl]propyl]-4,5-dimethyl-2,4-dihydro-3H-1,2,4-triazol-3-one; KCZ, ketoconazole; NA, not applicable; t<sub>1/2</sub>, half-life; V<sub>ss</sub>, steady-state volume of distribution.

<sup>a</sup> Mean of two monkeys.

### Table 5

Comparing pharmacokinetic parameters of E2074 between WT and mdr1a/1b KO mice

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>WT</th>
<th>mdr1a/1b KO</th>
<th>KO/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.25–1</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>0.23 ± 0.04</td>
<td>0.24 ± 0.04</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>8.3</td>
<td>3.5</td>
<td></td>
</tr>
</tbody>
</table>

AUC, area under the plasma concentration curve; E2074, 2-[(2R)-2-fluoro-3-[[3-fluorobenzyl]oxy]-8-azabicyclo[3.2.1]oct-8-yl]propyl]-4,5-dimethyl-2,4-dihydro-3H-1,2,4-triazol-3-one; KO, knockout; K<sub>p,brain</sub>, brain to plasma concentration ratio; t<sub>max</sub>, time to reach C<sub>max</sub>; WT, wild type.
abolished in monkeys. These results demonstrated that the nonlinear PK profile of E2074 in monkeys was caused by the saturation of first-pass intestinal metabolism.

E2074 is a P-gp substrate (flux ratio: 3.14) based on the result in mouse P-gp-overexpressing cells. P-gp is present in the intestine and brain blood capillaries, and it acts as a barrier to drug absorption and brain penetration. The brain penetration and intestinal absorption of fexofenadine are restricted by P-gp in mice (Tahara et al., 2005). As KCZ is an inhibitor of P-gp and CYP3A4, the contribution of P-gp to the absorption of E2074 in the intestine was elucidated using mdr1a/1b KO mice.

There were no differences in the plasma concentration–time profile, \( C_{\text{max}} \) or AUC between KO and WT mice; however, the \( K_{p,\text{brain}} \) in KO mice was 3.5-fold higher than that in the WT mice (Fig. 7; Table 5). These results indicated that P-gp did not affect the intestinal absorption or systemic exposure of E2074, but it restricted the brain penetration of the drug in mice.

Intestinal absorption can be estimated by simultaneously measuring plasma concentrations in portal and systemic veins (Kuze et al., 2009). The intestinal absorption of E2074 was estimated in double-cannulated rats (Fig. 5), and the absorption was estimated to be almost complete (116%) in these rats (unpublished data), which further supports the very minor at most contribution of P-gp to E2074 absorption. These results suggest that the effect of P-gp on E2074 absorption in intestine is limited, and the saturation of P-gp transport activity would not be the cause of nonlinear PK profiles in monkeys as observed in rats and dogs.

In conclusion, we clarified the cause of nonlinear PK profiles of E2074 in animals, identifying the saturation of hepatic metabolism in rats and dogs, and the saturation of intestinal metabolism in monkeys as the primary mechanisms.

Acknowledgments
The authors thank Drs. Tatsuhiko Onogi (director of Department of Pharmacology), Fumihiro Ozaki, and Motohiro Socjima (Department of Medicinal Chemistry) for the synthesis of E2074, and Drs. Nancy Wong and Anne Cooper for useful discussions.

Authorship Contributions
Participated in research design: Nagaya, Takenaka. Conducted experiments: Nagaya. Performed data analysis: Nagaya. Wrote or contributed to the writing of the manuscript: Nagaya, Takenaka, Kusano, Yoshimura.

References

Species Difference of Nonlinear Pharmacokinetics of E2074


Address correspondence to: Yoko Nagaya, Drug Metabolism and Pharmacokinetics Japan, Eisai Product Creation Systems, Eisai Co., Ltd., 1-3, Tokodai 5-Chome Tsukuba-shi, Ibaraki 300-2635, Japan. E-mail: y-nagaya@hhc.eisai.co.jp