CYP3A4 Induction in the Liver and Intestine of Pregnane X Receptor/CYP3A-Humanized Mice: Approaches by Mass Spectrometry Imaging and Portal Blood Analysis^[S]

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ABSTRACT

Induction of cytochrome P450 enzyme 3A (CYP3A) in response to pregnane X receptor (PXR) activators shows species-specific differences. To study the induction of human CYP3A in response to human PXR activators, we generated a double-humanized mouse model of PXR and CYP3A. CYP3A-humanized mice generated by using a mouse artificial chromosome (MAC) vector containing the entire genomic human CYP3A locus (hCYP3A-MAC mouse line) were bred with PXR-humanized mice in which the ligand-binding domain of mouse PXR was replaced with that of human PXR, resulting in double-humanized mice (hCYP3A-MAC/hPXR mouse line). Oral administration of the human PXR activator rifampicin increased hepatic expression of CYP3A4 mRNA and triazolam (TRZ) 1'- and 4-hydroxylation activities, CYP3A probe activities, in the liver and intestine microsomes of hCYP3A-MAC/hPXR mice. The plasma concentration of TRZ after oral dosing was significantly decreased by rifampicin treatment in hCYP3A-MAC/hPXR mice but not in hCYP3A-MAC mice. In addition, mass spectrometry imaging analysis showed that rifampicin treatment increased the formation of hydroxy TRZ in the intestine of hCYP3A-MAC/hPXR mice after oral dosing of TRZ. The plasma concentration of 1'- and 4-hydroxy TRZ

in portal blood was also increased by rifampicin treatment in hCYP3A-MAC/hPXR mice. These results suggest that the hCYP3A-MAC/hPXR mouse line may be a useful model to predict human PXR-dependent induction of metabolism of CYP3A4 substrates in the liver and intestine.

SIGNIFICANCE STATEMENT

We generated a double-humanized mouse line for CYP3A and PXR. Briefly, CYP3A-humanized mice generated by using a mouse artificial chromosome vector containing the entire genomic human CYP3A locus were bred with PXR-humanized mice in which the ligand-binding domain of mouse PXR was replaced with that of human PXR. Expression of CYP3A4 and metabolism of triazolam, a typical CYP3A substrate, in the liver of CYP3A/PXR-humanized mice were enhanced in response to rifampicin, a typical human PXR activator. Enhancement of triazolam metabolism in the intestine of CYP3A/PXR-humanized mice was firstly shown by combination of mass spectrometry imaging of sliced intestine and liquid chromatography with tandem mass spectrometry analysis of metabolite concentration in portal blood after oral dosing of triazolam.

Introduction

Cytochrome P450 enzyme 3A4 (CYP3A4) is mainly expressed in the liver and intestine and contributes significantly to the presystemic elimination of drugs after oral administration

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(Thummel et al., 1996; von Richter et al., 2001). Because CYP3A4 is involved in the metabolism of about 50% of therapeutic drugs, inhibition or induction of CYP3A4 by concomitant drugs results in clinically important drug–drug interactions (DDIs) (Pelkonen et al., 1998). Thus, regulatory agencies including the U.S. Food and Drug Administration, the European Medicines Agency, and the Pharmaceuticals and Medical Devices Agency of Japan have made recommendations in their DDI guidance documents for various methodologies to assess DDI potential (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf>; http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf; <http://www.pmda.go.jp/files/000228122.pdf>).

Pregnane X receptor (PXR, NR1I2), a member of the nuclear receptor superfamily, regulates the induction process of CYP3A4 (Willson and Kliewer, 2002). PXR is activated by a broad range of chemicals such as steroid hormones, herbals, and antibiotics (Mackowiak and Wang, 2016). Activated PXR translocates into the nucleus and forms a heterodimer with the retinoid X receptor α (RXR α). The PXR-RXR α heterodimer binds to DNA response elements and activates the transcription of target genes including CYP3A4 (Goodwin et al., 1999; Toriyabe et al., 2009).

Interestingly, there are significant species differences in response to PXR activators between humans and rodents (Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Jones et al., 2000; Moore et al., 2000; Quattrochi and Guzelian, 2001). The antibiotic rifampicin (RIF) activates human PXR but is a weak activator of rodent PXR. In contrast, pregnenolone 16 α -carbonitrile (PCN) potentially activates rodent PXR but is a weak activator of human PXR. Because such differences lead to a problem for studies using rodents to predict DDIs in humans, some humanized mouse lines in which there are responses to human PXR activators have been developed (Xie et al., 2000; Cheung et al., 2006; Ma et al., 2008; Igarashi et al., 2012).

Hasegawa et al. (2011) generated a multiple humanized mouse line that combines humanization of PXR and constitutive androstane receptor (CAR) and replacement of the mouse *Cyp3a* cluster with a human genomic region carrying CYP3A4 and CYP3A7 (huPXR/huCAR/huCYP3A4/3A7). The huPXR/huCAR/huCYP3A4/3A7 mice showed a CYP3A4 induction response to human PXR activators. In the huPXR/huCAR/huCYP3A4/3A7 mice, plasma concentrations of triazolam (TRZ), a typical CYP3A4 substrate, were decreased by oral administration of RIF. However, the induction of intestinal CYP3A4 mRNA expression was negligible when RIF was orally administered. In addition, the area under the plasma concentration–time curves (AUCs) of TRZ metabolites were decreased by RIF treatment. Therefore, there is no direct evidence for enhancement of CYP3A4-mediated metabolism in the intestine of huPXR/huCAR/huCYP3A4/3A7 mice treated orally with human PXR activators.

In this study, we generated a double-humanized mouse line for CYP3A and PXR and examined CYP3A4 induction in the liver and intestine of the double-humanized mice in response to RIF. To demonstrate the functional induction of intestinal CYP3A4 by RIF, we determined the metabolite concentrations in portal blood after oral administration of TRZ and visualized the metabolite formation in the sliced small intestine by mass spectrometry imaging.

Materials and Methods

Materials. TRZ and RIF were purchased from Wako Pure Chemicals (Osaka, Japan). Pregnenolone 16 α -carbonitrile (PCN) was purchased from Toronto Research Chemicals (North York, ON, Canada). Both 1'-hydroxy TRZ and 4-hydroxy TRZ were supplied by Nihon Upjohn

(Tokyo, Japan). All other reagents were purchased from commercial sources.

Animals. The generation and characterization of CYP3A-humanized mice and PXR-humanized mice were described elsewhere (Igarashi et al., 2012; Kazuki et al., 2019). Briefly, a trans-chromosomal mouse line with a mouse artificial chromosome (MAC) vector containing the entire human CYP3A gene cluster (CYP3A4, CYP3A5, CYP3A7, and CYP3A43) and their regulatory regions (promoters and enhancers) was crossed with Cyp3a-knockout (Cyp3a-KO) mice to produce CYP3A-humanized mice with a Cyp3a-knockout background (hCYP3A-MAC mouse line).

The double-humanized mice of CYP3A and PXR (hCYP3A-MAC/hPXR mouse line) and PXR-humanized mice with a Cyp3a-knockout background (Cyp3a-KO/hPXR mouse line) were generated by mating the hCYP3A-MAC mouse line with a PXR-humanized mouse line in which the ligand-binding domain of mouse PXR was replaced with that of human PXR. Each mouse line was backcrossed to the C57BL/6 or ICR strain at least six generations.

The primer sequences for the genomic polymerase chain reaction (PCR) analyses are described in Supplemental Table 1. Primer sets for the genotyping of hCYP3A-MAC, Cyp3a-KO (double KO of Cyp3a57-59 and Cyp3a13), and hPXR knock-in are described in Supplemental Table 2. All experiments were done using male mice between 10 and 11 weeks of age.

Animals were kept in a temperature-controlled environment with a 12-hour light/dark cycle. The light-cycle hours were between 7:00 AM and 7:00 PM. They received a standard diet (CE-2; CLEA, Tokyo, Japan). The present study was conducted in accordance with the guidelines for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Taiho Pharmaceutical Co., Tottori University and Chiba University.

Analysis of PXR-Specific Induction in the Liver. We treated hCYP3A-MAC mice and hCYP3A-MAC/hPXR mice intraperitoneally with RIF (10 mg/kg, an hPXR activator), PCN (100 mg/kg, an mPXR activator), or a vehicle (corn oil) for 4 consecutive days. Sixteen hours after the final treatment, liver pieces were collected.

Analysis of CYP3A4 Induction by RIF in the Liver and Intestine. We treated hCYP3A-MAC mice orally with RIF (30 mg/kg) or a vehicle (0.5% carboxymethyl cellulose) for 4 consecutive days. Sixteen hours after the final treatment, the liver and small intestine were collected.

mRNA Analysis. Total RNA was isolated from tissue pieces of livers and small intestines (1-cm segments collected at 10 cm from the pyloric region) by using the RNeasy Mini Kit (Qiagen, Germany). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). We determined the CYP3A4 mRNA levels by quantitative real-time PCR carried out on a CFX Connect Real-time System (Bio-Rad Laboratories, Hercules, CA). The expression levels of CYP3A4 mRNA were determined by TaqMan probe-based real-time PCR using TaqMan Gene Expression Assays (Hs00604506_m1; Thermo Fisher Scientific). The mRNA expression levels were normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression detected by 20 $\times$ Pre-Developed TaqMan Assay Reagent mouse GAPDH (Thermo Fisher Scientific).

TRZ Hydroxylation Activity In Vitro. Microsomal fractions of the liver and intestine were prepared as described elsewhere (Kazuki et al., 2013). Incubations were performed in a total volume of 250 μ l containing 0.1 mM EDTA, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 mM NADP⁺, 2 mM glucose-6-phosphate, 1 IU/ml glucose-6-phosphate dehydrogenase, 4 mM MgCl₂), and a substrate (200 μ M TRZ).

ABBREVIATIONS: AUC, area under the plasma concentration–time curve; CAR, constitutive androstane receptor; P450, cytochrome P450 enzyme; DDI, drug–drug interaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; h, human; KO, knockout; LC-MS/MS, liquid chromatography with tandem mass spectrometry; MAC, mouse artificial chromosome; PCN, pregnenolone 16 α -carbonitrile; PCR, polymerase chain reaction; PXR, pregnane X receptor; RIF, rifampicin; TRZ, triazolam.

The microsomal protein concentrations and incubation times used were selected from the linear range of formation of TRZ metabolites.

The mixtures were preincubated for 1 minute at 37°C, and reactions were initiated by the addition of 25 μ l of the NADPH-generation system. Incubation was stopped by the addition of 100 μ l of acetonitrile followed by the addition of 50 μ l of 1 μ g/ml oxazepam in methanol as an internal control.

Determination of 1'-hydroxy TRZ and 4-hydroxy TRZ was performed using a Hitachi L-7000 model HPLC system (Tokyo, Japan) consisting an L-7400 UV detector and a CAPCELL PAK C18 UG120 column (4.6 $\times$ 250, 5 mm; Shiseido, Tokyo, Japan). The mobile phase consisted of 10 mM potassium phosphate buffer (pH 7.4), acetonitrile, and methanol (60/30/10, v/v/v), with a flow rate of 1.0 ml/min. The eluent was monitored at a wavelength of 220 nm.

Immunoblot Analysis. Microsomal fractions (1 μ g) of small intestines were separated by 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and probed using a mouse monoclonal anti-CYP3A4 antibody (1:1000 dilution, sc-53850; Santa Cruz Biotechnology, Dallas, TX) and a rabbit monoclonal anti-GAPDH antibody (1:10,000 dilution, ab181602; Abcam, Cambridge, United Kingdom). The secondary antibodies used were horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:5000 dilution, ab6728; Abcam) and goat anti-rabbit IgG (1:2000 dilution, ab6721; Abcam) for anti-CYP3A4 and anti-GAPDH, respectively. The primary and secondary antibodies were diluted with Can Get Signal Solution (TOYOBO, Osaka,

Japan). CYP3A4 and GAPDH proteins were detected by an enhanced chemiluminescence method (GE Healthcare, Buckinghamshire, United Kingdom) and LAS-4000 (Fujifilm, Tokyo, Japan). Intensities of bands were determined using ImageQuant TL (GE Healthcare).

Pharmacokinetic Analysis. CYP3A-MAC/hPXR and hCYP3A-MAC mice were orally treated with TRZ (1 mg/kg) after oral administration of RIF (30 mg/kg per day) or a vehicle (0.5% carboxymethyl cellulose) for 3 consecutive days. Blood samples were collected from suborbital veins at 15, 30, 60, 120, 240, and 360 minutes after TRZ administration. Plasma was separated by centrifugation and stored at -80°C. The concentrations of TRZ, 1'-hydroxy TRZ and 4-hydroxy TRZ were determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS) as described elsewhere (Kazuki et al., 2019).

The area under the plasma concentration-time curve (AUC) from time 0 to the last data point (AUC_{0-t}) was calculated using the linear trapezoidal method. $AUC_{0-\infty}$ was calculated by combining AUC_{0-t} and AUC_{extra} . AUC_{extra} represents an extrapolated value obtained by C_t/k_{el} , where C_t is the plasma concentration at the last data point and k_{el} represents the terminal elimination rate constant determined by log-linear regression analysis of the measured plasma concentrations of the terminal elimination phase. Maximum plasma concentration (C_{max}) and time to C_{max} (t_{max}) were obtained directly from the observed values. Apparent half-life was obtained as $\ln 2/k_{el}$.

Mass Spectrometry Imaging. The hCYP3A-MAC/hPXR mice were orally treated with RIF (10 mg/kg) or a vehicle (0.5% carboxymethyl

Fig. 1. CYP3A4 mRNA and CYP3A marker activities in the liver of hCYP3A-MAC and hCYP3A-MAC/hPXR mice. Mice ($N = 3$ /group except for $N = 5$ /hCYP3A-MAC treated with oil and $N = 4$ /hCYP3A-MAC treated with RIF) were treated by intraperitoneal injection of a vehicle (corn oil), PCN (100 mg/kg), or RIF (10 mg/kg) for 4 days. (A) CYP3A4 mRNA expression level was normalized by GAPDH. (B) Formation rates of 1'-hydroxy TRZ (1'-OH) and 4-hydroxy TRZ (4-OH) from TRZ (200 μ M) were determined by using liver microsomes as CYP3A marker activities. Data are expressed as mean with S.D. Statistical analysis was firstly performed with multivariate ANOVA. Then one-way ANOVA with Scheffe's F test was performed for mRNA, 1'-OH, and 4-OH in hCYP3A-MAC and hCYP3A-MAC/hPXR mice, respectively. ** $P < 0.01$ compared with the mRNA value of hCYP3A-MAC mice (hCYP3A/mPXR) treated with a vehicle. †† $P < 0.01$ compared with 1'-OH of hCYP3A-MAC mice treated with a vehicle. ‡‡ $P < 0.01$ compared with 4-OH of hCYP3A-MAC mice treated with a vehicle.

Fig. 2. CYP3A4 mRNA and metabolic activity of CYP3A4 probe substrate in the liver and intestine of hCYP3A-MAC/hPXR mice. Mice ($N = 3/\text{group}$) were orally treated with either a vehicle (0.5% CMC) or RIF (30 mg/kg) for 3 days. (A) The CYP3A4 mRNA expression level was normalized by GAPDH. (B) Formation rates of 1'-hydroxy TRZ (1'-OH) and 4-hydroxy TRZ (4-OH) from TRZ (200 μM) were determined by using liver and intestine microsomes. Data are expressed as mean with S.D. Statistical analysis was performed with two-tailed t test. * $P < 0.05$ compared with the mRNA value in the liver of hCYP3A-MAC/hPXR mice treated with a vehicle (Welch's t test). † $P < 0.05$ compared with 1'-OH and 4-OH, respectively, in the liver of hCYP3A-MAC/hPXR mice treated with a vehicle (Welch's t test). ††, ††† $P < 0.01$ compared with 1'-OH and 4-OH, respectively, in the intestine of hCYP3A-MAC/hPXR mice treated with a vehicle (Student's t test).

cellulose) for 3 consecutive days. On day 4, the mice were orally administered TRZ (10 mg/kg). At 10 minutes after TRZ administration, the liver and small intestine were quickly removed and frozen in liquid nitrogen.

Serial sections were sliced to a thickness of 20 μm using a cryostat (CM 1950; Leica Microsystems, Wetzlar, Germany). Sections were thaw-mounted onto an indium-tin-oxide-coated glass slide (Sigma-Aldrich, St. Louis, MO) and dried at room temperature. Before the mass spectrometry imaging, the samples were kept at -80°C . We used α -cyano-4-hydroxycinnamic acid as the matrix-assisted laser desorption ionization matrix, deposited by iMLayer (Shimadzu Corp., Kyoto, Japan). Mass imaging data were obtained using iMScope TRIO (Shimadzu). The mass range was set to m/z 300–400 with a spatial resolution of 20 μm .

Analysis of CYP3A-Dependent Induction in the Intestine. CYP3A-MAC/hPXR and Cyp3a-KO/hPXR mice were orally treated with TRZ (1 mg/kg) after oral administration of RIF (30 mg/kg per day) or a vehicle (0.5% carboxymethyl cellulose) for 3 consecutive days. Blood samples were collected from portal veins at 10 minutes after TRZ administration. Plasma was separated by centrifugation and stored at -80°C . The concentrations of TRZ, 1'-hydroxy TRZ, and 4-hydroxy TRZ were determined by LC-MS/MS as described elsewhere (Minegishi et al., 2019).

Statistical Analysis. Data are expressed as mean with S.D. Data were analyzed by using Statcel 4 (OMS, Saitama, Japan) and R 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria). Comparisons of two groups were performed using a two-tailed t test. Comparisons of multiple groups were performed by multivariate ANOVA followed by one-way ANOVA with a post hoc test. $P < 0.05$ was considered statistically significant.

Results

Human PXR-Dependent Induction of Hepatic CYP3A in hCYP3A-MAC/hPXR Mice. We treated hCYP3A-MAC/hPXR and hCYP3A-MAC mice intraperitoneally with the human PXR ligand RIF and rodent PXR ligand PCN. After PCN treatment, CYP3A4 mRNA was significantly increased in the liver of hCYP3A-MAC mice but not in hCYP3A-MAC/hPXR mice (Fig. 1A). By contrast, RIF treatment did not increase CYP3A4 mRNA in hCYP3A-MAC mice, whereas an increase of CYP3A4 mRNA was found in hCYP3A-MAC/hPXR mice, though the increase was not statistically significant.

Consistent with CYP3A4 expression, TRZ 1'- and 4-hydroxylation activities—marker activities of CYP3A—increased significantly in the liver microsomes of hCYP3A-MAC mice after PCN treatment but not after RIF treatment (Fig. 1B). In hCYP3A-MAC/hPXR mice, TRZ 1'- and 4-hydroxylation activities increased after RIF treatment but not after PCN treatment. These results showed that the response of human CYP3A4 expression to PXR activators in the liver of hCYP3A-MAC/hPXR mice mimicked the response in hPXR.

Effect of RIF on CYP3A4 Expression in the Liver and Intestine of hCYP3A-MAC/hPXR Mice. Next, we examined the induction of CYP3A4 in the liver and intestine of hCYP3A-MAC/hPXR mice after oral treatment with RIF. A significant increase of CYP3A4 mRNA levels induced by RIF

Fig. 3. Plasma concentration–time profiles of TRZ (A and B) and 1'-hydroxy TRZ (C and D) in hCYP3A-MAC/hPXR (A and C) and hCYP3A-MAC (B and D) mice. Mice (*N* = 3/group for hCYP3A-MAC/hPXR mice and *N* = 4/group for hCYP3A-MAC mice) were given an oral dose of the vehicle or RIF (30 mg/kg) for 3 days followed by a 1 mg/kg oral dose of TRZ. Values for the vehicle are indicated as open circles and those for RIF are indicated as closed circles. Each point represents the mean with S.D.

was observed in the liver of hCYP3A-MAC/hPXR mice (Fig. 2A). Hepatic CYP3A activity was also significantly induced by RIF in hCYP3A-MAC/hPXR mice (Fig. 2B). In the intestine, a significant increase of CYP3A activity was observed, but the magnitude of increase was less than that in the liver. No increase of CYP3A4 mRNA with RIF

treatment was found in the intestine (Fig. 2A), although CYP3A4 protein was marginally increased by RIF in the intestine of CYP3A-MAC/hPXR mice (Supplemental Fig. 1).

Effect of RIF on Pharmacokinetics of TRZ in hCYP3A-MAC/hPXR Mice. To assess whether RIF can affect the pharmacokinetics of TRZ and its metabolites in

TABLE 1
Pharmacokinetic parameters of TRZ in hCYP3A-MAC/hPXR and hCYP3A-MAC mice
hCYP3A-MAC/hPXR and hCYP3A-MAC mice were administered the vehicle or RIF followed by a 1-mg/kg oral dose of TRZ. Data represent the mean $\pm$ S.D. (*N* = 3/group for hCYP3A-MAC/hPXR mice and *N* = 4/group for hCYP3A-MAC mice).

Group	<i>t</i> _{max}	<i>C</i> _{max}	Half-life	AUC _{0-∞}
	<i>h</i>	<i>nM</i>	<i>h</i>	<i>nM·h</i>
hCYP3A-MAC/hPXR				
Vehicle	0.42 $\pm$ 0.14	515 $\pm$ 190	1.10 $\pm$ 0.14	1050 $\pm$ 242
RIF	0.50 $\pm$ 0.43	235 $\pm$ 55	0.39 $\pm$ 0.05 ^a	250 $\pm$ 71 ^a
hCYP3A-MAC				
Vehicle	0.38 $\pm$ 0.14	229 $\pm$ 81	1.08 $\pm$ 0.13	448 $\pm$ 157
RIF	0.44 $\pm$ 0.13	180 $\pm$ 22	1.21 $\pm$ 0.44	370 $\pm$ 82

AUC_{0-∞}, area under the plasma concentration–time curve from time 0 to infinity; hCYP3A-MAC, human cytochrome P450 3A enzyme mouse artificial chromosome; PXR, pregnane X receptor; RIF, rifampicin.
^a*P* < 0.01 compared with the vehicle control (Student's *t* test).

TABLE 2

Pharmacokinetic parameters of 1'-hydroxy TRZ in hCYP3A-MAC/hPXR and hCYP3A-MAC mice. We administered to hCYP3A-MAC/hPXR and hCYP3A-MAC mice the vehicle or RIF followed by a 1-mg/kg oral dose of TRZ. Data represent mean $\pm$ S.D. ($N = 3$ /group for hCYP3A-MAC/hPXR mice and $N = 4$ /group for hCYP3A-MAC mice).

Group	$t_{\max}$	$C_{\max}$	Half-life	$AUC_{0-\infty}$
	h	nM	h	$nM \cdot h$
hCYP3A-MAC/hPXR				
Vehicle	1.33 $\pm$ 0.58	978 $\pm$ 200	3.25 $\pm$ 0.50	4893 $\pm$ 1062
RIF	0.58 $\pm$ 0.38	1250 $\pm$ 414	0.64 $\pm$ 0.14 ^a	1737 $\pm$ 27 ^b
hCYP3A-MAC				
Vehicle	0.63 $\pm$ 0.25	747 $\pm$ 165	2.19 $\pm$ 0.43	2618 $\pm$ 738
RIF	0.88 $\pm$ 0.25	712 $\pm$ 47	2.07 $\pm$ 0.38	2313 $\pm$ 562

$AUC_{0-\infty}$, area under the plasma concentration-time curve from time 0 to infinity; hCYP3A-MAC, human cytochrome P450 3A enzyme mouse artificial chromosome; hPXR, human pregnane X receptor; RIF, rifampicin.

^a $P < 0.001$ compared with the vehicle control (Student's t test for half-life and Welch's t test for AUC).

^b $P < 0.05$ compared with the vehicle control (Student's t test for half-life and Welch's t test for AUC).

hCYP3A-MAC/hPXR mice, hCYP3A-MAC mice and hCYP3A-MAC/hPXR mice were orally administered TRZ after oral daily doses of the vehicle or RIF for 3 days. Because the plasma concentration of 4-hydroxy TRZ in hCYP3A-MAC mice was lower than the quantification limit (250 nM), the plasma concentration-time curves for TRZ and 1'-hydroxy TRZ are shown in Fig. 3, and the pharmacokinetic parameters are summarized in Tables 1 and 2.

The $AUC_{0-\infty}$ value of TRZ was significantly decreased by RIF treatment in hCYP3A-MAC/hPXR mice (76% decrease). The half-life of TRZ was also decreased by RIF. On the other hand, no significant effects of RIF on the $AUC_{0-\infty}$ value and half-life of TRZ were observed in hCYP3A-MAC mice. These results showed that the decreases of the $AUC_{0-\infty}$ value and half-life of TRZ were human PXR dependent.

Induction of Intestinal CYP3A by RIF in hCYP3A-MAC/hPXR Mice. To investigate the enhancement of TRZ metabolism in the intestine of hCYP3A-MAC/hPXR mice, we tried to visualize the metabolism of TRZ in the intestine by mass spectrometry imaging. As shown Fig. 4A, the signal intensity of hydroxy TRZ increased in the sliced intestine of hCYP3A-MAC/hPXR mice treated with RIF.

Furthermore, we investigated whether the enhancement of TRZ metabolism in the intestine of hCYP3A-MAC/hPXR mice treated with RIF is CYP3A dependent. The hCYP3A-MAC/hPXR mice and Cyp3a-KO/hPXR mice were orally administered the vehicle or RIF for 3 days. At 10 minutes after oral administration of TRZ, the plasma concentrations of TRZ and its metabolites in portal blood were determined.

As shown in Fig. 4B, the plasma concentration of TRZ in portal blood was decreased in hCYP3A-MAC/hPXR mice. The plasma concentrations of 1'-hydroxy TRZ and 4-hydroxy TRZ in portal blood, which showed first-pass metabolism in the small intestine, were increased by RIF treatment in hCYP3A-MAC/hPXR mice (Fig. 4C). We still found a significant decrease of TRZ concentration in the portal blood of Cyp3a-KO/hPXR mice treated with RIF (Supplemental Fig. 2A). The plasma concentrations of 1'-hydroxy TRZ and 4-hydroxy TRZ in portal blood were also increased by RIF treatment in Cyp3a-KO/hPXR mice, although the concentrations were lower than those in hCYP3A-MAC/hPXR mice (Supplemental Fig. 2B).

Discussion

We previously reported an hCYP3A-MAC mouse model with a mouse PXR background (Kazuki et al., 2019; Minegishi et al.,

2019). In contrast to the hCYP3A-MAC mouse model, the present hCYP3A-MAC/hPXR mouse model was improved as a mouse line with a human PXR-like response. Therefore, the hCYP3A-MAC/hPXR mouse model would be a useful tool for the study of CYP3A4 induction by human PXR activators.

The present study clearly showed that plasma concentrations of TRZ were decreased by RIF only in the hCYP3A-MAC/hPXR mice (Fig. 3, A and B), suggesting that oral clearance of TRZ was enhanced in response to hPXR activation by RIF. In hCYP3A-MAC/hPXR mice, hepatic CYP3A4 mRNA levels and TRZ metabolism in the liver microsomes were increased by oral treatment with RIF (Fig. 2), which was consistent with previous results obtained from humanized mouse models for PXR accomplished using different strategies (Ma et al., 2008; Hasegawa et al., 2011). Plasma elimination of TRZ after oral dosing was also enhanced by RIF in hCYP3A-MAC/hPXR mice (Table 1). These findings suggested induction of CYP3A4-mediated metabolism of TRZ in the liver of hCYP3A-MAC/hPXR mice. In addition, TRZ metabolism and CYP3A4 protein in the intestinal microsomes were also increased by oral treatment with RIF (Fig. 2B; Supplemental Fig. 1). The results of mass spectrometry imaging analysis demonstrated that hydroxy TRZ in the sliced intestine of hCYP3A-MAC/hPXR mice was increased by treatment with RIF (Fig. 4A). Moreover, the plasma concentrations of TRZ and its metabolites in portal blood were decreased and increased, respectively, by RIF in hCYP3A-MAC/hPXR mice (Fig. 4, B and C). Although metabolites found in blood of the portal vein were not always derived from the mesenteric vein, the concentrations of metabolites at 10 minutes after oral administration would reflect the intestinal metabolism. Because these experiments were performed for hCYP3A-MAC/hPXR mice, we cannot exclude the possibility that the induction of intestinal metabolism is hPXR-independent. However, plasma concentrations of 1'-hydroxy TRZ at the early phase after TRZ dosing were increased by RIF treatment in hCYP3A-MAC/hPXR mice but not in hCYP3A-MAC mice (Fig. 3, C and D). In addition, RIF could not induce CYP3A in Pxr-null mice (Ma et al., 2007). These findings suggested that TRZ metabolism in the intestine of hCYP3A-MAC/hPXR mice was enhanced in response to hPXR activation by RIF. Therefore, our study has shown that the overall metabolism of TRZ in the liver and intestine was functionally enhanced by oral administration of RIF in hCYP3A-MAC/hPXR mice.

In agreement with previously reported findings in huPXR/huCAR/huCYP3A4/3A7 mice (Hasegawa et al., 2011), the

AUC_{0-∞} value and half-life of 1'-hydroxy TRZ were also decreased by RIF treatment in hCYP3A-MAC/hPXR mice (Table 2). Notably, our results indicated a trend for plasma concentrations of 1'-hydroxy TRZ at 15 and 30 minute after TRZ dosing to be increased by RIF treatment in hCYP3A-MAC/hPXR mice (Fig. 3C). These results taken together with results showing enhanced formation of 1'-hydroxy TRZ in the intestine of hCYP3A-MAC/hPXR mice treated with RIF (Fig. 4) suggested that formation of 1'-hydroxy TRZ in intestinal metabolism was enhanced and subsequently further metabolism of 1'-hydroxy TRZ was enhanced in the liver of hCYP3A-MAC/hPXR mice treated with RIF.

Previously, Hasegawa et al. (2011) reported that huPXR/huCAR/huCYP3A4/3A7 mice showed a CYP3A4 induction response to human PXR activators, but the induction of intestinal CYP3A4 mRNA expression was not significant when RIF was orally administered. Ma et al. (2007, 2008)

also reported a PXR-humanized mouse model and a double transgenic mouse model expressing human PXR and CYP3A4, but they showed no data for the induction of CYP3A4 mRNA in the intestine. Consistent with these findings, our study using hCYP3A-MAC/hPXR mice also has shown that the induction of intestinal CYP3A4 mRNA expression was not significant when RIF was orally administered (Fig. 2A). On the other hand, expression of intestinal CYP3A protein was increased in hCYP3A-MAC/hPXR mice (Supplemental Fig. 1) as well as previous mouse models (Ma et al., 2007, 2008; Hasegawa et al., 2011) when RIF was orally administered. Therefore, it may be difficult to detect the induction of CYP3A4 at the mRNA level in the intestine of mouse models treated with RIF.

Metabolic activities of TRZ in intestinal microsomes were increased by RIF in hCYP3A-MAC/hPXR mice (Fig. 2B), but we could not exclude the possibility that TRZ was metabolized by other genes that were induced by RIF in hCYP3A-MAC/hPXR

Fig. 4. Induction of TRZ metabolism in the small intestine of hCYP3A-MAC/hPXR mice. (A) Imaging mass spectrometry of TRZ and its hydroxy metabolites in the small intestine. We gave hCYP3A-MAC/hPXR mice ($N = 1/\text{group}$) an oral dose of the vehicle or RIF (30 mg/kg) for 3 days followed by a 10-mg/kg oral dose of TRZ. At 10 minutes after TRZ dosing, the small intestine was removed and analyzed as described in the *Materials and Methods* section. (B and C) Plasma concentrations of TRZ (B) and its metabolites 1'-hydroxy TRZ and 4-hydroxy TRZ (C) in portal blood of hCYP3A-MAC/hPXR mice. Mice ($N = 4/\text{group}$) were given an oral dose of the vehicle or RIF (30 mg/kg) for 3 days followed by a 1 mg/kg oral dose of TRZ. At 10 minutes after TRZ dosing, blood samples were collected from portal veins. Data are expressed as mean with S.D. Statistical analysis was performed with Student's *t* test. * $P < 0.05$ compared with the vehicle control. ^{††} $P < 0.01$ compared with 1'-OH of hCYP3A-MAC/hPXR mice treated with a vehicle. ^{**} $P < 0.01$ compared with 4-OH of hCYP3A-MAC/hPXR mice treated with a vehicle.

mice. Therefore, we investigated the induction of intestinal CYP3A4 by RIF in hCYP3A-MAC/hPXR mice by comparing the plasma concentrations of TRZ and its metabolites in portal blood between hCYP3A-MAC/hPXR mice and Cyp3a-KO/hPXR mice. In our present study, the plasma concentration of 1'-hydroxy TRZ and 4-hydroxy TRZ in portal blood after 10 minutes of dosing was detected in both Cyp3a-KO/hPXR and hCYP3A-MAC/hPXR mice (Fig. 4C; Supplemental Fig. 2B). We still observed increases of 1'-hydroxy TRZ and 4-hydroxy TRZ in the portal blood in Cyp3a-KO/hPXR mice by RIF. These results suggest that RIF-inducible enzymes other than CYP3A contribute to TRZ metabolism in Cyp3a-KO/hPXR mice. It has been reported that expression levels of some genes including mouse Cyp2c enzymes could be increased by KO of *Cyp3a* genes and by treatment with PXR activators (van Waterschoot et al., 2009; Minegishi et al., 2019). In addition, an anti-CYP2C antibody inhibited TRZ hydroxylation activities in Cyp3a-KO mice but not in wild-type mice (Minegishi et al., 2019). These findings implied that the mouse Cyp2c enzymes were involved in TRZ metabolism in Cyp3a-KO/hPXR mice. To exclude the possibility of contribution of enzymes other than CYP3A to TRZ metabolism in mice with a Cyp3a-KO background, further modifications such as a combination with KO of other genes are needed.

The hCYP3A-MAC/hPXR mice used in our study have not only the *CYP3A4* gene but also the *CYP3A5*, *CYP3A7*, and *CYP3A43* genes. *CYP3A5* shows polymorphic expression in the adult liver (Kuehl et al., 2001). Because hCYP3A-MAC/hPXR mice have a 6986A>G mutation in the *CYP3A5**3 allele, the expression level of CYP3A5 protein is negligible. Recently, we generated CYP3A-MAC mice with *CYP3A5**1 by genome editing technology (Abe et al., 2017). Because CYP3A5 could be induced by RIF (Burk et al., 2004), further studies for comparison of induction between CYP3A4 and CYP3A5 in vivo may be accomplished by generation of a mouse model containing *CYP3A5**1 and hPXR.

In conclusion, our study demonstrated that oral treatment with RIF to hCYP3A-MAC/hPXR mice enhanced TRZ metabolism in the intestine as well as the liver in response to hPXR activation. The hCYP3A-MAC/hPXR mouse line will be a useful in vivo model for predicting the induction of hepatic and intestinal metabolism of CYP3A substrates via human PXR activation.

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Authorship Contributions

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References

- Abe S, Kobayashi K, Oji A, Sakuma T, Kazuki K, Takehara S, Nakamura K, Okada A, Tsukazaki Y, Senda N, et al. (2017) Modification of single-nucleotide polymorphism in a fully humanized CYP3A mouse by genome editing technology. *Sci Rep* 7:15189.
- Blumberg B, Sabbagh W Jr, Juguilon H, Bolado J Jr, van Meter CM, Ong ES, and Evans RM (1998) SRX, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* 12:3195–3205.
- Burk O, Koch I, Raucy J, Hustert E, Eichelbaum M, Brockmüller J, Zanger UM, and Wojnowski L (2004) The induction of cytochrome P450 3A5 (CYP3A5) in the human liver and intestine is mediated by the xenobiotic sensors pregnane X receptor (PXR) and constitutively activated receptor (CAR). *J Biol Chem* 279:38379–38385.
- Cheung C, Yu AM, Chen CS, Krausz KW, Byrd LG, Feigenbaum L, Edwards RJ, Waxman DJ, and Gonzalez FJ (2006) Growth hormone determines sexual dimorphism of hepatic cytochrome P450 3A4 expression in transgenic mice. *J Pharmacol Exp Ther* 316:1328–1334.
- Goodwin B, Hodgson E, and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 56:1329–1339.
- Hasegawa M, Kapelyukh Y, Tahara H, Seibler J, Rode A, Krueger S, Lee DN, Wolf CR, and Scheer N (2011) Quantitative prediction of human pregnane X receptor and cytochrome P450 3A4 mediated drug-drug interaction in a novel multiple humanized mouse line. *Mol Pharmacol* 80:518–528.
- Igarashi K, Kitajima S, Aisaki K, Tanemura K, Taquahashi Y, Moriyama N, Ikeno E, Matsuda N, Saga Y, Blumberg B, et al. (2012) Development of humanized steroid and xenobiotic receptor mouse by homologous knock-in of the human steroid and xenobiotic receptor ligand binding domain sequence. *J Toxicol Sci* 37:373–380.
- Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, et al. (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* 14:27–39.
- Kazuki Y, Kobayashi K, Aueviriyavit S, Oshima T, Kuroiwa Y, Tsukazaki Y, Senda N, Kawakami H, Ohtsuki S, Abe S, et al. (2013) Trans-chromosomal mice containing a human CYP3A cluster for prediction of xenobiotic metabolism in humans. *Hum Mol Genet* 22:578–592.
- Kazuki Y, Kobayashi K, Hirabayashi M, Abe S, Kajitani N, Kazuki K, Takehara S, Takiguchi M, Satoh D, Kuze J, et al. (2019) Humanized UGT2 and CYP3A transchromosomal rats for improved prediction of human drug metabolism. *Proc Natl Acad Sci USA* 116:3072–3081.
- Kliwer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterström RH, et al. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* 92:73–82.
- Kuehl P, Zhang J, Lin Y, Lamba J, Assem J, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, et al. (2001) Sequence diversity in CYP3A promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. *Nat Genet* 27:383–391.
- Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, and Kliwer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* 102:1016–1023.
- Ma X, Cheung C, Krausz KW, Shah YM, Wang T, Idle JR, and Gonzalez FJ (2008) A double transgenic mouse model expressing human pregnane X receptor and cytochrome P450 3A4. *Drug Metab Dispos* 36:2506–2512.
- Ma X, Shah Y, Cheung C, Guo GL, Feigenbaum L, Krausz KW, Idle JR, and Gonzalez FJ (2007) The PREGNANE X receptor gene-humanized mouse: a model for investigating drug-drug interactions mediated by cytochromes P450 3A. *Drug Metab Dispos* 35:194–200.
- Mackowiak B and Wang H (2016) Mechanisms of xenobiotic receptor activation: direct vs. indirect. *Biochim Biophys Acta* 1859:1130–1140.
- Minegishi G, Kazuki Y, Yamasaki Y, Okuya F, Akita H, Oshimura M, and Kobayashi K (2019) Comparison of the hepatic metabolism of triazolam in wild-type and Cyp3a-knockout mice for understanding CYP3A-mediated metabolism in CYP3A-humanized mice in vivo. *Xenobiotica* 49:1303–1310.
- Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, et al. (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* 275:15122–15127.
- Pelkonen O, Mäenpää J, Taavitsainen P, Rautio A, and Raunio H (1998) Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica* 28:1203–1253.
- Quattrochi LC and Guzelian PS (2001) Cyp3A regulation: from pharmacology to nuclear receptors. *Drug Metab Dispos* 29:615–622.
- Thummel KE, O'Shea D, Paine MF, Shen DD, Kunze KL, Perkins JD, and Wilkinson GR (1996) Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. *Clin Pharmacol Ther* 59:491–502.
- Toriyabe T, Nagata K, Takada T, Aratsu Y, Matsubara T, Yoshinari K, and Yamazoe Y (2009) Unveiling a new essential cis element for the transactivation of the CYP3A4 gene by xenobiotics. *Mol Pharmacol* 75:677–684.
- van Waterschoot RA, Rooswinkel RW, Wagenaar E, van der Kruijsen CM, van Herwaarden AE, and Schinkel AH (2009) Intestinal cytochrome P450 3A plays an important role in the regulation of detoxifying systems in the liver. *FASEB J* 23:224–231.

- von Richter O, Greiner B, Fromm MF, Fraser R, Omari T, Barclay ML, Dent J, Somogyi AA, and Eichelbaum M (2001) Determination of in vivo absorption, metabolism, and transport of drugs by the human intestinal wall and liver with a novel perfusion technique. *Clin Pharmacol Ther* **70**:217–227.
- Willson TM and Kliewer SA (2002) PXR, CAR and drug metabolism. *Nat Rev Drug Discov* **1**:259–266.
- Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS, and Evans RM (2000) Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature* **406**:435–439.

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