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The Unique Human N10-Glucuronidated Metabolite Formation from Olanzapine in Chimeric NOG-TKm30 Mice with Humanized Livers[S]

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ABSTRACT

Olanzapine is an antipsychotic agent with species-dependent pharmacokinetic profiles in both humans and animals. In the present study, the metabolic profiles of olanzapine in vitro and in vivo were compared in non-transplanted immunodeficient NOG-TKm30 mice and chimeric mice with humanized livers (hereafter humanized-liver mice). Hepatic microsomal fractions prepared from humanized-liver mice and humans mediated olanzapine N10-glucuronidation, whereas fractions from cynomolgus monkeys, marmosets, minipigs, dogs, rabbits, guinea pigs, rats, CD1 mice, and NOG-TKm30 mice did not. The olanzapine N10-glucuronidation activity in liver microsomes from humanized-liver mice was inhibited by hecogenin, a human UDP-glucuronosyltransferase (UGT) 1A4 inhibitor. In addition, hepatocytes from humanized-liver mice suggest that olanzapine N10-glucuronidation was a major metabolic pathway in the livers of humanized-liver mice. After a single oral dose of olanzapine (10 mg/kg body weight) to humanized-liver mice and control NOG-TKm30 mice, olanzapine N10-glucuronide isomers and olanzapine N4′-glucuronide were detected only in the plasma of humanized-liver mice. In contrast, the area under the curve for N4′-demethylolanzapine, 2-hydroxymethylolanzapine, and 7-hydroxyolanzapine glucuronide was higher in NOG-TKm30 mice than that in humanized-liver mice. The cumulative excreted amounts of olanzapine N10-glucuronide isomers were high in the urine and feces from humanized-liver mice, whereas the cumulative excreted amounts of 2-hydroxymethylolanzapine were higher in NOG-TKm30 mice than in humanized-liver mice. Thus, production of human-specific olanzapine N10-glucuronide was observed in humanized-liver mice, which was consistent with the in vitro glucuronidation data. These results suggest that humanized-liver mice are useful for studying drug oxidation and conjugation of olanzapine in humans.

SIGNIFICANCE STATEMENT

Human-specific olanzapine N10-glucuronide isomers were generated in chimeric NOG-TKm30 mice with humanized livers (humanized-liver mice), and high UGT1A4-dependent N10-glucuronidation was observed in the liver microsomes from humanized-liver mice. Hence, humanized-liver mice may be a suitable model for studying UGT1A4-dependent biotransformation of drugs in humans.

Introduction

Interspecies differences in drug metabolism between humans and animals are important to note in drug development as they can prevent the accurate prediction of the clearance and oral bioavailability of drugs during preclinical testing using various experimental animals, including mice, rats, dogs, and monkeys. Species differences have been observed in isoform composition, expression, and catalytic activities of drug-metabolizing enzymes, including cytochrome P450s (P450s) and UDP-glucuronosyltransferases (UGTs) (Martignoni et al., 2006; Oda et al., 2015). The glucuronidation pathway transforms small lipophilic molecules, such as steroids, bilirubin, hormones, and drugs, into water-soluble, excretable metabolites. Human UGT1A4 reportedly converts a range of tertiary amine substrates to the respective quaternary glucuronides (Fredenhagen et al., 2017; Meech et al., 2019).

A chimeric humanized-liver mouse model (termed humanized-liver mice) possesses various human drug-metabolizing enzymes, including P450 and UGT (Uehara et al., 2022c). This chimeric mouse model was established by transplanting human hepatocytes into NOD/Shi-*scid* interleukin 2 receptor gamma null mice expressing the herpes simplex virus thymidine kinase mutant30 transgenic NOG mice (NOG-TKm30) or the herpes simplex virus thymidine kinase transgene (TK-NOG) at the Central Institute for Experimental Animals (Kawasaki, Japan) (Hasegawa et al., 2011; Uehara et al., 2022a). We previously characterized the drug-metabolizing properties of humanized-liver mice and found them to be useful experimental animals for studying the profiles of drug dispositions and their metabolite formation in humans, although the metabolism of humans and humanized-liver mice exhibit several differences. Methyl-hydroxylation and subsequent oxidation of tolbutamide in humanized-liver mice are mediated by multiple drug-metabolizing

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ABBREVIATIONS: AUC, area under the curve; LC-MS/MS, liquid chromatography tandem mass spectrometry; NOG, NOD/Shi-Scid interleukin 2 receptor gamma-null mice; P450, cytochrome P450; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; TK, HSVtk; TKm30, herpes simplex virus thymidine kinase mutant 30 clone; UGT, UDP-glucuronosyltransferase.

enzymes, including P450, aldehyde dehydrogenase, and aldehyde oxidase, similar to those reported in humans (Uehara et al., 2021b). Humanized-liver mice exhibits high BIBX1382 clearance and efficient oxidative metabolite production, consistent with human drug metabolism and disposition (Uehara et al., 2021c). Additionally, hepatocytes isolated from humanized-liver mice (hereafter Hu-Liver cells) provide a relevant and robust model for in vitro preclinical studies, including P450 induction and drug metabolism (Uehara et al., 2019; Bachour-El Azzi et al., 2022).

Olanzapine is one of the most effective antipsychotic drugs with a thienobenzodiazepinyl structure, and has been approved for the treatment of schizophrenia, bipolar disorder, and treatment-resistant depression. The disposition and metabolism of olanzapine after a single oral dose in humans, rhesus monkeys, dogs, and mice have been reported (Kassahun et al., 1997; Mattiuz et al., 1997). However, olanzapine metabolism differs among these four species, with the major metabolite in the urine found to be strictly species-dependent. In humans, olanzapine is extensively metabolized to conjugate and oxidative metabolites, and olanzapine N10-glucuronide is the major metabolite found in plasma, urine, and feces, accounting for 25% of the total olanzapine dose (Kassahun et al., 1997; Kassahun et al., 1998). In the human liver, N10- and N4′-glucuronidation, N4′-emethylation, N4′-oxygenation, and 2-hydroxylation are mediated by UGT1A4, CYP1A2, flavin-containing monooxygenase 3 (FMO3), and CYP2D6, respectively (Ring et al., 1996; Erickson-Ridout et al., 2011; Okubo et al., 2016). Meanwhile, olanzapine N10-glucuronide is absent from the urine of these experimental animals following administration of a single oral dose of olanzapine, except for trace amounts in dog urine (Mattiuz et al., 1997). The major urinary metabolites after a single oral administration of olanzapine were N4′-demethyl-2-carboxy olanzapine (17% of the dose), 7-hydroxy-N-oxide olanzapine (8% of the dose), and 7-hydroxyolanzapine/2-hydroxymethylolanzapine/2-carboxyolanzapine (10%, 4%, and 2% of the dose, respectively) for rhesus monkeys, dogs, and mice, respectively (Mattiuz et al., 1997).

Based on previous studies, olanzapine was selected as a suitable model drug for analyzing UGT-mediated drug metabolism in humanized-liver mice. In this study, we investigated the biotransformation of olanzapine to oxidative and conjugated metabolites using in vivo and in vitro metabolic assays in humanized-liver mice. Herein, we report that humanized-liver mice are useful for studying olanzapine metabolism in humans.

Materials and Methods

Materials. Olanzapine and hecogenin were obtained from Tokyo Chemical Industry (Tokyo, Japan), olanzapine N10-glucuronide from BOC Sciences (Shirley, NY), and 8-cyclopentyl-1,3-dimethylxanthine (8-CPT), N4′-demethylolanzapine, olanzapine N4′-oxide, and 2-hydroxymethylolanzapine from Toronto Research Chemicals (Toronto, Canada). β-NADP$^+$, D-glucose 6-phosphate, and D-glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). Alamethicin and uridine 5′-diphosphoglucuronic acid were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human UGT1A4 expressed in baculovirus-infected insect cells was obtained from Corning Life Sciences (Woburn, MA). Pooled liver microsomal fractions from humans (31 men and 19 women, aged 25–78 years), guinea pigs (Hartley Albino, 37 males, approximately 9–10 weeks old), rats (Sprague Dawley, 711 males, approximately 8 weeks old), and mice (CD1, 2370 males, approximately 11–12 weeks old) were purchased from Sekisui-XenoTech (Lenexa, KS). Pooled liver microsomes from cynomolgus monkeys (six males, sexually mature), marmosets (14 males, sexually mature), minipigs (Göttingen, two males, ~11 months old), dogs (five males, >12 months old), and rabbits (eight males, >9 months old) were purchased from Corning Life Sciences.

Animals, Hepatocytes, and Liver Microsomes. Humanized-liver mice were generated by transplanting cryopreserved human hepatocytes [35-year-old Asian male (donor A), Lonza Walkersville, Inc. (Walkersville, MD); 12-year-old Caucasian female (donor B), Lonza Walkersville, Inc.] via the spleen into liver-injured NOG-TKm30 mice following administration of Val-ganciclovir using a previously described method (Uehara et al., 2022a). Briefly, NOG-TKm30 mice (6 to 8 weeks old) were given 0.2 mg/ml valganciclovir in drinking water for 2 days to ablate hepatocytes expressing the HSVtk mutant30 transgene. Ten days after valganciclovir administration, liver-injured mice with high aminotransferase levels (exceeding 600 U/L) in plasma received human hepatocyte transplantation. Humanized-liver mice were generated by a single injection of cryopreserved primary human hepatocytes (0.80×10^6 cells/mouse) into the spleen of NOG-TKm30 mice. Eleven weeks after the transplantation, the degree of human hepatocyte chimerism in the humanized liver (replacement index) was estimated using plasma human albumin levels measured with a human ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX). Fifteen of 48 NOG-TKm30 mice engrafted human hepatocytes exhibited a replacement index >90%. Typical lobular structure, a portal triad comprising the portal vein, bile duct, and hepatic artery, was reportedly conserved within liver of humanized-liver mice with a high humanization level >70% (Uehara et al., 2022a). In addition, the distribution pattern of major human P450 proteins in the liver of humanized-liver mice was reportedly similar to that of the mature human liver (Uehara et al., 2022a). All humanized-liver mice used in this study exhibited high replacement index values (>90%). All experimental animals reported in this study were housed in a pathogen-free environment (ambient temperature, 24°C; humidity, 55%) under a 12-hour light/dark cycle with sterilized water and CLEA Rodent Diet CA-1 (CLEA Japan, Tokyo, Japan) provided ad libitum. Hu-Liver cells were isolated from humanized-liver mice using a previously described method (Uehara et al., 2019). High purity (>90%) Hu-Liver cells were confirmed by fluorescence-activated cell sorting. Moreover, the viability of Hu-Liver cells was >79%, as determined using the trypan blue exclusion method. Control NOG-TKm30 mouse hepatocytes were also prepared for comparison with Hu-Liver cells in in vitro metabolic assays. Liver microsomes from humanized-liver mice (four males, 20 weeks old) and control NOG-TKm30 mice (four females, 20 weeks old) were prepared as previously described (Uehara et al., 2021a). Protein concentrations in the microsomal fractions were determined using the Bradford assay kit (Takara Bio, Shiga, Japan). All experimental procedures were conducted in accordance with the guidelines provided by the Central Institute for Experimental Animals (CIEA) and were approved by the Animal Care and Use Committee of the CIEA (20060 A).

In Vitro Drug-Metabolizing Enzyme Assay Using Liver Microsomal Fractions. For glucuronidation, olanzapine (0.017–3.0 mM) was incubated at 37°C for 20 minutes with liver microsomes (0.50 mg/ml) in 0.25 ml volume containing 100 mM potassium phosphate buffer (pH 7.4), alamethicin (100 μg/mg protein), and magnesium chloride (5 mM) in the presence of uridine 5′-diphosphoglucuronic acid (5 mM). Prior to enzyme reaction, the reaction mixture containing liver microsomes, potassium phosphate buffer, and alamethicin were placed on ice for 30 minutes to achieve pore formation in the microsomal vesicles by alamethicin. For oxidation, olanzapine (0.5 or 2.5 mM) was incubated at 37°C for 20 minutes with liver microsomes (0.5 mg/ml) in 0.25 ml volume containing 100 mM potassium phosphate buffer (pH 7.4) in the presence of an NADPH regenerating system (0.25 mM NADP$^+$, 2.5 mM glucose 6-phosphate, and 0.25 U/ml glucose 6-phosphate dehydrogenase, respectively). After preincubating for 3 minutes in a water bath at 37°C, enzymatic reactions were initiated by adding the NADPH-regenerating system or uridine 5′-diphosphoglucuronic acid. The enzymatic reactions were terminated by addition of an equal volume of acetonitrile. The incubation samples were vortex-mixed and centrifuged (20,000 × g, 10 minutes, 4°C), and the supernatant was collected for high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. Linearity for formation of olanzapine N10-glucuronide was determined over a protein concentration (0.2–0.6 mg/ml) and time (5–20 minutes) range at 0.5 mM substrate using liver microsomes from humanized-liver mice. Substrate concentrations for enzyme assay using liver microsomal fraction were selected according to kinetic parameters for olanzapine N10-glucuronidation by human liver microsomes (0.5 mM, substrate concentration around K_m; 2.5 mM, substrate concentration at V_{max}) (Erickson-Ridout et al., 2011).

In Vitro Drug Metabolism Assay Using Hepatocytes. Olanzapine (0.10 mM) was incubated at 37°C for 120 minutes with Hu-Liver cells (0.5×10^5 cells/ml) in 0.2 ml containing Williams' Media E (Gibco, Grand Island, NY) at 37°C and 5% CO_2. After preincubation (for 3 minutes) in a shaking water bath at 37°C, the catalytic reactions were initiated by adding the substrate. At designated time points (0, 10, 20, 40, 60, 90, and 120 minute), 20 μl aliquots were

removed from the samples and immediately mixed with 20 μl acetonitrile to precipitate proteins. The analyte samples were centrifuged (20,000 × g, 10 minutes, 4°C), and the supernatant was collected for LC-MS/MS analysis.

Measurement of mRNA Expression. Human P450 and UGT mRNA levels were analyzed in the livers of humanized-liver mice by performing real-time reverse-transcription polymerase chain reaction (real-time RT-PCR), as reported previously (Uehara et al., 2022c). Briefly, total RNA was extracted from the livers of humanized-liver mice using an RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). PCR amplification was performed in

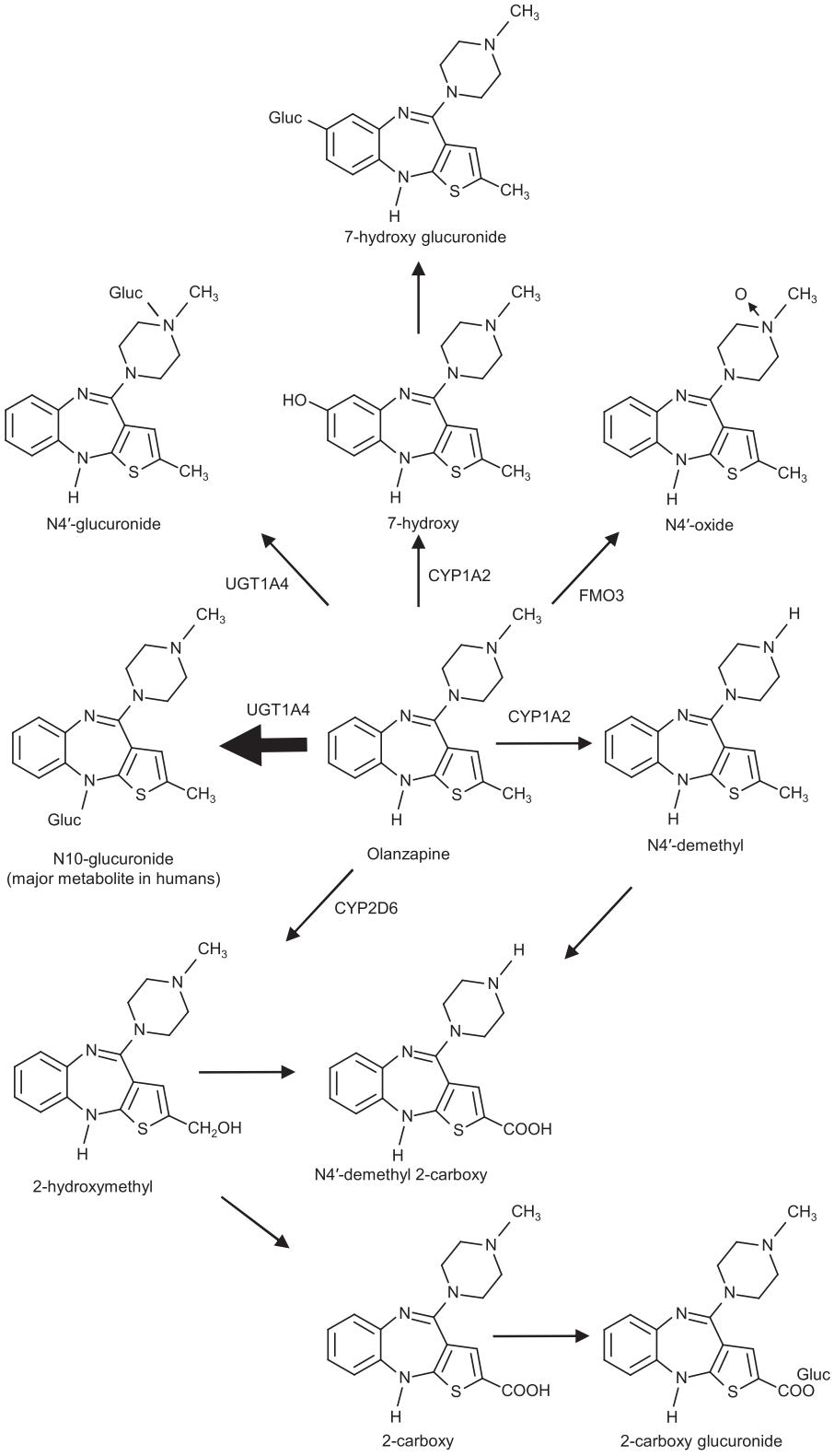

Fig. 1. Olanzapine metabolic pathways in humans. This figure was drawn based on the data of Kassahun et al. (1997), with slight modifications and with permission from ASPET.

a total volume of 10 μl with a TaqMan probe with FAM-labeled minor groove binder (Thermo Fisher Scientific) and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative expression levels were determined by normalizing the raw data to that of the human glyceraldehyde-3-phosphate dehydrogenase endogenous control (Hs99999905_m1, VIC/MGB probe, Thermo Fisher Scientific).

Olanzapine Administration. Olanzapine suspended in corn oil containing 10% (v/v) DMSO (1 mg/ml) was orally administered to humanized-liver mice (four males, 20 weeks old, donor B) and control NOG-TKm30 mice (four males, 16 weeks old) at a single dose of 10 mg/kg body weight. Blood (approximately 15 μl at each time point) was collected from the orbital vein of the mice using capillary tubes under anesthesia with isoflurane inhalation at 0.25, 0.5, 1, 2, 4, 7, 24, and 48 hours after oral administration. Heparin sodium was used as the anticoagulant. Plasma was harvested from whole blood by centrifugation at 3,500 × *g* for 20 minutes at 4°C. The obtained plasma, urine, and fecal samples were stored at approximately –70°C until further analysis. Urine and fecal samples were collected during the post-dose period of 0–48 hours. Fecal samples were homogenized with 10 volumes of acetonitrile/water (1:1) and centrifuged at 20,000 × *g* for 10 minutes; the supernatants were collected and stored until further analysis.

Quantitative Analysis of Olanzapine and Its Metabolites. The concentrations of olanzapine and its metabolites in the plasma, urine, and fecal samples of control NOG-TKm30 and humanized-liver mouse blood were determined by LC-MS/MS. Analyte samples (5.0 μl each) from the plasma, urine, and fecal solutions were mixed with three volumes of acetonitrile containing 8-CPT and centrifugated at 20,000 × *g* for 10 minutes at 4°C. Quantitative measurements were performed using an ultra-high-performance liquid chromatography system (Shimadzu Nexera, Shimadzu, Kyoto, Japan), an analytical column (YMC-Triart C18, 100 × 3.0 mm i.d., particle size 3 mm, YMC, Kyoto, Japan), and a mass spectrometer (AB SCIEX QTRAP 5500, AB Sciex, Darmstadt, Germany). Mobile phase A was composed of 10 mM ammonium acetate (pH6.0), and mobile phase B was composed of methanol; both were delivered at 0.3 ml/min. The gradient conditions were as follows:0–12 minute, 10% B; 12–15 minute, 90% B; 15–18 minutes, 10% B. The analytical column was maintained at an ambient temperature of approximately 40°C and the sample chamber of the autosampler (Shimadzu) was maintained at 4°C. Mass spectrometry was conducted using electrospray ionization in positive ion mode. Quantitative multiple reaction monitoring analysis of the parent compound and its metabolites was performed using the following transitions: *m/z* 313 to 256 (olanzapine), *m/z* 489 to 313 (olanzapine N10-glucuronide), *m/z* 299 to 256 (N4′-demethylolanzapine), m/z 329 to 213 (olanzapine N4′-oxide), *m/z* 329 to 272 (2-hydroxymethylolanzapine), m/z 489 to 282 (olanzapine N4′-glucuronide), *m/z* 329 to 286 (N4′-demethyl 2-carboxy olanzapine), m/z 343 to 312 (2-carboxyolanzapine), m/z 519 to 286 (2-carboxyolanzapine glucuronide), m/z 329 to 272 (7-hydroxyolanzapine), m/z 505 to 329 (7-hydroxyolanzapine glucuronide), and m/z 249 to 192 (internal standard 8-CPT). The plasma concentrations of olanzapine N4′-glucuronide, N4′-demethyl 2-carboxy olanzapine, 2-carboxyolanzapine, 2-carboxyolanzapine glucuronide, 7-hydroxyolanzapine, and 7-hydroxyolanzapine glucuronide were expressed as peak responses as authentic reference substances were unavailable. Olanzapine N10-glucuronide was detected as an isomer, as described previously (Franklin, 1998) (Supplemental Fig. 1). The lower limit of quantitation was 0.20 ng/ml for olanzapine, 1.0 ng/ml for olanzapine N10-glucuronide isomer 1, 2.0 ng/ml for olanzapine N10-glucuronide isomer 2, 0.20 ng/ml for N4′-demethylolanzapine, 0.40 ng/ml for olanzapine N4′-oxide, and 0.20 ng/ml for 2-hydroxymethylolanzapine. The calibration curves were linear in the range of 0.2–200 ng/ml ($r > 0.992$) for olanzapine, 1.0–2000 ng/ml ($r > 0.996$) for olanzapine N10-glucuronide isomer 1, 2.0–2000 ng/ml ($r > 0.996$) for olanzapine N10-glucuronide isomer 2, 0.20–1000 ng/ml ($r > 0.995$) for N4′-demethylolanzapine, 0.40–200 ng/ml ($r > 0.991$) for olanzapine N4′-oxide, and 0.20–200 ng/ml ($r > 0.992$) for 2-hydroxymethylolanzapine. Quantification of other olanzapine metabolites were determined as values from specific multiple reaction monitoring transition with signal peaks over 4000 counts.

Data Analyses. Non-compartmental pharmacokinetics were calculated using the PKPlus module in GastroPlus software Version 2.5 (Simulations Plus, Inc., Lancaster, CA). The area under the curve (AUC) from 0 to t h (AUC_{0-t}) was estimated using the linear trapezoidal rule, and the ratio of the AUC was calculated as the ratio of the olanzapine metabolite AUC value to that of olanzapine. Pharmacokinetic parameters are reported as the mean of four animals per group. Statistical analyses of the urinary and fecal excretion levels of olanzapine and its metabolites and pharmacokinetic parameters were conducted using GraphPad Prism (GraphPad Prism Software, La Jolla, CA) with an unpaired *t* test with or without Welch's corrections. Kinetic parameters for in vitro olanzapine N10-glucuronidation were estimated using KaleidaGraph (Synergy Software, Reading, PA).

Results

In Vitro Metabolism of Olanzapine in Liver Microsomes and Hepatocytes from NOG-TKm30 mice, Humanized-Liver Mice, and Humans. Olanzapine is extensively metabolized via phase I and phase II reactions (Fig. 1), and olanzapine N10-glucuronide is the major

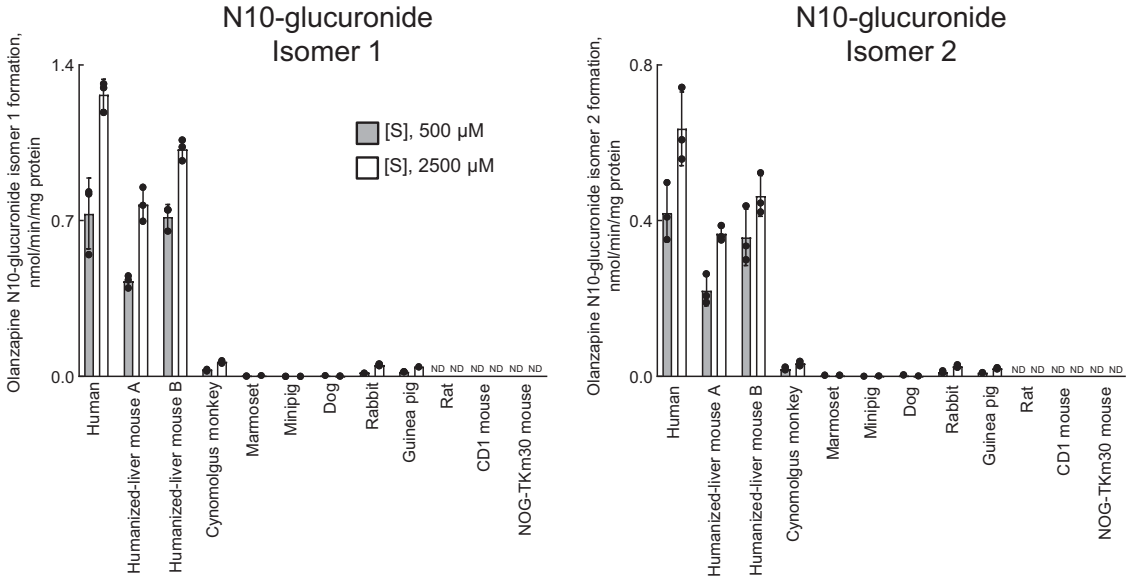

Fig. 2. Olanzapine N10-glucuronidation activity of liver microsomes from humans, humanized-liver mice, cynomolgus monkeys, marmosets, minipigs, dogs, rabbits, guinea pigs, rats, CD1 mice, and NOG-TKm30 mice. Olanzapine (500 μM and 2500 μM) was incubated with pooled liver microsomes (0.50 mg/ml) at 37°C for 20 min. Data represent the mean ± S.D. of triplicate measurements from a single experiment. Olanzapine N10-glucuronidation activity in liver microsomes from rats, CD1 mice, and NOG-TKm30 mice was below the detection limit (0.00041 and 0.00082 nmol/min/mg protein for isomer 1 and 2, respectively).

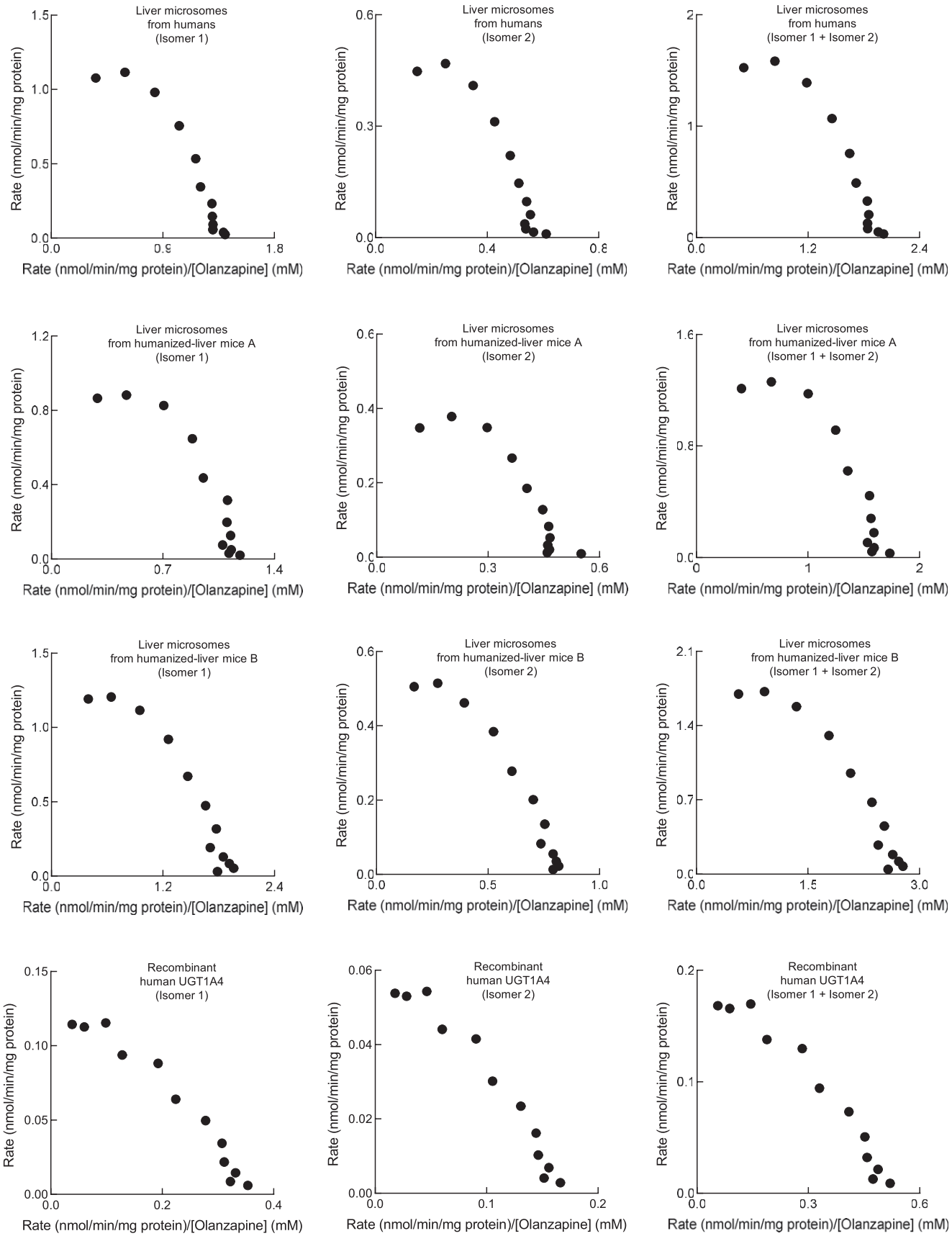

Fig. 3. Eadie-Hofstee plots (v versus $V/[S]$) for olanzapine N10-glucuronidation by liver microsomes and recombinant UGT1A4 enzyme. Olanzapine (0.017–3.0 mM) was incubated with pooled liver microsomes (0.50 mg/ml) from humans, humanized-liver mice, and NOG-TKm30 mice or recombinant human UGT1A4 (0.50 mg/ml) at 37°C for 20 min. Data represent the mean of duplicate determinations. Olanzapine N10-glucuronidation activity in liver microsomes from NOG-TKm30 mice was below the detection limit (0.00041 and 0.00082 nmol/min/mg protein for isomer 1 and 2, respectively).

TABLE 1

Kinetic parameters of olanzapine N10-glucuronidation by liver microsomes and recombinant human UGT1A4

Enzyme source	Isomer	K_m	V_{max}	K_{si}	V_{max}/K_m
Liver microsomes		*mM*	*nmol/min/mg protein*	*mM*	*μl/min/mg protein*
Human	Isomer 1	1.4 ± 0.3	2.3 ± 0.4	4.5 ± 2.0	1.6
	Isomer 2	2.2 ± 0.9	1.3 ± 0.4	2.5 ± 1.4	0.59
	Isomer 1 + 2	2.7 ± 0.5	5.4 ± 0.9	1.8 ± 0.5	2.0
Humanized-liver mouse A	Isomer 1	2.0 ± 1.0	2.5 ± 1.0	2.1 ± 1.4	1.3
	Isomer 2	2.4 ± 1.1	1.2 ± 0.5	1.5 ± 0.9	0.50
	Isomer 1 + 2	1.2 ± 0.3	2.6 ± 0.5	4.6 ± 2.3	2.2
Humanized-liver mouse B	Isomer 1	1.5 ± 0.1	2.9 ± 0.2	3.0 ± 0.4	1.9
	Isomer 2	1.4 ± 0.1	1.2 ± 0.1	3.5 ± 0.3	0.86
	Isomer 1 + 2	0.89 ± 0.14	2.9 ± 0.3	7.3 ± 2.6	3.3
NOG-TKm30 mouse	Isomer 1	NA	NA	NA	NA
	Isomer 2	NA	NA	NA	NA
	Isomer 1 + 2	NA	NA	NA	NA
Recombinant UGT enzyme					
UGT1A4	Isomer 1	0.42 ± 0.06	0.16 ± 0.01	9.6 ± 3.5	0.38
	Isomer 2	0.42 ± 0.06	0.077 ± 0.006	9.6 ± 3.5	0.18
	Isomer 1 + 2	0.43 ± 0.06	0.24 ± 0.01	9.4 ± 3.4	0.56

Kinetic parameters were determined by non-linear regression analysis (mean ± standard error, $n = 12$ substrate concentrations of 0.017–3.0 mM) employing the equation for substrate inhibition, $v = V_{max} \times [S]/(K_m + [S] + [S]^2/K_{si})$. Data represent the mean of duplicate determinations.

metabolic product found in human plasma and urine (Kassahun et al., 1997). To investigate the differences in olanzapine metabolism, we examined the activities of N10-glucuronidation, N4′-demethylation, N4′-oxygenation, and 2-hydroxylation of olanzapine in liver microsomes from humanized-liver mice, humans, cynomolgus monkeys, marmosets, minipigs, dogs, rabbits, guinea pigs, rats, and mice. N10-glucuronide showed two distinct peaks under optimal HPLC conditions (Supplemental Fig. 1), suggesting the presence of two 10-N-glucuronide isomers. The two peaks of 10-N-glucuronide at 8.5 and 8.9 minutes were designated isomer 1 and 2, respectively. Kassahun et al. reported that although the type of isomeric relationship of the conjugates was unclear, the two 10-N-glucuronide isomers do not appear to be regioisomers (Kassahun et al., 1998). N4′-demethylation, N4′-oxygenation, and 2-hydroxylation of olanzapine in the liver microsomes were observed in all species (Supplemental Fig. 2). In contrast, the large species differences in N10-glucuronide isomer formation activities in liver microsomes were as follows: elevated levels in humans and humanized-liver mice; low levels in cynomolgus monkeys, rabbits, and guinea pigs; negligible amounts in marmosets, minipigs, and dogs; and below the detection limit in rats, CD1 mice, and NOG-TKm30 mice (Fig. 2). Notably, liver microsomes from humanized-liver mice and humans displayed substantial N10-glucuronidation at a low substrate concentration (500 μM; Fig. 2). Eadie–Hofstee plots were constructed to identify the appropriate kinetic model and estimate the kinetic parameters as previously described (Miners et al., 2021). The most appropriate empirical model was selected based on the coefficient of determination (R^2) magnitude and the χ^2 test. Kinetic data for olanzapine N10-glucuronidation with liver microsomes from humans and humanized-liver mice were best described by the substrate inhibition model (Fig. 3, Supplemental Fig. 3). Human and humanized-liver mouse liver microsomes had similar K_m values (1.4 and 1.5–2.0 mM for isomer 1 and 2.2 and 1.4–2.4 mM for isomer 2, respectively) and high V_{max} values (2.3 and 2.5–2.9 nmol/min/mg protein for isomer 1 and 1.3 and 1.2 nmol/min/mg protein for isomer 2) for olanzapine N10-glucuronidation (Table 1 and Supplemental Fig. 3). Moreover, human and humanized-liver mouse liver microsomes efficiently catalyzed olanzapine N10-glucuronidation with similar V_{max}/K_m values (1.6 and 1.3–1.9 μl/

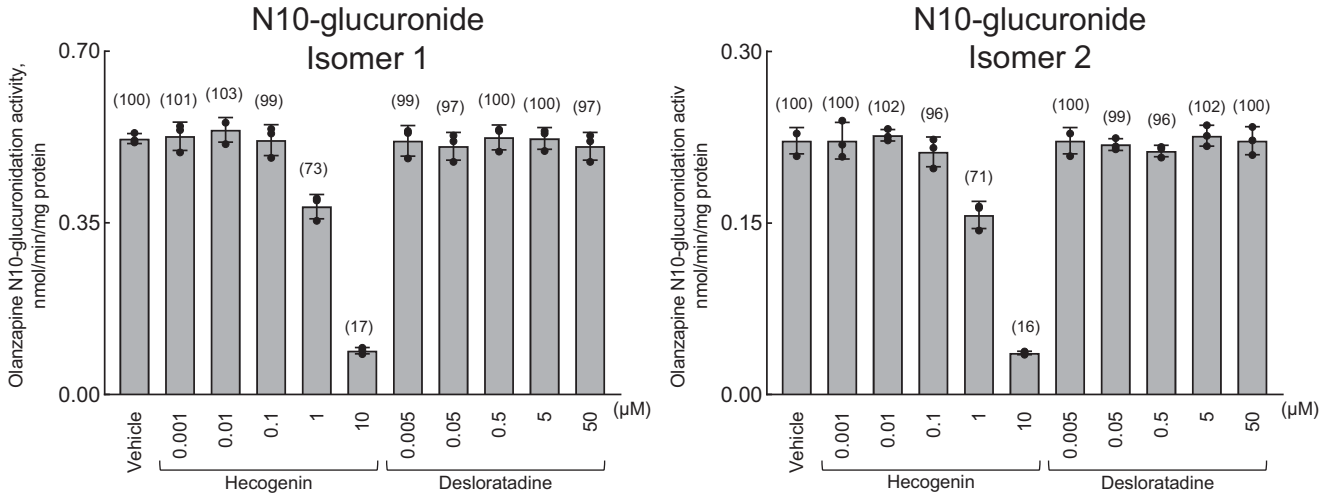

Fig. 4. Effects of UGT inhibitors on olanzapine N10-glucuronidation in liver microsomes. Olanzapine (500 mM) was incubated with pooled liver microsomes (0.50 mg/ml) from humans, humanized-liver mice, and NOG-TKm30 mice at 37°C for 20 min in the presence of hecogenin (an inhibitor of UGT1A4) and desloratadine (an inhibitor of UGT2B10). Data represent the mean of triplicate measurements. The numbers in parentheses are the percentages of the control (vehicle). Data represent the mean ± S.D. of triplicate measurements from a single experiment.

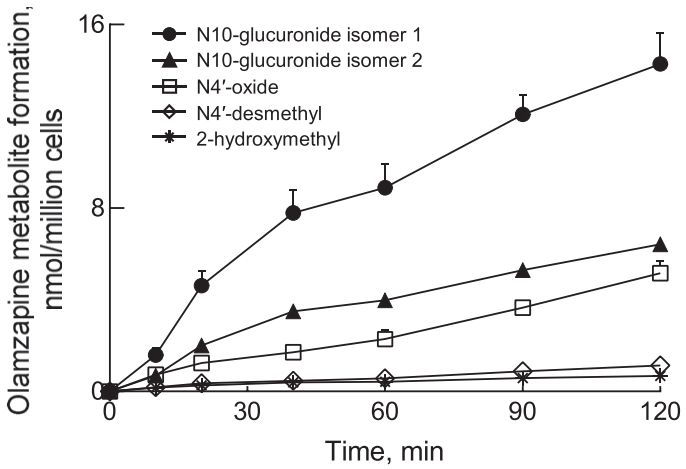

Fig. 5. Formation of olanzapine metabolites in Hu-Liver cells. Cryopreserved Hu-Liver cells (three lots from donor B) were incubated with 100 μM olanzapine at 37°C for 120 min at 5.0 × 10^5 cells/ml. Olanzapine metabolites in media collected at 0, 10, 20, 40, 60, 90, and 120 min were measured using LC-MS/MS. Data represent the mean ± S.D.

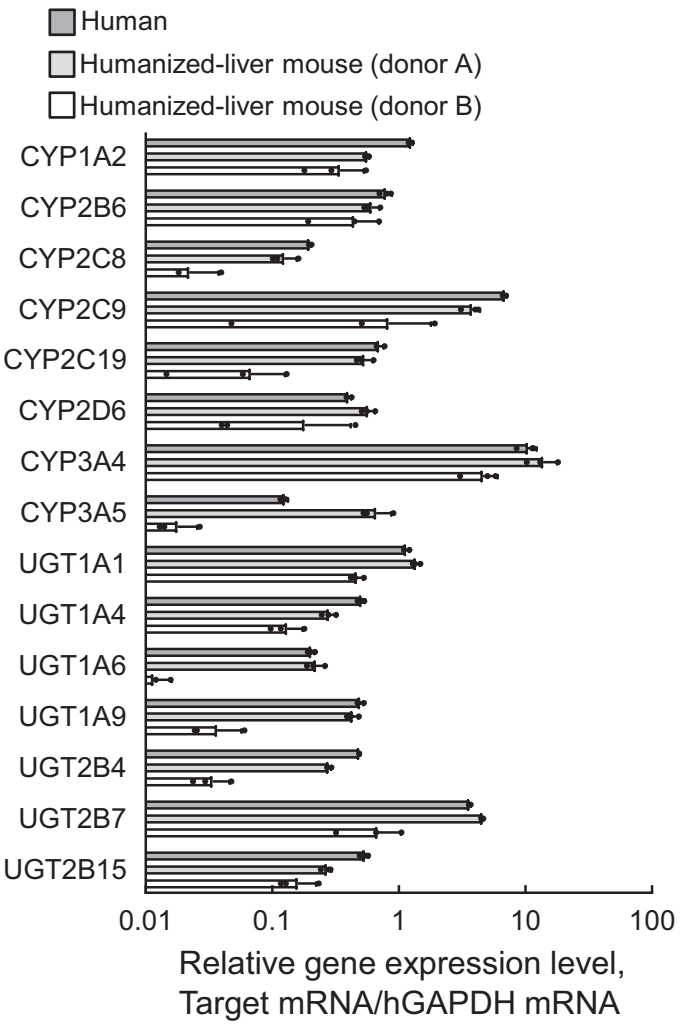

Fig. 6. Expression levels of UGT and P450 genes in the liver of humans and humanized-liver mice. The mRNA expression levels of target genes in the livers of humanized mice and humans were measured using real-time reverse transcription-polymerase chain reaction. Raw data were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase mRNA. Data represent the mean ± S.D. of three humanized-liver mice. Human (pool of 50) expression levels of *UGT* and *P450* genes in the liver of humans were obtained from Uehara et al. (2022a).

min/mg protein for isomer 1 and 0.59 and 0.50–0.86 μl/min/mg protein for isomer 2, respectively; Table 1). In addition, olanzapine N10-glucuronidation in humanized-liver microsomes was inhibited (~16% and 17% of control) by hecogenin, a human UGT1A4 inhibitor (Fig. 4). In contrast, this activity in humanized-liver mouse microsomes was 96~102% of control activity in the presence of desloratadine, a human UGT2B10 inhibitor (Fig. 4), indicating that UGT2B10, another enzyme known to primarily glucuronidate tertiary amines, excludes involvement of UGT2B10 in olanzapine glucuronidation in humanized-livers. The olanzapine metabolism assay using Hu-Liver cells revealed that olanzapine N10-glucuronide isomers were produced at a faster rate in Hu-Liver cells than other metabolites (Fig. 5). Moreover, the human P450 and UGT mRNA levels in the livers of humanized-liver mice were comparable to those in the human liver (Fig. 6). These results suggest that olanzapine metabolism in the liver of humanized-liver mice is similar to that in humans, in terms of high UGT1A4-mediated olanzapine N10-glucuronidation activity.

In Vivo Metabolism of Olanzapine in NOG-TKm30 Mice and Humanized-Liver Mice. Olanzapine was orally administered to NOG-TKm30 mice and humanized-liver mice, and olanzapine metabolites in the plasma, urine, and fecal samples were analyzed using LC-MS/MS (Figs. 7, 8, and 9). Urine samples from humanized-liver mice and control NOG-TKm30 mice were analyzed by mass detection of m/z 313.0, 489.0, 299.0, 329.0, 343.0, 519.0, and 505.0 for the unchanged drug, olanzapine N10-glucuronide/olanzapine N4′-glucuronide, N4′-demethylolanzapine, olanzapine N4′-oxide/2-hydroxymethylolanzapine/N4′-demethyl 2-carboxy olanzapine/7-hydroxyolanzapine, 2-carboxyolanzapine, 2-carboxyolanzapine glucuronide, and 7-hydroxyolanzapine glucuronide, respectively. Twelve peaks with retention times of 6.7, 7.0, 7.2, 7.4, 8.5, 8.9, 9.0, 9.1, 9.6, 10.0, 10.6, and 11.8 minutes were identified in the urine samples of humanized-liver mice and/or control NOG-TKm30 mice (Fig. 7). When the ions at m/z 505.0 (6.7 minutes), 329.0 (7.0 minutes), 343.0 (7.2 minutes), 519.0 (7.4 minutes), 329.0 (9.1 minutes), and 489.0 (9.6 minutes) were subjected to collision-induced dissociation, product ions were obtained at m/z 329, m/z 243/286, m/z 243/286/312, m/z 286/343, m/z 272/298, and m/z 256/282/313, respectively (Supplemental Fig. 4). We inferred that the MS peaks detected with m/z 505.0 at 6.7 minutes, m/z 329.0 at 7.0 minutes, m/z 343.0 at 7.2 minutes, m/z 519.0 at 7.4 minutes, m/z 329.0 at 9.1, and m/z 489.0 at 9.6 minutes corresponded to 7-hydroxyolanzapine glucuronide, N4′-demethyl 2-carboxy olanzapine, 2-carboxyolanzapine, 2-carboxyolanzapine glucuronide, 7-hydroxyolanzapine, and olanzapine N4′-glucuronide, respectively (Fig. 7).

The plasma concentrations of olanzapine in samples obtained from humanized-liver mice were higher than those obtained from NOG-TKm30 mice 4–48 hours after oral administration (Fig. 9). C_{max} (80 ng/ml) and $AUC_{0\text{-inf}}$ (1300 ng·h/ml) in humanized-liver mice were higher than those in control NOG-TKm30 mice (C_{max}, 67 ng/ml and $AUC_{0\text{-inf}}$, 360 ng·h/ml). The plasma concentrations, C_{max}, and $AUC_{0\text{-last}}$ of each metabolite in the control NOG-TKm30 and humanized-liver mice are shown in Fig. 9 and Table 2, respectively. Additionally, the plasma concentrations of N10-glucuronide isomers and olanzapine N4′-glucuronide were observed in humanized-liver mice, but were not in control NOG-TKm30 mice (Fig. 9). Conversely, N4′-demethylolanzapine and 2-hydroxymethylolanzapine levels were higher in control NOG-TKm30 mice than in humanized-liver mice. The $AUC_{0\text{-last}}$ values for olanzapine N10-glucuronide (3700 and 1700 ng·h/ml for isomers 1 and 2, respectively), olanzapine N4′-glucuronide, and 2-carboxyolanzapine glucuronide in humanized-liver mice were higher than those in control NOG-

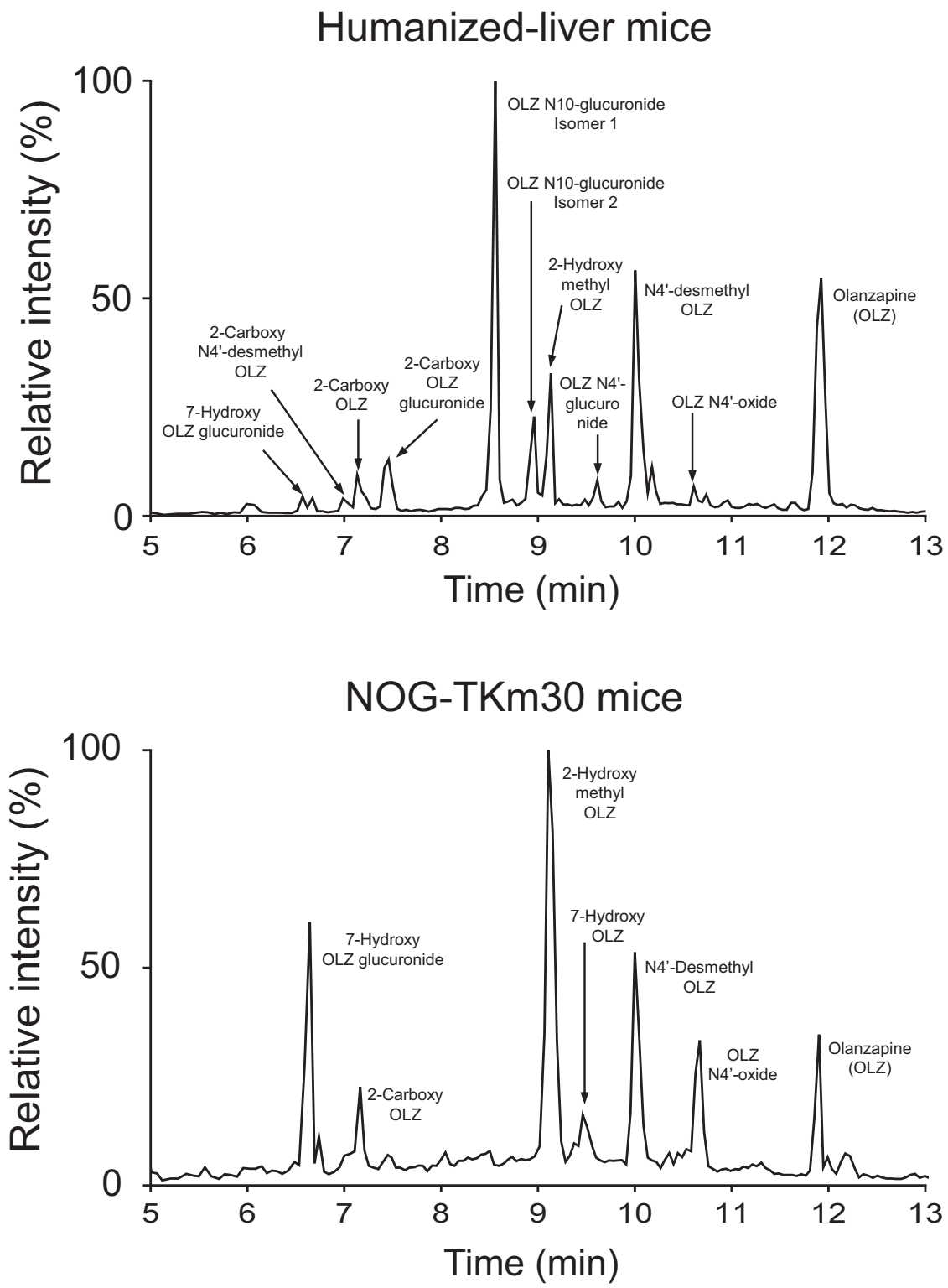

Fig. 7. Chromatographic profile of olanzapine and its metabolites in urine after oral administration of olanzapine (10 mg/kg) to humanized-liver mice and NOG-TKm30 mice. Urine samples were analyzed by mass detection of m/z 313.0 (olanzapine), 489.0 (N10-glucuronide, N4′-glucuronide), 299.0 (N4′-demethyl), 329.0 (N4′-oxide, 2-hydroxymethyl, N4′-demethyl-2-carboxy), 343.0 (2-carboxy), and 519.0 (2-carboxy glucuronide).

TKm30 mice (Table 2). In contrast, the $AUC_{0\text{-last}}$ values of N4′-demethylolanzapine (420 ng·h/ml) and 2-hydroxymethylolanzapine (120 ng·h/ml) in control NOG-TKm30 mice were lower than those in humanized-liver mice (180 and 66 ng·h/ml, respectively; Table 2). Olanzapine, olanzapine N10-glucuronide, N4′-demethylolanzapine, olanzapine N4′-oxide, and 2-hydroxymethylolanzapine in the urine of humanized-liver mice after the administration of olanzapine were 1.3–3.0%, 15.0–21.0%, 0.7–1.2%, 0.3–0.7%, and 1.1–2.2% of the administered dose, respectively (Fig. 8),

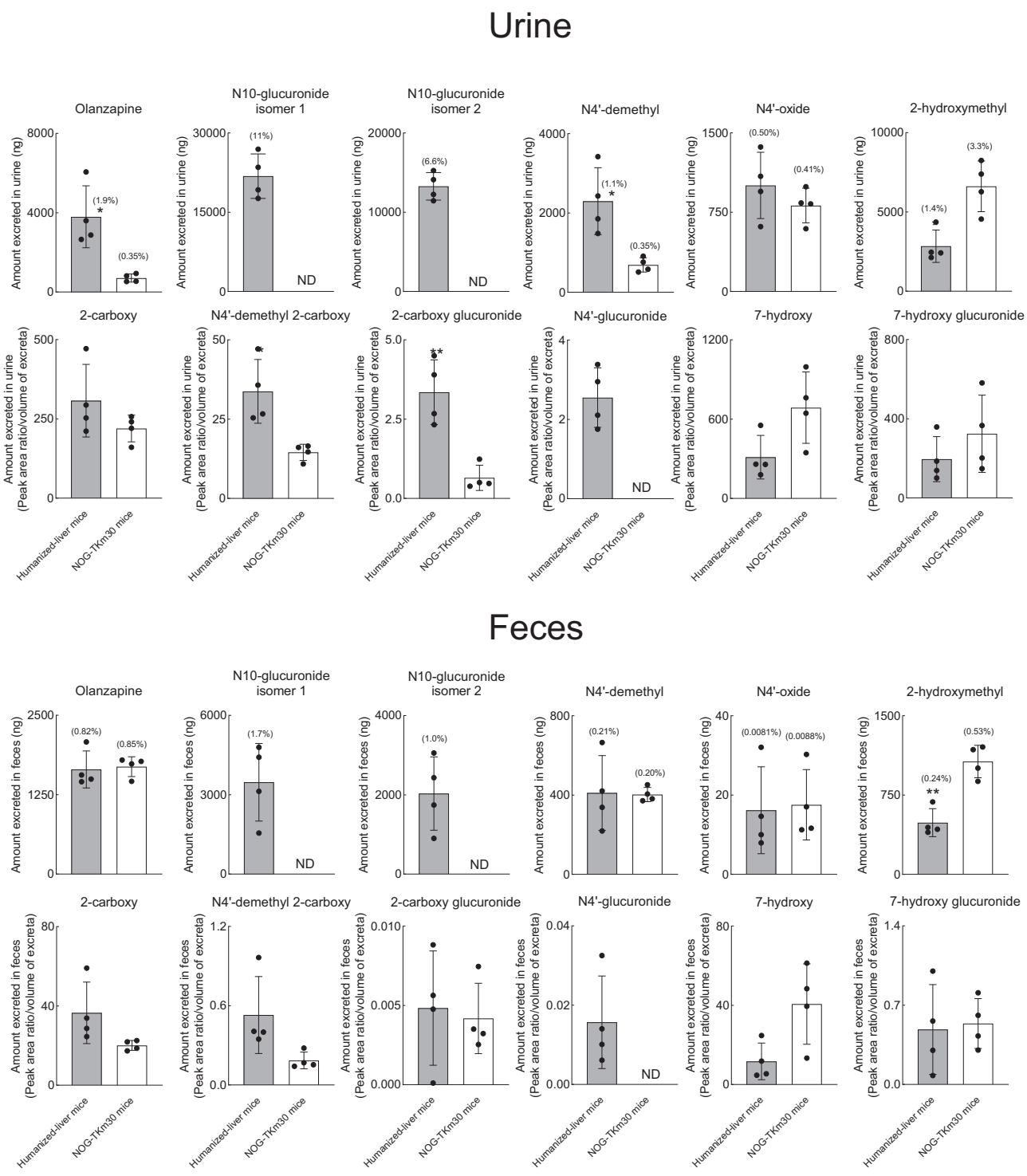

Fig. 8. Cumulative amounts of olanzapine and its metabolites excreted in the urine and feces of humanized-liver mice and NOG-TKm30 mice after oral administration of olanzapine (10 mg/kg). The amounts of olanzapine, N10-glucuronide, N4′-oxide, N4′-demethyl, 2-hydroxymethyl, 2-carboxy, N4′-demethyl 2-carboxy, 2-carboxy glucuronide, N4′-glucuronide, 7-hydroxy, and 7-hydroxyolanzapine glucuronide excreted in the urine and feces up to 48 h after administration differed ($P < 0.05$) between humanized-liver mice (gray bar) and NOG-TKm30 mice (open bar) based on unpaired Student's t test with Welch's correction (*). Data represent the mean ± SD ($n = 4$ mice). ND, not detected. The numbers in parentheses are the percentages of olanzapine and its metabolites excreted (% of dose) in urine and feces.

similar to the results reported for humans (7.3%, 13.2%, 0.6%, 3.4%, and 1.4% of the radioactivity in 4- to 72-hour urine samples, respectively) (Kassahun et al., 1997). These results confirmed that the in vivo metabolism of olanzapine in humanized-liver mice closely reflected the efficient olanzapine N10-glucuronidation in liver microsomes and hepatocytes in vitro. These results further suggested that the humanized-liver mouse model could be useful in predicting UGT1A4-dependent drug metabolism in humans.

TABLE 2

$AUC_{0\text{-last}}$ of metabolites observed after oral administration of olanzapine (10 mg/kg) to humanized-liver mice and NOG-TKm30 mice

Metabolite	C_{max}[a]		$AUC_{0\text{-last}}$[b]		Ratio[c]
	Humanized-liver mice	NOG-TKm30 mice	Humanized-liver mice	NOG-TKm30 mice	
Olanzapine N10-glucuronide isomer 1	180 ± 70	BDL	3700 ± 800	NA	NA
Olanzapine N10-glucuronide isomer 2	100 ± 70	BDL	1700 ± 100	NA	NA
N4′-Demethylolanzapine	6.2 ± 3.9**	19 ± 2	180 ± 100*	420 ± 60	0.43
Olanzapine N4′-oxide	1.6 ± 0.8	2.6 ± 1.1	28 ± 8	21 ± 2	1.3
2-Hydroxymethylolanzapine	4.0 ± 1.9**	15 ± 4	66 ± 19**	120 ± 10	0.55
2-Carboxy olanzapine	0.49 ± 0.25	1.2 ± 0.5	7.2 ± 1.3	5.8 ± 1.3	1.2
N4′-Demethyl-2-carboxyolanzapine	0.023 ± 0.011	0.029 ± 0.012	0.50 ± 0.11*	0.27 ± 0.04	1.9
Olanzapine 2-carboxyglucuronide	0.21 ± 0.07*	0.044 ± 0.005	0.24 ± 0.03**	0.12 ± 0.01	2.0
Olanzapine N4′-glucuronide	0.0044 ± 0.0011	BDL	0.11 ± 0.01	NA	NA
7-Hydroxyolanzapine	0.16 ± 0.03	0.16 ± 0.02	0.73 ± 0.26	0.77 ± 0.26	0.95
7-Hydroxyolanzapine glucuronide	0.18 ± 0.07	0.17 ± 0.03	1.5 ± 0.4	0.85 ± 0.12	1.8

[a]Units of C_{max} for olanzapine N10-glucuronide isomers, N4′-demethylolanzapine, olanzapine N4′-oxide, and 2-hydroxymethylolanzapine were ng/ml, and the C_{max} measurements for the other metabolites were relative values calculated using peak response.
[b]Units of $AUC_{0\text{-last}}$ for olanzapine N10-glucuronide isomers, N4′-demethylolanzapine, olanzapine N4′-oxide, and 2-hydroxymethylolanzapine were ng·h/ml, and the $AUC_{0\text{-last}}$ measurements for the other metabolites were relative values calculated using peak response.
[c]Ratios of $AUC_{0\text{-last}}$ in humanized-liver mice to those in control mice.
*$P < 0.05$ and **$P < 0.01$ versus the control mice.
$AUC_{0\text{-last}}$ values represent the mean ± S.D., $n = 4$ animals.
NA, not available; BDL, below detection limit.

Discussion

Chimeric TK-NOG mice with humanized livers provide a mouse model with human hepatocytes, which make up the major portion of the liver (Hasegawa et al., 2011; Uehara et al., 2022a). This animal model contains human phase I and phase II drug-metabolizing enzymes and transporters expressed in the liver (Uehara et al., 2022c). Hence, this model reflects the metabolism and excretion of drugs in humans, with several minor differences (Kamimura et al., 2015; Uehara et al., 2021b; Uehara et al., 2021c). This study on olanzapine metabolism using chimeric mice demonstrated that the major olanzapine metabolite, olanzapine N10-glucuronide, was detected at high levels in the plasma and urine after olanzapine administration, as previously reported in humans (Kassahun et al., 1997). These data indicate that in vivo experiments involving humanized-liver mice are a valuable tool for predicting olanzapine metabolism in humans.

Although the model drug olanzapine is reportedly metabolized by inducible human CYP1A2 and variable copy-number CYP2D6 and polymorphic FMO3 in different pathways, olanzapine clearance in patients was not affected by *CYP2D6* or *FMO3* genotypes or smoking behavior as a single factor as olanzapine clearance is mediated by multiple enzymes (Okubo et al., 2016). Olanzapine is extensively metabolized by these four species (>20 metabolites), with the major metabolite in the urine being strictly species-dependent. Olanzapine N10-glucuronide is the major metabolite detected in the plasma, urine, and fecal samples of humans administered a single oral dose of olanzapine (0.14–0.18 mg/kg) (Kassahun et al., 1997). However, this metabolite is absent in the urine of preclinical species when a single oral dose of olanzapine (5–15 mg/kg) is administered, with the exception of a trace amount in dog urine (Mattiuz et al., 1997). Moreover, olanzapine N4′-glucuronide has been detected only in humans (Kassahun et al., 1997; Mattiuz et al., 1997). In contrast, oxidative metabolites have been detected in the plasma and urine of humans, rhesus monkeys, dogs, and mice. In this study, two human olanzapine N-glucuronides were detected in the plasma, urine, and feces of humanized-liver mice (Figs. 8 and 9). Multiple oxidative metabolites, including N4′-demethylolanzapine, olanzapine N4′-oxide, 2-hydroxymethylolanzapine, and their derivatives, were also found in the plasma, urine, and/or feces of humanized-liver mice (Figs. 8 and 9). Furthermore, extensive olanzapine metabolic pathways (2-calboxylation and 7-hydroxylation) identified in humans were detected in humanized-liver mice. The in vivo urinary excretion ratios of metabolites associated with olanzapine metabolism in humanized-liver mice (olanzapine, 1.9%; olanzapine N10-glucuronide isomer 1, 11%; olanzapine N10-glucuronide isomer 2, 6.6%; N4′-demethylolanzapine, 1.1%; olanzapine N4′-oxide, 0.50%; 2-hydroxymethylolanzapine, 1.4%) are comparable to those in humans (olanzapine, 7.3%; olanzapine N10-glucuronide, 13.2%; N4′-demethylolanzapine, 0.6%; olanzapine N4′-oxide, 3.4%; 2-hydroxymethylolanzapine, 1.4%) (Kassahun et al., 1997). Hence, humanized-liver mice are a useful model for studying olanzapine metabolism in humans.

Human UGT1A4 exhibits some glucuronidase activity toward bilirubin, although it is more active on amines, steroids, and sapogenins. Human UGT1A4 is responsible for the formation of quaternary glucuronides from the tertiary amine substrates, such as trifluoperazine, lamotrigine, and olanzapine. Interestingly, human *UGT1A4* is functional, whereas rat and mouse *UGT1A4* are pseudogenes (Fujiwara et al., 2018). Therefore, the ability of aliphatic tertiary amines to form quaternary ammonium glucuronides (N-glucuronides) is greater in humans than in rodents (Franklin, 1998). The substrate inhibition kinetics of olanzapine N10-glucuronidation was observed in humanized-liver mouse liver microsomes and recombinant human UGT1A4 enzyme. In addition, olanzapine N10-glucuronidation activity in liver microsomes from humanized-liver mice was suppressed by hecogenin treatment. These results suggest that olanzapine N10-glucuronide is produced via human UGT1A4 expressed in the liver of humanized-liver mice. Our recent work showed that lamotrigine N2-glucuronide generated by UGT1A4 is the major metabolic pathway of lamotrigine in humanized-liver mice, similar to that reported in humans. However, lamotrigine N2-oxide was predominantly excreted in the urine of control NOG-TKm30 mice (Uehara et al., 2021a). Notably, a secondary amine N10 of olanzapine is preferentially conjugated in humanized-liver. This contrasts glucuronidation of a tertiary amine N2 of lamotrigine N2-glucuronidation. It is, therefore, to further investigate in vivo metabolism of various primary, secondary, and tertiary amines to confirm if the humanized-liver mouse is a valuable model for studying UGT1A4-dependent drug metabolism.

Conversely, studies using humanized-liver mouse models do not always accurately reflect human metabolism (Bateman et al., 2014). Indeed, the urinary excretion data for in vivo olanzapine metabolism in humanized-liver mice do not fully reflect that in humans

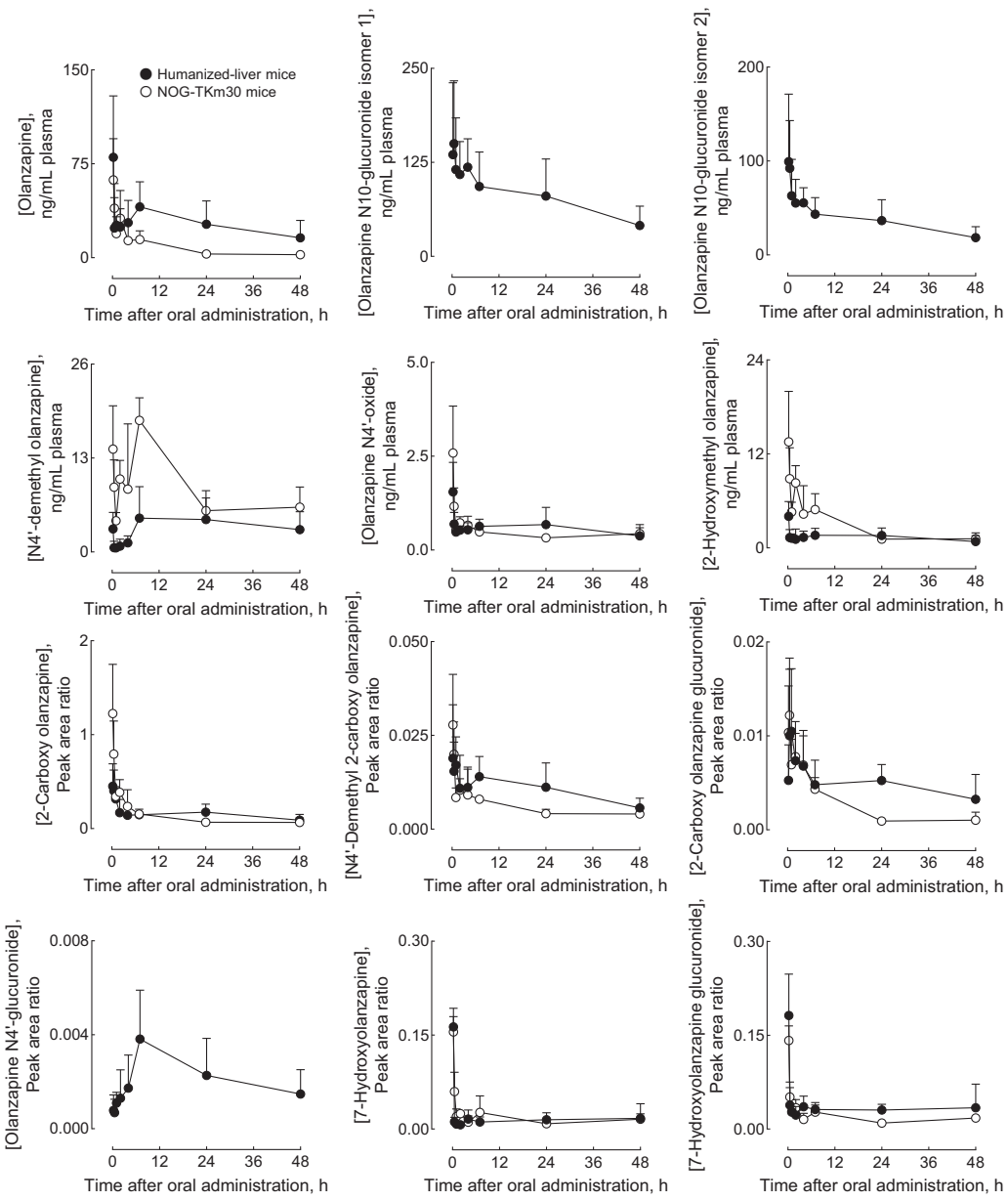

Fig. 9. Plasma concentrations of olanzapine and its metabolites in humanized-liver mice and NOG-TKm30 mice after oral administration of olanzapine (10 mg/kg). Plasma concentrations of olanzapine and its metabolites in humanized-liver mice (closed circles) and NOG-TKm30 mice (open circles) were determined using LC-MS/MS, as described in the *Materials and Methods* section. Data represent the mean ± S.D. (n = 4 mice). Olanzapine N10-glucuronide and olanzapine N4′-glucuronide in plasma from NOG-TKm30 mice was below the detection limit (olanzapine N10-glucuronide isomer 1, < 1.0 ng/mL; olanzapine N10-glucuronide isomer 2, < 2.0 ng/ml). For 2-carboxy olanzapine, N4′-demethyl-2-carboxy olanzapine, olanzapine 2-carboxy glucuronide, olanzapine N4′-glucuronide, 7-hydroxy olanzapine, and 7-hydroxyolanzapine glucuronide, no standard curves were used and that therefore the data are semi-quantitative.

(Kassahun et al., 1997), although the rank order of metabolite excretion is comparable. This may be due to the drug-metabolizing activity of residual mouse hepatocytes (Uehara et al., 2022c) and/or extrahepatic mouse tissues interfering with the metabolic profile of drugs (Uehara et al., 2022b). The principal urinary metabolites in mice are 7-hydroxyolanzapine glucuronide (10% of the dose), 2-hydroxymethylolanzapine (4% of the dose), and 2-carboxyolanzapine (2% of the dose) (Mattiuz et al., 1997). Although the in vitro generation of 7-hydroxyolanzapine by human liver microsomes has been reported (Ring et al., 1996; Korprasertthaworn et al., 2015), 7-hydroxyolanzapine and its glucuronide were not detected in the plasma, urine, or feces of humans in a previous in vivo study (Kassahun et al., 1997), likely due to the minor in vivo contribution of clearance. In contrast, 7-hydroxyolanzapine and its glucuronide were detected in the plasma, urine, and feces of humanized-liver mice and control NOG-TKm30 mice (Figs. 8 and 9). The early rapid decline of the 7-hydroxyolanzapine glucuronide in humanized-liver mice and NOG-TKm30 mice may be due to distribution in the tissue compartment. To test this hypothesis, it is necessary to analyze the pharmacokinetics and pharmacodynamics following administration of 7-hydroxyolanzapine glucuronide to these animals. Therefore, humanized-liver mice are a suitable animal model for analyzing drug metabolism in humans; however, enzyme activity in residual mouse hepatocytes and/or extrahepatic organs must be considered.

Unique human olanzapine N10-glucuronide was detected at elevated levels in hepatocytes from humanized-liver mice (Hu-Liver cells). Phase I and phase II drug-metabolizing activities in Hu-Liver cells are comparable to, or higher than, those in primary human hepatocytes (Bachour-El Azzi et al., 2022). The in vitro metabolism of lamotrigine and tolbutamide in Hu-Liver cells reflects the human metabolism (Uehara et al., 2021a; Uehara et al., 2021b). To evaluate the toxicological potential of the major metabolites of new drug candidates in humans, animals that generate the same major metabolites as humans must be selected, and the comparison of exposure and toxicity between these animal species and humans must be performed according to the introduction of metabolites in safety testing (MIST) guidance by the Food and Drug Administration (US Food and Drug Administration, 2016). Humanized-liver TK-NOG mice can be used repeatedly for in vivo drug metabolism experiments as the humanized liver (replacement index of >70%) can be stably maintained for >2 months without exogenous drug treatments (Uehara et al., 2022a). However, generation of humanized mice requires considerable time. Nevertheless, in vivo and in vitro metabolic profiling and toxicity studies using humanized-liver mice could provide valuable findings during the initial stages of drug research and development.

In conclusion, the findings of this study demonstrate that a unique human metabolite, olanzapine N10-glucuronide, is generated in humanized-liver mice. Moreover, the human UGT1A4 protein expressed in the transplanted liver of humanized-liver mice is functional in vivo. The critical differences in the metabolism of olanzapine in humanized-liver mice and non-transplanted NOG-TKm30 mice reflect the in vivo interspecies phenotypic differences in N-glucuronidation between humans and mice, suggesting that the humanized-liver mouse model is useful for studying human UGT1A4-dependent olanzapine metabolism in humans.

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Authorship Contributions

Participated in research design: Uehara, Suemizu.
Conducted experiments: Uehara, Higuchi, Yoneda, Kato.
Contributed new reagents or analytic tools: Higuchi, Yoneda, Kato.
Performed data analysis: Uehara, Higuchi.
Wrote or contributed to writing the manuscript: Uehara, Yamazaki, Suemizu.

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