Inter-individual Variations in Metabolism and Pharmacokinetics of 
3-(6-methylpyridine-3-yl-sulfanyl)-6-(4H-[1,2,4]triazole-3-yl-sulfanyl)-
N-(1,3-thiazole-2-yl)-2-pyridine carboxamide,
a Glucokinase Activator, in Rats Caused by the Genetic Polymorphism
of CYP2D1

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Running title: ROLE OF CYP2D1 IN POLYMORPHIC PHARMACOKINETICS IN RATS

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Nonstandard Abbreviations:

AUC_{0-\infty}, area under the plasma concentration-time curve extrapolated to infinity; CL_{p}, total body (plasma) clearance; C_{max}, maximum observed concentrations after P.O. administration;

Cpd-B, (3-[(1S)-2-hydroxy-1-methylethoxy]-5-[4-(methylsulfonyl)phenoxy]-N-1,3-thiazol-2-yl) benzamide;

Cpd-D, 3-(6-methylpyridine-3-yl-sulfanyl)-6-(4H-[1,2,4]triazole-3-yl-sulfanyl)-N-(1,3-thiazole-2-yl)-
2-pyridine carboxamide; FM, fast metabolizer; GKA, glucokinase activator; HPLC, high-performance liquid chromatography; IV, intravenous; MS, mass spectrometry; PCR, polymerase chain reaction; PK, pharmacokinetics; P.O., oral; P450, cytochrome P-450; RT-PCR, reverse transcriptase polymerase chain reaction; SD, Sprague-Dawley; SM, slow metabolizer; $t_{1/2}$, terminal-phase half-life; Vd$_{ss}$, apparent volume of distribution at steady-state.
ABSTRACT

3-(6-methylpyridine-3-yl-sulfanyl)-6-(4H-[1,2,4]triazole-3-yl-sulfanyl)-N-(1,3-thiazole-2-yl)-2-pyridine carboxamide (Cpd-D) is a novel glucokinase activator that is being developed for the treatment of type 2 diabetes. Large inter-individual variations were observed in the pharmacokinetics of Cpd-D in male Sprague-Dawley (SD) rats, which were subsequently divided into two phenotypes; >6-fold longer terminal-phase half-life and ~10-fold larger \( \text{AUC}_{0-\infty} \) values were observed in slow metabolizers (SM) than in fast metabolizers (FM) after the oral administration of Cpd-D. The thiohydantoic acid analog (M2) was the predominant metabolite detected in the urine, bile, and plasma following the oral administration of \(^{14}\text{C}\)Cpd-D to the FM phenotypes of bile-duct cannulated SD rats. The liver microsomes prepared from FM phenotyped rats extensively formed M2 with the highest affinity (\( \text{K}_m = 0.09 \mu\text{M} \)) and largest \( \text{V}_{\text{max}}/\text{K}_m \) value in primary metabolism, while those from SM phenotypes had little capacity to form M2. Of the rat cytochrome P450 isoforms tested, the formation of M2 was only catalyzed by recombinant CYP2D1. Sequence substitutions (418A/421C and 418G/421T) were detected in the \text{CYP2D1} gene, and were designated F and S alleles, respectively. The genotype-phenotype correlation analysis indicated that two S alleles were homozygous (S/S) in the SM phenotypes, while the FM phenotypes were either homozygous for the F-alleles (F/F) or heterozygous (F/S). These results indicated that the \text{CYP2D1}
polymorphism caused by nucleotide substitutions (418A/421C vs. 418G/421T) was responsible for inter-individual variations leading to the polymorphism in the major metabolism and pharmacokinetics of Cpd-D in male SD rats.
Introduction

Large inter-individual variations in pharmacokinetics (PK) and circulating metabolite(s) following the oral administration of new drug candidates often complicate the interpretation of PK-pharmacodynamic relationships and safety profiling in preclinical species during the course of drug development. These variations add difficulty to the consistent and reliable evaluations of efficacy and safety assessments in preclinical species, and also confound the prediction of therapeutic indexes and design of clinical studies. Unless the mechanism responsible for variabilities in PK is deemed specific to a particular strain and/or species in preclinical species, the candidate could be withdrawn at the discovery stage in order to avoid complexities in later development and potential failure in clinical studies.

The significant impact of a genetic polymorphism in CYP1A2-dependent metabolism in dogs (Mise et al., 2004a; Tenmizu et al., 2004; Whiterock et al., 2007) on large variations in PK have been reported for the drug candidates GST-21 (Azuma et al., 2002), AC3933 (Mise et al., 2004b), and YM-64227 (Tenmizu et al., 2006). Genetic polymorphisms have also been identified in rats for metabolism by aldehyde oxidase (Adachi et al., 2007; Itoh et al., 2007b; Itoh et al., 2007a; Itoh et al., 2007c), N-acetyltransferase (Hein et al., 1991; Juberg et al., 1991), and UDP-glucuronosyltransferase (Iyanagi, 1991), and are responsible for the strain differences observed in metabolism catalyzed by the corresponding enzymes. Among
the cytochrome P450 (P450) isoforms, the CYP2D subfamily is known to consist of a group of highly polymorphic enzymes. In rats, the very low expression level of CYP2D2 in female Dark Agouti rats allowed them to be used as the model for the CYP2D6 poor metabolizer phenotype in humans (Yamamoto et al., 1998; Schulz-Utermoehl et al., 1999). CYP2D3 has also been shown to cause genetic polymorphisms in the \( p \)-hydroxylation of diazepam in Wistar rats (Saito et al., 2004; Sakai et al., 2005; Sakai et al., 2009).

3-(6-methylpyridine-3-yl-sulfanyl)-6-(4H-[1,2,4]triazole-3-yl-sulfanyl)-N-(1,3-thiazole-2-yl)-2-pyridine carboxamide (Cpd-D) is a novel small-molecule glucokinase activator (GKA) that enhances the catalytic activity of glucokinase, a unique hexokinase isoform that plays pivotal roles in glucose-sensing by pancreatic \( \beta \) cells and glucose metabolism by hepatocytes (Grimsby et al., 2003; Matschinsky et al., 2006). The binding of GKA to glucokinase was shown to allosterically stabilize the enzyme in a conformational state with markedly higher affinity for glucose (Kamata et al., 2004). GKA candidates have progressed to clinical studies in patients (Zhi et al., 2008; Bonadonna et al., 2010; Meininger et al., 2011) as well as healthy human subjects (Zhai et al., 2008; Migoya et al., 2009). Cpd-D undergoes \textit{in vitro} and \textit{in vivo} metabolism in rats (in-house data, Fig. 1) to generate either primary metabolites from hydrolysis of the amide linkage forming the carboxylic acid analog (M1), oxidative cleavage of the thiazole moiety forming the thiohydantoic acid analog (M2) and thiourea.
analog (M5), \( S \)-oxidations (M3 and M6), methyl hydroxylation of pyridine (M4), oxidation of the thiazole moiety (M7), and glucuronidation (M11 and M12) or secondary metabolites from combinations of primary metabolism (M8, M9, M10, and M13). During the course of preclinical assessments, large inter-individual variations were observed in the PK of Cpd-D in male SD rats, which were subsequently divided into two phenotypes: >6-fold longer terminal-phase half-life \((t_{1/2})\) and ~10-fold larger area under the plasma concentration-time curve extrapolated to infinity \((\text{AUC}_{0-\infty})\) values were observed in slow metabolizers (SM) than in fast metabolizers (FM) after the oral (P.O.) administration of Cpd-D. Therefore, the purpose of the present study was to elucidate the mechanism(s) underlying the inter-individual variation leading to polymorphisms in the PK of Cpd-D in male SD rats from the genetic basis of intra-strain differences in the major metabolism of Cpd-D. Molecular analyses revealed that one of the major metabolisms of Cpd-D (the formation of M2) was selectively catalyzed by one of the allelic \( \text{CYP2D1} \) variants in the FM phenotypes, which accounted for the predominant formation of M2 after the P.O. administration of Cpd-D to the FM phenotypes of SD rats, and also for the 10-fold lower systemic exposure in the FM than SM phenotypes. To the best of our knowledge, this is the first study to report that a genetic polymorphism in \( \text{CYP2D1} \) affected metabolism and caused polymorphic variations in the PK profile of parent xenobiotics in male SD rats.
**Materials and Methods**

**Chemicals.**

Cpd-D [3-(6-methylpyridine-3-yl-sulfanyl)-6-(4\textsubscript{H}-[1,2,4]triazole-3-yl-sulfanyl)-N-(1,3-thiazole-2-yl)-2-pyridine carboxamide in Fig. 1] was synthesized at Tsukuba Research Institute, Banyu Pharmaceutical Co. Ltd. (Ibaraki, Japan). [\textsuperscript{14}C]Cpd-D [3-(6-methylpyridine-3-yl-sulfanyl)-6-(4\textsubscript{H}-[1,2,4]triazole-[3-\textsuperscript{14}C]-3-yl-sulfanyl)-N-(1,3-thiazole-2-yl)-2-pyridine carboxamide (Specific activity = 4.81 MBq/mg)] was synthesized by Merck Research Laboratories (Rahway, NJ). Metabolites M1-M6 were synthesized at Tsukuba Research Institute, Banyu Pharmaceutical Co. Ltd. (Ibaraki, Japan) as synthetic reference standards: M1, 3-(6-methylpyridine-3-yl-sulfanyl)-6-(4\textsubscript{H}-[1,2,4]triazole-3-yl-sulfanyl)-2-pyridine carboxylic acid; M2, \{[3-(6-methylpyridin-3-yl)sulfanyl]-6-(4\textsubscript{H}-1,2,4-triazol-3-yl-sulfanyl)pyridin-2-yl\} carbonyl carbamothioyl]amino]acetic acid; M3, 3-(6-methylpyridine-3-yl-sulfinyl)-6-(4\textsubscript{H}-[1,2,4]triazole-3-yl-sulfanyl)-N-(1,3-thiazole-2-yl)-2-pyridine carboxamide; M4, 3-(6-hydroxymethylpyridine-3-yl-sulfanyl)-6-(4\textsubscript{H}-[1,2,4]triazole-3-yl-sulfanyl)-N-(1,3-thiazole-2-yl)-2-pyridine carboxamide; M5,
N-carbamothioyl-3-[(6-methylpyridin-3-yl)sulfanyl]-6-(4H-1,2,4-triazol-3-yl-sulfanyl)-pyridine-2-carboxamide; M6,
3-(6-methylpyridine-3-yl-sulfanyl)-6-(4H-[1,2,4]triazole-3-yl-sulfinyl)-N-(1,3-thiazole-2-yl)-2-pyridine carboxamide. Structures of metabolites M7-M13 were preliminarily assigned as shown in Fig. 1 according to the fragmentations obtained from liquid chromatography-tandem mass spectrometry analyses (unpublished in-house data).

β-NADP⁺, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Microsomes prepared from human B-lymphoblastoid cells containing cDNA-expressed CYP1A2, CYP2B1, CYP2C6, CYP2C11, CYP2C13, CYP2D2, and CYP3A2 were obtained from BD Gentest Corporation (Wobum, MA). Microsomes prepared from human B-lymphoblastoid cells containing cDNA-expressed CYP2A1, CYP2C12, CYP2D1, and CYP3A1 were prepared in-house at Merck Research Laboratories (West Point, PA). All other chemicals and solvents were of the highest grade commercially available.

Animals. Eight- to twelve-week-old male Sprague-Dawley (Crl:CD), Wistar (Crlj:WI), and Fischer (F344/DuCrlCrlj) rats were purchased from Charles River Japan, Inc. (Yokohama, Japan). Rats were acclimated for at least one week to the laboratory conditions (room temperature, 22 ± 2°C; humidity; 55 ± 15%; 12-h light/dark cycle) with free access to both
food (CE-2; CLEA Japan, Inc. Tokyo, Japan) and water. Rats were fasted for approximately 17 h before the experiments, and food was given 4 h after the administration of Cpd-D. Water was given *ad libitum* during the experiments. The experiments described herein were carried out with the approval of the Institutional Animal Care and Use Committee.

**In Vivo Study of Cpd-D in Rats.** In the PK experiments, Cpd-D was orally administered to rats at 1 and 3 mg/kg (5 ml/kg in 0.5% methylcellulose suspension) for the P.O. administration protocol or was injected into the jugular vein at 1 mg/kg (1 ml/kg in 50% polyethylene glycol 400) under isoflurane anesthesia for the intravenous (IV) administration protocol. Blood was collected into heparinized capillary tubes from the tail vein at the following time points: 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after the IV administration; 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after the P.O. administration. Plasma was separated from whole blood by centrifugation and stored at -80°C until analyzed.

In the *in vivo* metabolite profiling experiments, [14C]Cpd-D (3.7 MBq) was orally administered to bile-duct cannulated male SD rats at 3 mg/kg (5 ml/kg in 0.5% methylcellulose suspension). Blood was collected into heparinized capillary tubes from the tail vein at 1 h postdose during the consecutive collection of urine and bile for 48 h. Plasma was separated from whole blood by centrifugation, and all samples were stored at -80°C until analyzed.
In the phenotyping of male SD rats based on the metabolic ratio (excretion ratio of M2 to Cpd-D) in the urine, Cpd-D was orally administered to rats at 3 mg/kg (5 ml/kg in 0.5% methylcellulose suspension). Blood was collected into heparinized capillary tubes from the tail vein 1, 2, 4, 6, 8, and 24 h after the administration during the consecutive collection of urine for 24 h postdose. Plasma was separated from whole blood by centrifugation, and all samples were stored at -80°C until analyzed.

In the genotype-phenotype correlation experiments, male SD rats were orally administered Cpd-D at 30 and 100 mg/kg/day for 5 days. Plasma was collected pre, 1, 2, 4, 6, 8, and 24 h postdose on Day 1 in order to determine plasma concentrations, as described in the section on the Analytical Procedure to calculate AUC\textsubscript{0-\infty} for phenotyping. Rats were sacrificed under isoflurane anesthesia 24 h after the last dose, and livers and blood samples were taken for the rat P450 cDNA sequencing experiments, as described in the section on CYP2D1 cDNA Sequencing.

**Preparation of Liver Microsomes from Phenotyped SD Rats.** Rats that were administered 3 mg/kg Cpd-D orally were phenotyped according to the plasma concentration of Cpd-D and metabolic ratio (M2/Cpd-D) in urine, followed by a wash-out for 1 week, and were then sacrificed to prepare liver microsomes. The livers were immediately removed, rinsed in cold 1.15% KCl, weighed, minced, and homogenized in 3 volumes of cold 1.15%
KCl using a Teflon tissue homogenizer. The homogenates were individually centrifuged at 9,000g for 20 min at 4°C. To prepare liver microsomes, the 9,000g supernatants were then centrifuged at 105,000g for 60 min at 4°C. The microsomal pellets were re-suspended in 10 ml of 0.1 M potassium phosphate buffer (pH 7.4) and the microsomes were stored at -80°C. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA).

**In Vitro Metabolism of Cpd-D.** Reaction mixtures containing 0.1 M potassium phosphate buffer (pH 7.4), 1 mM β-NADP+, 10 mM glucose 6-phosphate, 1.0 unit/ml glucose 6-phosphate dehydrogenase, 3 mM magnesium chloride, and rat liver microsomes prepared individually (0.05 mg protein/ml) as described above were pre-incubated for 5 min in a final volume of 0.8 ml at 37 °C, and the reaction was started with the addition of 8 µl of Cpd-D (at the designated final concentration). After a 10-min incubation, aliquots (0.1 ml) were taken from the incubation mixture, and mixed with three volumes of ice-cold ethanol containing 100 nM of the internal standard Cpd-B [(3-[(1S)-2-Hydroxy-1-methylethoxy]-5-[4-(methylsulfonyl) phenoxy]-N-1,3-thiazol-2-yl]benzamide, compound 6g in Iino et al. (2009)]. The resultant sample was then centrifuged at 12,000g for 10 min at 4°C, and an aliquot of the supernatant was injected into the high-performance liquid chromatography (HPLC)-mass spectrometry (MS) system to
determine the formation of the main primary metabolites (M2, M3, M5 and M6), as described in the section on the Analytical Procedure.

In the *in vitro* metabolism experiments with recombinant rat P450 isoforms, microsomes from rat P450 cDNA-expressed B-lymphoblastoid cells (10 pmol P450/incubation) were pre-incubated in the presence of an NADPH generating system [0.1 M potassium phosphate buffer (pH7.4), 1 mM β-NADP⁺, 10 mM glucose 6-phosphate, 1.0 unit/ml glucose 6-phosphate dehydrogenase, 3 mM magnesium chloride] for 5 min in a final volume of 0.25 ml, and the reaction was started with the addition of 5 µl of Cpd-D at final concentrations of 0.1, 1, and 10 µM. Incubations were terminated after 10 min with 3 volumes of ice-cold methanol. After centrifugation, the supernatant was analyzed by HPLC-MS to determine the thiohydantoic acid metabolite (M2), as described in the section on the Analytical Procedure.

**CYP2D1 cDNA Sequencing.** To detect the single nucleotide polymorphism in CYP2D1, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) from the livers of male SD rats that had previously been phenotyped as SM (n=8) and FM (n=8), as described in the section on the *In Vivo* Study of Cpd-D in Rats. Each rat CYP2D1 cDNA was produced from total RNA by reverse transcriptase polymerase chain reaction (RT-PCR). The sequences of the polymerase chain reaction (PCR) primers used in the present study were as follows: CYP2D1F (5’- ATGGCTGGACTTCTCGCTAC -3’, nucleotide
position 140–159 in the CYP2D1 cDNA sequence) and CYP2D1R (5’-GTCTTCTGACCTTGGAAGAC-3’, nucleotide position 778–797 in the CYP2D1 cDNA sequence), as reported previously (Hiroi et al., 1998). The PCR conditions used were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 sec, 72°C for 1 min, and finally 94°C for 30 sec, 68°C for 10 min. The amplified CYP2D1 products were cloned into the plasmid pCR2.1 vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Sequencing was performed on an ABI PRISM 377 DNA Sequencer (Applied BioSystems, Foster City, CA) and revealed mutations in the CYP2D1 gene.

**Analytical Procedure.** To determine Cpd-D and/or the thiohydantoic acid metabolite (M2) in samples from the *in vivo* experiments, samples were deproteinized with 3 volumes of acetonitrile/0.1% formic acid. HPLC and MS were performed on an Alliance 2790 series (Waters, Milford, MA) and API-3000 tandem quadrupole mass spectrometer (Applied Biosystems, Foster City, CA), respectively. Separation by HPLC was performed on a Waters Symmetry Shield RP8 column (5 µm, 2.1×150 mm) at 40°C at a flow rate of 0.3 ml/min. The mobile phase used was acetonitrile/water (80/20) containing 0.1% formic acid. The combinations of the precursor ion and its product ion were monitored at a capillary voltage of 5000 V and turbo probe temperature of 450°C in the multiple reaction monitoring mode of the transitions *m/z* 428.11 → 328.02 for Cpd-D and *m/z* 462.30 → 387.14 for M2.
Radiochromatograms for [14C]Cpd-D and its major metabolites were obtained by the HP1100 gradient HPLC system (Hewlett Packard, Palo Alto, CA) with the flow scintillation analyzer 625TR (Perkin Elmer, Waltham, MA). Separation was achieved on an Inertsil ODS-3 column (4.6 mm × 25 cm, 5 µm; GL Science; Tokyo, Japan) using a mobile phase consisting of 5 mM ammonium acetate containing 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a constant flow rate of 1.0 ml/min. The gradient was as follows: 0 min 10% B; 60 min, 40% B; 65 min, 90% B; 75 min, 90% B; and 75.1 min, 10% B. The system was equilibrated for 10 min at 10% B prior to the next injection. The 625TR detector was operated in the homogeneous liquid scintillation counting mode with a 500-µl flow cell at a scintillator (Ultima Flo) flow rate of 1.6 ml/min.

To determine Cpd-D and its primary metabolites in the incubated samples with liver microsomes and recombinant rat CYP isoforms, samples were deproteinized with 3 volumes of acetonitrile/0.1% formic acid containing Cpd-B as an internal standard. HPLC and MS were performed on a Surveyor including an autosampler/solvent delivery manager and TSQ Quantum tandem quadrupole mass spectrometer, respectively (Thermo Fisher Scientific Inc., Waltham, MA). Separation by HPLC was performed on an Inertsil ODS-3 column (5 µm, 2.1 × 150 mm, GL Science; Tokyo, Japan) at 40°C at a flow rate of 0.3 mL/min. The mobile phases used were acetonitrile/water (10/90) containing 0.1% formic acid (A) and
acetonitrile/water (90/10) containing 0.1% formic acid (B). The analytes were eluted for the column with an initial holding of 31% B for 0.5 min, followed by a linear gradient from 31% B to 44% B in 4 min, then returned to the initial gradient for 0.1 min, followed by isocratic elution at 31% B for 1.8 min. The combinations of the precursor ion and its product ion were monitored in the multiple reaction monitoring (MRM) mode with the following transitions: m/z 428.10 → 328.05 for Cpd-D, m/z 462.06 → 300.98 for M2, m/z 444.04 → 344.16 for M3, m/z 404.08 → 328.06 for M5, m/z 444.07 → 317.04 for M6, and m/z 347.11 → 233.04 for Cpd-B (an internal standard). The detection parameters used were as follows: capillary temperature, 280°C; spray voltage, 3800 V; polarity, positive; ion source, ESI.

Data Analysis. Kinetics parameters (Vmax and Km) were determined by nonlinear curve-fitting using GraphPad Prism 4 (GraphPad Software, San Diego, CA) to the Michaelis-Menten kinetics (one-site) for the relationship between the substrate (Cpd-D) concentration vs. the formation rate of each primary metabolite (M2, M3, M5 or M6) in the sample incubated and analyzed as described in previous sections. The determined kinetics parameters were also used to calculate metabolic intrinsic clearance (Vmax/Km) for each metabolic pathway. By summing the Vmax/Km obtained in each phenotyped sample of liver microsomes, total metabolic intrinsic clearance (ΣVmax/Km value) was calculated to compare the total metabolic capability of the liver microsomes between the two phenotypes of
the male SD rats.

The PK parameters of Cpd-D were calculated by WinNonlin Standard version 1.5 (Pharsight Corporation, Mountain View, CA). The maximum observed concentrations after the P.O. administration (C_{max}) were directly obtained from actual values. The area under the plasma concentration-time curve extrapolated to infinity (AUC_{0-\infty}) was determined by the trapezoidal (log/linear) rule. Terminal-phase rate constants (\lambda_z) were estimated using a least-squares regression analysis of the plasma concentration-time data during the terminal log-linear phase. The terminal-phase half-life (t_{1/2}) was calculated as 0.693/\lambda_z. The total body (plasma) clearance (CL_p) was calculated as Dose/AUC_{0-\infty}. The apparent volume of distribution at steady state (V_{dss}) was calculated as MRT_{inf} \cdot CL_p, where MRT_{inf} is the mean residence time based on the AUC_{0-\infty} and area under the moment curve extrapolated to infinity.

**Statistical Analysis.** All values are expressed as the mean \pm S.D. To test for significant differences in parameters between the phenotypes, an unpaired Student’s t test was performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA). Differences were considered significant if the probability (P value) was less than 0.05 (P < 0.05).
Results

Pharmacokinetics. The individual plasma concentrations of Cpd-D in male SD rats ($n$ = 6 each dosing group) after the IV (1 mg/kg) and P.O. (1 and 3 mg/kg) administrations of Cpd-D are shown in Figure 2A. Marked inter-individual variations were observed in the PK of Cpd-D, which resulted in two populations in the systemic exposure following both routes of administration. Rats were divided into two groups: slow metabolizer (SM) and fast metabolizer (FM) phenotypes with higher and lower systemic exposures, respectively. The mean value of $\text{AUC}_{0-\infty}$ after the IV administration was ~4-fold higher in SM rats than in FM rats (Table 1), which resulted in a ~4-fold lower value for $\text{CL}_p$ in the SM phenotype (4.9 ml/min/kg) than in the FM phenotype (18 ml/min/kg). As the values for $\text{Vd}_{ss}$ were similar between both phenotypes (0.28-0.29 l/kg), the longer terminal-phase half-life ($t_{1/2}$) in the SM phenotype of male SD rats (1.66 h in SM rats vs. 0.26 h in FM rats) was attributed to the smaller $\text{CL}_p$ values in the SM phenotype than in the FM phenotype. Consistent with the smaller $\text{CL}_p$ in SM rats, the $\text{C}_{\max}$ values of Cpd-D were ~5-fold higher in the SM phenotype (2.9 and 6.0 μM at 1 and 3 mg/kg P.O., respectively) than in the FM phenotype (0.5 and 1.3 μM at 1 and 3 mg/kg P.O., respectively), which results in a ~10-fold larger value for $\text{AUC}_{0-\infty}$ after the P.O. administrations of Cpd-D at both doses in the SM phenotype than in the FM phenotype. Whereas similar polymorphic variations in PK were also observed in outbred
Wistar strain (Crlj:WI) rats, PK was similar among inbred male Fischer 344 (F344/DuCrI/Crlj) rats after both the P.O. (n=12) and IV (n=6) administrations: the observed values of AUC\(_{0-\infty}\) (0.51-0.82 µM-h) and C\(_{\text{max}}\) (0.7-0.95 µM) after the P.O. dose at 3 mg/kg, and of AUC\(_{0-\infty}\) (1.96-2.91 µM-h) and CL\(_p\) (13.5-20.0 ml/min/kg) after the IV dose at 1 mg/kg to male Fischer rats (data not shown) were similar to those in the FM phenotype of male SD rats (Table 1).

The in vivo profiles of metabolites formed from \([^{14}\text{C}]\text{Cpd-D}\) (3 mg/kg P.O.) in the urine, bile, and plasma samples were compared between the SM and FM phenotypes of bile-duct cannulated male SD rats (Fig. 2B). Radioactivities were recovered (as a % of the dose) in bile (51.7±3.5 and 72.0±1.8% in the SM and FM phenotypes, respectively), urine (32.7±3.9 and 16.6±3.5% in the SM and FM phenotypes, respectively), and feces (11.9±6.9 and 8.4±3.9% in the SM and FM phenotypes, respectively) 48 h postdose in bile-duct cannulated rats. In the SM phenotype of male SD rats, Cpd-D underwent multiple metabolic steps to form M1 (urine), M2 (urine and bile), M9 (urine), and M10 (urine and bile), and the concentration of M2 in the plasma was markedly lower than that of Cpd-D 1 h postdose. In contrast, M2 was the predominant metabolite detected in the urine, bile, and plasma samples from FM phenotyped rats. The level of circulating M2 was markedly higher than that of Cpd-D following the P.O. administration (3 mg/kg) of Cpd-D to FM rats (Fig. 3A). Therefore, each rat could be accurately phenotyped by plotting the AUC\(_{0-\infty}\) value of Cpd-D in
the plasma against the metabolic ratio of the excreted amount of M2 to that of Cpd-D in the urine collected 24 h postdose (Fig. 3B). These results suggested that inter-individual variations in the formation of the thiohydantoic acid metabolite (M2) by oxidative cleavage of the thiazole moiety (Fig. 1) accounted for the polymorphic PK variations in Cpd-D in male SD rats.

**Cpd-D Metabolism in Liver Microsomes from Phenotyped Male SD Rats.** The initial velocities for the NADPH-dependent primary metabolism of Cpd-D in liver microsomes obtained from the SM and FM phenotypes (AUC$_{0-\infty}$ > 20 µM·h and < 5µM·h after 3 mg/kg the P.O. administration, respectively) of male SD rats were plotted against the substrate concentrations (Fig. 4A), and the calculated *in vitro* kinetic parameters for major primary metabolism (M2, M3, M5 and M6) are listed in Table 2. Consistent with the aforementioned *in vivo* results, the formation of M2 was predominant at low Cpd-D concentrations, followed by that of M5, in liver microsomes from FM phenotyped rats. Due to the high- (0.09 µM) and low-affinity (1.66 µM) characteristics for the formation of M2 and M5, respectively (Table 2), the relative contribution of M2 and M5 to the overall metabolism of Cpd-D in FM phenotyped rats may have depended on the substrate concentration: the simulation from the kinetic parameters indicated that the formation of M2 and M5 accounted for 83 and 16%, respectively, of overall metabolism at 0.01 µM, and the contribution of the
formation of M5 to overall metabolism became more dominant (56%) than that of M2 (20%) at 10 µM. In contrast, the formation of M2 was too low to be analyzed for kinetics under the conditions used for primary metabolism in the SM phenotypes. In order to compare the total metabolic capabilities in livers from both phenotypes, total metabolic intrinsic clearance ($\Sigma V_{\text{max}}/K_m$ value) was calculated by summing $V_{\text{max}}/K_m$ values in each sample of phenotyped liver microsomes. The value of $\Sigma V_{\text{max}}/K_m$ was markedly lower (11.2 µl/min/mg microsomal protein) in liver microsomes from SM than from FM phenotyped rats (193 µl/min/mg microsomal protein). In liver microsomes from SM phenotyped rats, the formation of M3, M5, and M6 had the kinetics with similar affinities ($K_m$ values, 5.6~15 µM) and may have contributed to overall metabolism at a wide range of substrate concentrations.

**Metabolism of Cpd-D in Recombinant P450 Isoforms.** To identify the P450 isoform(s) involved in the formation of M2, which may have been responsible for the metabolism of Cpd-D in the FM phenotypes both in vivo (Figs. 2 and 3) and in vitro in the low substrate concentration range (Fig. 4A), metabolism was examined in the recombinant rat P450 isoforms (Fig. 4B). The results demonstrated that, of the rat P450 isoforms tested, the formation of M2 was only catalyzed by recombinant CYP2D1. These results suggested that the CYP2D1-mediated formation of M2 may have accounted for inter-individual variations leading to the polymorphism of PK observed in male SD rats.
Genotype-Phenotype Correlation. Total RNA isolated from male SD rat livers phenotyped SM (AUC$_{0-24h}$; >127 and >424 µM·h after the 30 and 100 mg/kg P.O. administrations, respectively) and FM (AUC$_{0-24h}$; <6.8 and <26.3 µM·h after the 30 and 100 mg/kg P.O. administrations, respectively) were subjected to DNA sequence analyses for the CYP2D1 gene (Table 3). Sequence polymorphisms were detected at nucleotides 418 and 421 in the CYP2D1 gene: 418A/421C and 418G/421T were designated the F and S alleles, respectively. Genotype-phenotype correlation analysis indicated that two S alleles were homozygous (S/S) in the SM phenotypes of male SD rats, while the FM phenotypes were either homozygous for the F-allele (F/F) or heterozygous (F/S) (Table 3).
Discussion

Polymorphisms in the systemic exposure of Cpd-D (i.e. ~10-fold difference in AUC\(_{0-\infty}\)) and the distinct profiles of the main circulating thiohydantoic acid metabolite (M2) between the two phenotypes following the P.O. administration of Cpd-D to male SD rats resulted in complicated preclinical evaluations for the development of Cpd-D. The results of the \textit{in vitro} kinetic experiments revealed that M2 was only formed in liver microsomes obtained from the fast metabolizer phenotype, and, among the isoforms tested, was selectively catalyzed by recombinant CYP2D1. These results implied that a genetic polymorphism in CYP2D1 for the formation of M2 caused inter-individual variations that led to the polymorphism in the PK observed in male SD rats. Nucleotide sequence analysis revealed that two allelic \textit{CYP2D1} variants with 418A/421C and 418G/421T (designated as the F- and S-alleles, respectively), resulting in Ile123/Leu124 and Val123/Phe124, respectively, were either homozygous for the F-allele (F/F) or heterozygous (F/S) in the fast metabolizer phenotypes, while the slow metabolizer phenotypes were homozygous for the S-allele (S/S) for the polymorphic metabolism and PK of Cpd-D in male SD rats.

Six isoforms (CYP2D1, CYP2D2, CYP2D3, CYP2D4, CYP2D5, and CYP2D18) have been identified in rats by genomic analysis (Gonzalez, 1996; Nelson et al., 1996), and four isoforms (CYP2D1, CYP2D2, CYP2D3, and CYP2D4) account for the distinct functionality
of the rat CYP2D isoforms due to strong similarities in the amino acid sequences (>95%) of CYP2D1 and CYP2D5 as well as CYP2D4 and CYP2D18 (Gonzalez et al., 1987; Matsunaga et al., 1989). Of the four rat CYP2D isoforms, substrate specificity (Wan et al., 1997; Hiroi et al., 2002; Grobe et al., 2012), region-selective metabolism (Suzuki et al., 1992; Masubuchi et al., 1993; Masubuchi et al., 1994; Narimatsu et al., 1994), and a study on ligand-binding specificities by homology modeling (Venhorst et al., 2003) suggested that CYP2D2 functionally conserves human CYP2D6, and the markedly lower expression of CYP2D2 in female Dark Agouti rats than in Wistar and SD rats allowed female Dark Agouti rats to be used as a model of the CYP2D6 poor metabolizer phenotype in humans (Yamamoto et al., 1998; Schulz-Utermoehl et al., 1999). Homology modeling of rat and human CYP2D isoforms indicated that the negative electrostatic potential on the active site surface conferred common characteristics on both CYP2D2 and CYP2D6, which was distinct from the neutral electrostatic potentials calculated for the other rat CYP2D isoforms (Venhorst et al., 2003). This common feature in both CYP2D2 and CYP2D6 may exclude Cpd-D from the favored binding mode (or orientation) for oxidative cleavage of the thiazole moiety (M2 formation) because the formation of M2 was not catalyzed by either CYP2D2 (Fig. 4B) or CYP2D6 (data not shown).

The genotype-phenotype analysis performed in this study (Table 3) demonstrated that the
slow metabolizer (SM) phenotype of male SD rats had two nucleotide-substituted alleles with 418G/421T, which was designated as S-allele (S/S), while the fast metabolizer (FM) phenotype of these rats were either homozygous for the F-allele with 418A/421C (F/F) or heterozygous (F/S). Since the FM phenotype of the SD rats generated M2 as the main circulating metabolite, which may reflect the \textit{in vivo} metabolic profile in humans (data not shown), and the SD strain accumulated safety background data in rats, the high throughput Taq-man PCR genotyping assay for \textit{CYP2D1} with rat blood samples was used to group SD rats into different phenotypes prior to the onset of preclinical studies in order to facilitate the consistent and rigorous profiling of PK-pharmacodynamic relationships and safety assessments of Cpd-D.

The nucleotide substitutions at positions 418 and 421 in the S-allele (418G/421T) and F-allele (418A/421C) resulted in the amino acid residues Val123/Phe124 and Ile123/Leu124, respectively, and these nucleotide sequences were detected in the allelic \textit{CYP2D1} variants (IID1) (Ishida et al., 1988; Matsunaga et al., 1989) and IID1\textsubscript{v} (or db1) (Gonzalez et al., 1987; Matsunaga et al., 1989), respectively. These allelic variants in \textit{CYP2D1} were found to have different bufuralol 1'-hydroxylase and debrisoquine 4-hydroxylase activities in studies on COS cells transfected with IID1 and IID1\textsubscript{v} cDNA (Matsunaga et al., 1989; Matsunaga et al., 1990b): bufuralol 1'-hydroxylase activity was more than 10-fold higher in COS cells.
expressing IID1 than in those expressing IID1v, while both transfectants had similar activities towards debrisoquine 4-hydroxylation (Matsunaga et al., 1990b).

The amino acid residue Val123 is common among the rat CYP2D isoforms [CYP2D1 (IID1), CYP2D2, CYP2D3 and CYP2D4] (Gonzalez et al., 1987; Matsunaga et al., 1989; Matsunaga and Gonzalez, 1990; Matsunaga et al., 1990a), but different from the allelic CYP2D1 variant (IID1v) and CYP2D6, which possess Ile123 and Phe at corresponding position 120 (Kimura et al., 1989), respectively. Metabolism of oxidative cleavage of the thiazole ring of Cpd-D (M2 formation) was only observed in rat liver microsomes from the FM phenotype of male SD rats (Fig. 4A) with a F-allele (Ileu123/Leu124), and the formation of M2 in human liver microsomes was not catalyzed by CYP2D6, but by CYP3A4 and CYP2C isoforms (data not shown). Similar isoform-selective metabolism was observed for the \(N\)-oxidation of \(R\)-mianserin catalyzed by \textit{Saccharomyces cerevisiae} expressing IID1v, but not by CYP2D2, CYP2D3, CYP2D4, or CYP2D6 (Chow et al., 1999). Therefore, the unique amino acid residue(s) of Ile123 or the combination of both Ile123 and Leu124 in the F-allelic \textit{CYP2D1} variant or \textit{CYP2D1} IID1v, which are distinct from the other rat CYP2D isoforms and CYP2D6, at least partially conferred metabolic capabilities forming M2 from Cpd-D and catalyzing the \(N\)-oxidation of \(R\)-mianserin to CYP2D1 (IID1).

In conclusion, the present study demonstrated that the markedly large inter-individual
variations leading to two phenotypes for the systemic exposure and/or PK profiles of Cpd-D in male SD rats may be attributed to the oxidative cleavage of the thiazole moiety of Cpd-D (M2 formation) selectively catalyzed by the CYP2D1 F-allelic variant (418A/421C) in the fast metabolizer phenotype of male SD rats, either homozygous for the F-alleles or heterozygous. Based on the present study, a high throughput Taq-man PCR CYP2D1 genotyping assay with blood samples was developed and the assay has been used to group SD rats into two phenotypes prior to preclinical studies for consistent and reliable evaluations during the preclinical development of Cpd-D.
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Authorship Contribution

Participated in the research design: Hasegawa, Eiki, and Chiba

Conducted experiments: Hasegawa

Performed data analysis: Hasegawa, Eiki, and Chiba

Wrote or contributed to the writing of the manuscript: Hasegawa, Eiki, and Chiba
References


Hiroi T, Imaoka S, Chow T, and Funae Y (1998) Tissue distributions of CYP2D1, 2D2, 2D3 and 2D4 mRNA in rats detected by RT-PCR. *Biochim Biophys Acta* **1380**:305-312.


Subfamily: Analysis of the Molecular Basis of the Debrisoquine 4-Hydroxylase Deficiency in DA Rats.

Biochemistry 28:7349-7355.


SNP causing protein deletion. Xenobiotica 34:835-846.


Footnotes

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**Figure Legends**

**Figure 1.** Proposed metabolites of [14C]Cpd-D in rats *in vitro* and *in vivo*. The structures of the primary metabolites (M1-M6) were confirmed using synthesized authentic standards. Unpublished in-house data.

**Figure 2.** Pharmacokinetics and metabolite profiles of Cpd-D in the bile, urine, and plasma from the two phenotypes of male SD rats. (A) Individual plasma concentrations of Cpd-D in male SD rats after the IV (1 mg/kg, *n* = 6) and P.O. (1 and 3 mg/kg, *n* = 6 each) administrations of Cpd-D. PK profiles were grouped by the two phenotypes, designated as slow (red symbols) and fast (blue symbols) metabolizers. (B) HPLC radiochromatograms of metabolites in the urine (0-24 h), bile (0-24 h), and plasma (1 h) after the P.O. administration of [14C]Cpd-D at 3 mg/kg to slow and fast metabolizer phenotyped bile-duct cannulated male SD rats.

**Figure 3.** The thiohydantoic acid metabolite (M2) as a phenotyping marker for the fast and slow metabolizers of Cpd-D in male SD rats. (A) Individual plasma concentrations of Cpd-D and its thiohydantoic acid metabolite (M2) after the P.O. administration of Cpd-D at 3 mg/kg to slow and fast metabolizer phenotyped male SD rats (*n* = 4 each). (B) AUC0-∞ of
Cpd-D vs. metabolic ratio of M2 to Cpd-D in urine collected 24 h postdose following the P.O. administration of Cpd-D at 3 mg/kg to slow and fast metabolizer phenotyped male SD rats. Each symbol represents values from the same rat.

**Figure 4.** Predominant *in vitro* formation of the thiohydantoic metabolite (M2) from Cpd-D in liver microsomes from fast metabolizer phenotyped male SD rats and recombinant CYP2D1. (A) Formation of the main primary metabolites of Cpd-D in NADPH-fortified liver microsomes from slow (red symbols) and fast (blue symbols) metabolizer phenotyped male SD rats. Rat liver microsomes (0.05 mg microsomal protein/ml) were incubated at 37°C for 10 min with the designated concentrations of Cpd-D in the presence of an NADPH-generating system, and data represent the mean±S.D. (n=3). (B) Formation of the thiohydantoic acid metabolite (M2) by recombinant rat P450-expressing microsomes. Cpd-D (0.1, 1 and 10 μM) was incubated for 10 min at 37°C with cDNA-expressing rat P450 isoforms (10 pmol/incubation) in the presence of an NADPH-generating system.
Table 1. Pharmacokinetic parameters (mean ± S.D., n = 3 each phenotype) in the slow (SM) and fast metabolizer (FM) phenotypes of male SD rats after the IV and P.O. administrations of Cpd-D.

<table>
<thead>
<tr>
<th></th>
<th>Slow Metabolizer (SM)</th>
<th>Fast Metabolizer (FM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IV (1 mg/kg)</td>
<td>P.O. (3 mg/kg)</td>
</tr>
<tr>
<td>AUC0–∞ (μM·h)</td>
<td>± 8.04 ± 0.34</td>
<td>± 5.86 ± 0.97</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>± 1.66 ± 0.27</td>
<td>± 0.97 ± 0.26</td>
</tr>
<tr>
<td>Cmax (μM)</td>
<td>± 2.91 ± 0.55</td>
<td>± 5.96 ± 2.42</td>
</tr>
<tr>
<td>CLp (ml/min/kg)</td>
<td>± 4.86 ± 0.20</td>
<td>± 2.42 ± 0.28</td>
</tr>
<tr>
<td>Vdss (l/kg)</td>
<td>± 0.03 ± 0.03</td>
<td>± 0.01 ± 0.01</td>
</tr>
</tbody>
</table>

a not applicable.

Significantly different from Slow Metabolizers (*P<0.05, **P<0.01 and ***P<0.001).
Table 2. *In vitro* kinetic parameters for the formations of major primary metabolites of Cpd-D in liver microsomes from slow and fast metabolizer phenotyped male SD rats (mean ± S.D., n = 3).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Slow Metabolizer (SM)</th>
<th>Fast Metabolizer (FM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}^a$</td>
<td>$K_m^b$</td>
</tr>
<tr>
<td>M2</td>
<td>-c</td>
<td>-</td>
</tr>
<tr>
<td>M3</td>
<td>20.2±3.4</td>
<td>15.0±5.0</td>
</tr>
<tr>
<td>M5</td>
<td>42.0±5.7</td>
<td>5.61±1.74</td>
</tr>
<tr>
<td>M6</td>
<td>20.7±2.3</td>
<td>8.72±2.50</td>
</tr>
</tbody>
</table>

$^a$ pmol/min/mg microsomal protein.

$^b$ μM

$^c$ not analyzed, due to the very low formation of M2.

Significantly different from Slow Metabolizers (*P<0.05).
Table 3. Comparison of the *CYP2D1* nucleotide sequence at 418-421 between the slow and fast metabolizer phenotypes of PK of Cpd-D in male SD rats.

<table>
<thead>
<tr>
<th>PK Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>CYP2D1</em> Sequence at 418-421</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>GTCT</td>
</tr>
<tr>
<td>Slow</td>
<td>GTCT</td>
</tr>
<tr>
<td>Fast</td>
<td>ATCC</td>
</tr>
<tr>
<td>Slow</td>
<td>GTCT</td>
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<tr>
<td>Fast</td>
<td>G/A-T/C</td>
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<td>Fast</td>
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<tr>
<td>Slow</td>
<td>GTCT</td>
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<tr>
<td>Fast</td>
<td>G/A-T/C</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rats with AUC values less than 6.8 and 26.3 μM·h at the 30 and 100 mg/kg P.O. administrations, respectively, were designated as “Fast” metabolizer phenotypes; and those with AUC values exceeding 127 and 424 μM·h at the 30 and 100 mg/kg P.O. administrations, respectively, were designated as “Slow” metabolizer phenotypes.
Fig. 1
**Fig. 2**

**A**  
IV  
1 mg/kg  
Slow metabolizer  
Fast metabolizer  
P.O.  
1 mg/kg  
3 mg/kg  

**B**  
Slow metabolizer  
Fast metabolizer  

Urine (0-24 h)  
Bile (0-24 h)  
Plasma (1 h)  

Radioactivity (CPM)  
Retention Time (min)
Fig. 3

A

Plasma Concentration (μM)

Cpd-D

0 1 2 3 4

Thiohydantoic acid metabolite (M2)

Time (h)

0 4 8 12 16 20 24

B

AUC of Cpd-D (μM·h)

Slow metabolizer

Fast metabolizer

Metabolic Ratio in Urine (M2/Cpd-D)

0 20 40 60

0 20000 40000 60000
Fig. 4

A

Formation of thiohydantoic acid (M2) metabolite (nM)

Metabolic Activity (pmol/min/mg protein)

Cpd-D Concentration (µM)

CYP1A2
CYP2A1
CYP2B1
CYP2C6
CYP2C11
CYP2C12
CYP2C13
CYP2D1
CYP2D2
CYP3A1
CYP3A2

B

Formation of thiohydantoic acid (M2) metabolite (nM)

0.1 µM
1
10