Interindividual 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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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 interindividual 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 maximum observed concentrations after administration by mouth 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 after the oral administration of [14C]Cpd-D in the FM and SM phenotypes of bile-duct cannulated SD rats. The liver microsomes prepared from FM phenotyped rats extensively formed M2 with the highest affinity (Km = 0.09 μM) and largest Vmax/Km value in primary metabolism, whereas 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 versus 418G/421T) was responsible for interindividual variations leading to the polymorphism in the major metabolism and pharmacokinetics of Cpd-D in male SD rats.

Introduction

Large interindividual variations in pharmacokinetics (PK) and circulating metabolite(s) after the oral administration of new drug candidates can sometimes 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in PK is deemed specific to a particular strain and/or species in the preclinical species, the candidate could be withdrawn at the discovery stage to avoid complexities in later development and potential failure in clinical studies.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; Itsh et al., 2007a,b,c), N-acetyltransferase (Hein et al., 1991; Juberg et al., 1991), and UDP-glucuronosyltransferase (Iyana, 1991) and are responsible for the strain differences observed in metabolism catalyzed by the corresponding enzymes. Among the cytochrome P450 (P450) isomorphs, 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). CYP2D 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, 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.

ABBREVIATIONS: AUC0∞, area under the plasma concentration-time curve extrapolated to infinity; CI0, terminal-phase half-life; CLp, total body (plasma) clearance; Cpd-B, (3-[15S]-hydantoic acid, 2-pyridine carboxamide; 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; MS, mass spectrometry; PCR, polymerase chain reaction; PK, pharmacokinetics; P450, cytochrome P-450; RT-PCR, reverse transcriptase polymerase chain reaction; SD, Sprague-Dawley; SM, slow metabolizer; t1/2, terminal-phase half-life; Vmax, apparent volume of distribution at steady-state.
activity of glucokinase, a unique hexokinase isoform that plays pivotal roles in glucose sensing by pancreatic β 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 in vitro and 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 interindividual 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 (AUC$_{0\rightarrow\infty}$) values were observed in slow metabolizers (SM) than in fast metabolizers (FM) after the oral administration of Cpd-D. Therefore, the purpose of the present study was to elucidate the mechanism(s) underlying the interindividual variation leading to polymorphisms in the PK of Cpd-D in male SD rats from the genetic basis of intrastrain 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 CYP2D1 variants in the FM phenotypes, which accounted for the predominant formation of M2 after the oral 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 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-(4H-[1,2,4]triazole-3-yl-sulfanyl)-N-(1,3-thiazole-2-yl)-2-pyridine carboxamide in Fig. 1] was synthesized at Banyu Tsukuba Research Institute, (Ibaraki, Japan). $[^{14}C]$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 (specific activity = 4.81 MBq/mg)] was synthesized by Merck Research Laboratories (Rahway, NJ). Metabolites M1–M6 were synthesized at Banyu Tsukuba Research Institute as synthetic reference standards: M1, 3-(6-methylpyridine-3-yl-sulfanyl)-6-(4H-[1,2,4]triazole-3-yl-sulfanyl)-2-pyridine carboxylic acid; M2, [(3-{[(6-methylpyridin-3-yl)sul- fanyl]-6-(4H-[1,2,4]triazol-3-yl-sulfanyl)pyridin-2-yl} carbonyl) carbamothioyl] amino]acetic acid; M3, 3-(6-methylpyridine-3-yl-sulfinyl)-6-(4H-[1,2,4]triazole-3-yl-sulfanyl)-N-(1,3-thiazole-2-yl)-2-pyridine carboxamide; M4, 3-(6-hydroxy-methylpyridine-3-yl-sulfanyl)-6-(4H-[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]triazole-3-yl-sulfanyl)-pyridine-2-carboxamide; M6, 3-(6-methylpyridine-3-yl-sulfanyl)-6-(4H-[1,2,4]triazole-3-yl-sulfanyl)-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.
concentrations, as described in administered Cpd-D at 30 and 100 mg/kg/day for 5 days. Plasma was collected were stored at heparinized capillary tubes from the tail vein 1, 2, 4, 6, 8, and 24 hours after the administration during the consecutive collection of urine for 24 hours postdose. 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 hours after the intravenous administration; 0.25, 0.5, 1, 2, 4, 6, 8, and 24 hours after the oral 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 rats at 1 mg/kg (5 ml/kg in 0.5% methylcellulose suspension) for the oral 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 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 hours after the intravenous administration; 0.25, 0.5, 1, 2, 4, 6, 8, and 24 hours after the oral administration. Plasma was separated from whole blood by centrifugation and stored at −80°C until analyzed.

In the in vitro metabolism experiments with recombinant rat CYP450 isozymes, microsomes from rat P450 cdna-expressed B-lymphoblastoid cells (10 pmol P450/incubation) were preincubated in the presence of an NADPH generating system (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) for 5 minutes 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 minutes with 3 volumes of ice-cold methanol. After centrifugation, 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 Analytical Procedure.

In the in vitro metabolism experiments with recombinant rat CYP450 isozymes, microsomes from rat P450 cdna-expressed B-lymphoblastoid cells (10 pmol P450/incubation) were preincubated in the presence of an NADPH generating system (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) for 5 minutes 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 minutes with 3 volumes of ice-cold methanol. After centrifugation, the supernatant was analyzed by HPLC-MS to determine the thiodydroxyacid metabolite (M2), as described in Analytical Procedure.

Analytical Procedure. To determine Cpd-D and/or the thiodydroxyacid 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), 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 45°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 minutes 10% B; 60 minutes, 40% B; 65 minutes, 90% B; 75 minutes, 90% B; and 75.1 minutes, 10% B. The system was equilibrated for 10 minutes at 10% B before the next injection. The 625TR detector was operated in the homogenous liquid scintillation counting mode with a 500-μl flow cell at a scintillator (Ultima Flo, Perkin Elmer, Waltham, MA) 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 isozymes, samples were deproteinized with 3 volumes of acetonitrile/0.1% formic acid containing Cpd-D 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.). Separation by HPLC was performed on an Inertisil ODS-3 column (5 μm, 2.1 × 150 mm, GL Science) 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 minutes, followed by a linear gradient from 31% B to 44% B in 4 minutes, then to the initial gradient for 0.1 minute, followed by isocratic elution at 31% B for 1.8 minutes. The combinations of the precursor ion and its product ion were monitored in the multiple reaction monitoring 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 parameter used was as follows: capillary temperature, 280°C; spray voltage, 3800 V; polarity, positive; ion source, ESI.

Data Analysis. Kinetics parameters (V_{max} and K_{m}) 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 versus 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 (V_{int}/K_{m}) for each metabolic pathway. By summing the V_{int}/K_{m} obtained in each phenotyped sample of liver microsomes, total metabolic intrinsic clearance (2V_{int}/K_{m} 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 oral administration (C_{max}) were directly obtained from actual values. The area under the plasma concentration-time curve extrapolated to infinity (AUC_{0-∞}) was determined by the trapezoidal (log-linear) rule. Terminal-phase rate constants (λz) 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/λz. The total body (plasma) clearance (CL_{p}) was calculated as dose/AUC_{0-∞}. The apparent volume of distribution at steady state (V_{d,ss}) was calculated as MRT_{ss}/CL_{p}, where MRT_{ss} is the mean residence time based on the AUC_{0-∞} and area under the moment curve extrapolated to infinity.

Statistical Analysis. All values are expressed as the mean ± 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). 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 intravenous (1 mg/kg) and oral (1 and 3 mg/kg) administrations of Cpd-D are shown in Fig. 2A. Marked interindividual variations were observed in the PK of Cpd-D, which resulted in two populations in the systemic exposure after both routes of administration. Rat divisions were into two groups: slow metabolizer (SM) and fast metabolizer (FM) phenotypes with higher and lower systemic exposures, respectively. The mean value of AUC_{0-∞} after the intravenous administration was ~4-fold higher in SM rats than in FM rats (Table 1), which resulted in a ~4-fold lower value for CL_{p} in the SM phenotype (4.9 ml/min/kg) than in the FM phenotype (18 ml/min/kg). As the values for V_{d,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 hours in SM rats versus 0.26 hour in FM rats) was attributed to the smaller CL_{p} values in the SM phenotype than in the FM phenotype. Consistent with the smaller CL_{p} in SM rats, the 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 PO, respectively) than in the FM phenotype (0.5 and 1.3 μM at 1 and 3 mg/kg PO, respectively), which results in a ~10-fold larger value for AUC_{0-∞} after the oral 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/DuCrIcrj) rats after both the oral (n = 12) and intravenous (n = 6) administrations: the observed values of AUC_{0-∞} (0.51–0.82 μM·h) and C_{max} (0.7–0.95 μM) after the oral dose at 3 mg/kg and of AUC_{0-∞} (1.96–2.91 μM·h) and CL_{p} (13.5–20.0 ml/min/kg) after the intravenous 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 [14C]Cpd-D (3 mg/kg oral dose) 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 hours 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 hour postdose. In contrast, M2 was the predominant metabolite detected in the urine,
The initial velocities for the NADPH-dependent primary formation of the thiohydantoic acid metabolite (M2) by oxidative (Fig. 3B). These results suggested that interindividual variations in the amount of M2 to that of Cpd-D in the urine collected 24 hours postdose of Cpd-D in the plasma against the metabolic ratio of the excreted metabolizer phenotyped male SD rats (administration of Cpd-D at 3 mg/kg to slow (red symbols) and fast (blue symbols) concentrations of Cpd-D and its thiohydantoic acid metabolite (M2) after the oral administration of Cpd-D at 3 mg/kg to slow and fast metabolizer phenotyped male SD rats. (A) Individual plasma concentrations of Cpd-D and its thiohydantoic acid metabolite (M2) after the oral administration of Cpd-D at 3 mg/kg to slow (red symbols) and fast (blue symbols) metabolizer phenotyped male SD rats (n = 4 each). (B) AUC_{0-24} of Cpd-D versus metabolic ratio of M2 to Cpd-D in urine collected 24 hours postdose after the oral 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.

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_{0-24} > 20 and < 5 μM·hour after 3 mg/kg oral 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. Because of 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. To compare the total metabolic capabilities in livers from both phenotypes, total metabolic characteristics for the formation of M2 and M5 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, whereas 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<sub>0-∞</sub>) and the distinct profiles of the main circulating thiohydantoic acid metabolite (M2) between the two phenotypes after the oral administration of Cpd-D to male SD rats resulted in complicated preclinical evaluations for the development of Cpd-D. The results of the 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 interindividual variations that led to the polymorphism in the PK observed in male SD rats. Nucleotide sequence analysis revealed that two allelic 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, whereas 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, 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), whereas the fast metabolizer (FM) phenotype of these rats were either homozygous for the F-allele with 418A/421C (F/F) or heterozygous (F/S). Because the FM phenotype of the SD rats generated M2 as the main circulating metabolite, which may reflect the 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 CYP2D1 with rat blood samples was used to group SD rats into different phenotypes before the onset of preclinical studies 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 in the 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 variants (IID1) (Ishida et al., 1988; Matsunaga et al., 1989) and IID1v (or db1) (Gonzalez et al., 1987; Matsunaga et al., 1989), respectively. These allelic variants in CYP2D1 were found to have different bufuralol 1'-hydroxylase and debrisoquine 4-hydroxylase activities in studies on COS cells transfected with IID1 and IID1v cDNA (Matsunaga et al., 1989, 1990b): bufuralol 1'-hydroxylase activity was more than 10-fold higher in COS cells expressing IID1 than in those expressing IID1v, whereas both transfectants had similar activities toward 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, 1990; Matsunaga and Gonzalez, 1990) 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 an 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 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 CYP2D1 variant or 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 interindividual 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. On the basis of 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 before preclinical studies for consistent and reliable evaluations during the preclinical development of Cpd-D.

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Authorship Contributions
Participated in the research design: Hasegawa, Eiki, Chiba.
Conducted experiments: Hasegawa.
Performed data analysis: Hasegawa, Eiki, Chiba.
Wrote or contributed to the writing of the manuscript: Hasegawa, Eiki, Chiba.

References

TABLE 2
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

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<td></td>
<td>V&lt;sub&gt;m&lt;/sub&gt;</td>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>pmol/min/mg microsomal protein</td>
<td>µM</td>
</tr>
<tr>
<td>M2</td>
<td>20.2 ± 3.4</td>
<td>15.0 ± 5.0</td>
</tr>
<tr>
<td>M3</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>

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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</th>
<th>CYP2D1 Sequence at 418–421</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>GTCT</td>
</tr>
<tr>
<td>Fast</td>
<td>ATCC</td>
</tr>
<tr>
<td>Slow</td>
<td>GTCT</td>
</tr>
<tr>
<td>Fast</td>
<td>G/ATCC/C</td>
</tr>
<tr>
<td>Slow</td>
<td>GTCT</td>
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<tr>
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<td>G/ATCC/C</td>
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<tr>
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<td>G/ATCC/C</td>
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<td>Slow</td>
<td>GTCT</td>
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<tr>
<td>Fast</td>
<td>G/ATCC/C</td>
</tr>
</tbody>
</table>

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Notes:
- *P < 0.05.