Pharmacokinetics and Metabolism of Delamanid, a Novel Anti-Tuberculosis Drug, in Animals and Humans: Importance of Albumin Metabolism In Vivo

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AUC_{0-24h}, area under the plasma concentration–time curve from 0 to 24 h; AUC_{0-t}, area under the plasma concentration–time curve calculated to the last observable concentration at time t; AUC_{\infty}, area under the plasma concentration–time curve from time zero to infinity; BID, *bis in die*; CYP, cytochrome P450; C_{\text{max}}, maximum plasma concentration; HIV/AIDS, human immunodeficiency virus/acquired immunodeficiency syndrome; HPLC, high-performance liquid chromatography; IS, internal standard; LC-MS/MS, liquid chromatography with tandem mass spectrometry; MDR-TB, multidrug-resistant tuberculosis; MRT_{0-\infty}, mean residence time from time zero to infinity; NADH, $\beta$-nicotinamide adenine dinucleotide, reduced form; NADPH, $\beta$-nicotinamide adenine dinucleotide phosphate, reduced form; SRM, selected reaction monitoring; TB, tuberculosis; $t_{1/2,z}$, terminal-phase elimination half-life; $t_{\text{max}}$, time to maximum (peak) plasma concentration.
Abstract

Delamanid, a new anti-tuberculosis drug, is metabolized to M1, a unique metabolite formed by cleavage of the 6-nitro-2,3-dihydroimidazo [2,1-b] oxazole moiety, in plasma albumin in vitro (Shimokawa et al., 2015). The metabolic activities in dogs and humans are higher than those in rodents. In this study, we characterized the pharmacokinetics and metabolism of delamanid in animals and humans. Eight metabolites (M1 to M8) produced by cleavage of the imidazooxazole moiety of delamanid were identified in the plasma after repeated oral administration by liquid chromatography-mass spectrometry analysis. Delamanid was initially catalyzed to M1 and subsequently metabolized by 3 separate pathways, which suggested that M1 is a crucial starting point. The major pathway in humans was hydroxylation of the oxazole moiety of M1 to form M2 and then successive oxidation to the ketone form (M3) mainly by CYP3A4. M1 had the highest exposure among the 8 metabolites after repeated oral dosing in humans, which indicated that M1 was the major metabolite. The overall metabolism of delamanid was qualitatively similar across nonclinical species and humans, but quantitatively different among the species. After repeated administration, the metabolites had much higher concentrations in dogs and humans than in rodents. The in vitro metabolic activity of albumin on delamanid probably caused the species differences observed. We determined that albumin metabolism is a key component of the pharmacokinetics and metabolism of delamanid. Nonhepatic formation of M1 and multiple separate pathways for metabolism of M1 suggest that clinically significant drug–drug interactions with delamanid and M1 are limited.
Introduction

Delamanid (OPC-67683, Deltyba™, nitro-dihydro-imidazooxazole derivative), (R)-2-methyl-6-nitro-2-[(4-[4-(4-(trifluoromethoxy)phenoxy)piperidin-1-yl]phenoxy)methyl]-2,3-dihydroimidazo[2,1-b]oxazole, was developed by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan) as a new anti-tuberculosis agent for the treatment of multidrug-resistant tuberculosis (MDR-TB) (Sasaki et al., 2006), and it reduces mortality in affected patients (Diacon et al., 2011; Gler et al., 2012; Skripconoka et al., 2013). The action mechanism of delamanid is the inhibition of mycolic acid synthesis in the bacterium Mycobacterium tuberculosis (Matsumoto et al., 2006). Delamanid received regulatory approval in the European Union, Japan, and Korea in 2014 as part of combination therapy with a regimen of World Health Organization (WHO)-recommended second-line drugs.

An estimated 9 million people became sick with TB and 1.5 million people died from TB or TB-related causes in 2013 (WHO, 2014). Current TB treatment is long and cumbersome and requires ≥6 months of multidrug chemotherapy that typically uses first-line drugs, such as rifampicin, isoniazid, ethambutol, and pyrazinamide. Furthermore, MDR-TB may result when TB therapy is discontinued, interrupted or when improper drug regimens, including monotherapy, are prescribed (Blumberg et al., 2003). MDR-TB treatment may require >2 years of chemotherapy with less effective and more toxic second-line drugs (Horsburgh et al., 2000; Shin et al., 2004). Furthermore, there are many patients with TB-HIV/AIDS co-infection, and an estimated 1.1 million new TB cases were reported among people with HIV/AIDS (WHO, 2014). TB is still a leading cause of morbidity and mortality among patients with HIV/AIDS (Joint United Nations
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Programme on HIV/AIDS, 2013). Therefore, new drugs, such as delamanid, have been sought to improve the therapeutic value of TB chemotherapy.

As described above, concomitant treatment is the cornerstone of TB treatment, and delamanid will be administered with various drugs, including medicines for TB and HIV/AIDS. Ritonavir, nelfinavir, saquinavir, and amprenavir, which are HIV protease inhibitors, inhibit cytochrome P450 (CYP) activity (Granfors et al., 2006). Conversely, rifampicin induces CYP2B6 and CYP3A4 (Dooley et al., 2012). Alterations caused by efavirenz, an anti-retroviral drug known to be a CYP3A4 inducer, in the pharmacokinetics of co-administered drugs, lead to the lack of efficiency or adverse drug reactions of the victim drug (Deeks and Perry, 2010). Therefore, when administered concomitantly, these drugs may either increase or decrease the plasma concentrations of other co-administered drugs and their metabolites.

Drug-metabolizing enzymes have a vital role in eliminating drugs from the body. About 75% of drug-transformation reactions, i.e., metabolism, involve catalysis by CYP isozymes, which suggests that the CYP complex of enzymes is very important in drug metabolism (Flockhart, 2007; Guengerich and Rendic, 2010; Wienkers and Heath, 2005). In humans, CYP isozymes perform highly specific reactions, such as oxidation of androgens to estrogens for biofunctional control (Sasahara et al., 2007), and broad-spectrum oxidations of xenobiotics, such as drug metabolism in the liver (Paul and Ortiz de Montellano P, 2005).

Since coenzyme-dependent metabolites were hardly detected in human liver microsomes, CYP enzymes have not been thought to contribute much to the metabolism of delamanid (Matsumoto et al., 2006). Our companion paper (Shimokawa et al., 2015)
DMD#064527 reported that plasma albumin was involved in the metabolism of delamanid. Delamanid was rapidly degraded to M1, a unique metabolite formed by cleavage of the 6-nitro-2,3-dihydroimidazo[2,1-b]oxazole moiety, by incubation in human plasma at 37°C, with a half-life of 0.64 h. Furthermore, these metabolic activities of albumin were much higher in dogs and humans than in rodents, a finding that suggests species differences. However, pharmacokinetic and metabolic profiles for delamanid including evaluation of albumin contribution, have not been clarified.

It is important to understand the pharmacokinetic and metabolic profiles for new drugs in animals and humans so that the impact on efficacy and safety can be interpreted or predicted. In this study, we investigated the metabolic pathways, interspecies pharmacokinetics, and CYP enzymes that catabolize delamanid and characterized the pharmacokinetics and metabolism of delamanid in animals and humans.
Materials and Methods

Materials

Delamanid and its metabolites, DM-6705 (M1), (4RS,5S)-DM-6720 (M2), (S)-DM-6718 (M3), DM-6704 (M4), DM-6706 (M5), (4R,5S)-DM-6721 (M6), (4S,5S)-DM-6722 (M7), and (S)-DM-6717 (M8) were supplied by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan, Supplemental Methods and Supplemental Fig. 1). Microsomes prepared from baculovirus-infected insect cells expressing recombinant human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 were obtained from Becton Dickinson and Company (Franklin Lakes, NJ). Human liver microsomes were obtained from XenoTech LLC (Lenexa, KS). Furafylline, ticlopidine, sulfaphenazole, quinidine, ketoconazole, Tris–HCl buffer (pH 7.4), β-nicotinamide adenine dinucleotide, reduced form (NADH), and β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) were purchased from Sigma-Aldrich Co. (St Louis, MO). Benzylpirvanol was purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). Human serum was prepared from blood samples of 3 healthy male volunteers after approval by the Institutional Ethics Committee. Mouse (Institute of Cancer Research; ICR), rat (Sprague–Dawley; SD), and dog (beagle) sera were supplied from Kitayama Labes Co. (Nagano, Japan). Phosphate buffer (pH 7.4) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Other reagents and solvents were either special or high-performance liquid chromatography (HPLC) grade.

Animals and Humans

Male ICR mice at 4 or 5 weeks of age were supplied from Japan SLC, Inc. (Shizuoka, Japan) or Charles River Laboratories Japan Inc. (Yokohama, Japan). Male SD
rats at 5 weeks of age were supplied from Charles River Laboratories Japan, Inc. Male and female beagle dogs at 6 months old were supplied from Covance Research Products, Inc. (Cumberland, VA). Environmental conditions were set to maintain an air-exchange rate of 13–17 times/h and maintained at 18°C to 26°C with 30% to 80% relative humidity in the housing room that was lighted for 12 h (7:00–19:00) daily. Animals were individually housed and allowed free access to tap water via an automatic water supply system. Male mice and rats were provided with pelleted food (CRF-1, sterilized by radiation; Oriental Yeast Co., Ltd., Tokyo, Japan) ad libitum. Male and female dogs were supplied with 300 g/day of pellet diet (DS-A; Oriental Yeast Co., Ltd. or CD-5M; Clea Japan, Inc., Tokyo, Japan).

The animal experimental protocols and procedures were reviewed in accordance with Guidelines for Animal Care and Use in Otsuka Pharmaceutical Co., Ltd. and approved by the in-house Animal Ethics Committee. The human trial protocol, available with the full text of another report (Gler et al., 2012), was approved by independent ethics committees and institutional review boards for all sites. All male and female patients provided written informed consent in their native language before enrollment. The trial was performed in accordance with the Good Clinical Practice guidelines of the International Conference on Harmonization (ICH-GCP), adhered to the ethical principles of the Declaration of Helsinki, and was monitored by an independent data and safety monitoring committee.

Investigation of Metabolites

After repeated dosing at 100 mg/kg/day for 14 days to 6 male mice and 3 male rats, blood was withdrawn into heparinized syringes at 8 h and 24 h. Blood from male
and female dogs (n = 3, each sex) was withdrawn into heparinized syringes at 24 h after the final oral dosing at 100 mg/kg/day for 13 weeks. After centrifugation at approximately 1700 × g for 10 min at 4°C, the supernatants were stored at −15°C or below until use. Plasma samples for each sex and at each sampling point were equally mixed. After the plasma sample was mixed with an equal volume of acetonitrile, the mixture was centrifuged at 12000 × g for 5 min at 4°C. A 5-μL aliquot of the supernatant was analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). In addition, another 0.6-mL aliquot of the plasma sample was extracted with 5 mL of ethyl acetate by shaking for 10 min. After centrifugation as above, the organic layer was evaporated to dryness at 40°C. The residue was dissolved in 0.1 mL of acetonitrile/water (100:1, v/v) and sonicated, and then a 5-μL aliquot of the resulting solution was analyzed by LC-MS/MS and monitored by ultraviolet (UV) detection at 254 nm. Liquid chromatography used a TSKgel ODS-80Ts column (150 mm × 2.0 mm i.d., 5 μm, Tosoh Corp., Yamaguchi, Japan) with a binary gradient solvent system consisting of A: water/acetic acid (100:1, v/v) and B: acetonitrile/acetic acid (100:1, v/v); the chromatography was performed using a Nanospace SI-2 HPLC system (Shiseido Co., Ltd., Tokyo, Japan). The column temperature was maintained at 40°C, and the flow rate was 0.2 mL/min. LC eluate was introduced directly into an API3000 triple-quadrupole mass spectrometer (AB SCIEX, Foster, CA), equipped with an electrospray ionization interface operated in positive-ion mode with the following operation parameters: gas temperature, 475°C; gas flow rate, 7 L/min; gas pressure, 70 psi; ion-spray voltage, 4.5 kV; nebulizer gas, 12 units; curtain gas, 8 units; and collision gas, 8 units. Nitrogen was used in the ion source and the collision cell. A full scan from m/z 200 to 700, a precursor
ion scan at m/z 352, and a product-ion scan from the protonated molecules ([M+H]+) of analytes were performed.

**Exposure to Delamanid and its Metabolites**

Animal blood samples in the single-dose pharmacokinetic study were collected as follows: 1, 2, 4, 6, 8, 12, 24, 32, 48, 72, 96, 144, 192, 288, and 480 h after the single dosing at 3 mg/kg in mice and rats (n = 3) and 2, 4, 6, 8, 12, 24, 32, 48, 72, 96, 144, 192, 240, 288, 384, 480, 576, and 768 h after the single dosing at 10 mg/kg in dogs (n = 4). In the repeated dose study, the animal blood was collected at 2, 4, 6, 8, and 24 h on day 1 (mice and rats, n = 3), at 13 weeks (mice, n = 3), and 26 weeks (rats, n = 3), and 2 and 6 h on day 1 (dogs, n = 4), and 1, 2, 6, 8, and 24 h at 39 weeks (dogs, n = 4) after oral dosing at 30 mg/kg/day. The human blood was withdrawn at 0, 2, 3, 4, 10, 12, 13, 14, and 24 h after oral dosing on days 1, 14, 28, and 56 after oral dosing at 100 mg bis in die (BID; twice a day) in human (n = 144).

The blood samples were immediately placed in an ice bath and centrifuged at 1800 g for >10 min at 4°C to obtain plasma. The plasma samples were stored at −15°C or below until assay. The plasma concentrations of delamanid and its metabolites were simultaneously determined by LC-MS/MS and validated according to Food and Drug Administration guidance, including selectivity, accuracy, precision, recovery, calibration curve, post-preparative stability, freeze–thaw stability, short-term stability, and long-term stability. The sample analyses were performed under the optimal conditions of stability. The following typical method for the analysis of animal samples was used: to a 0.1-mL aliquot of plasma sample in an ice-water bath, 20 μL of internal standard solution (1 μg/mL, in-house compound) and 0.1 mL of phosphate buffer (0.2 M, pH 7.0) were added.
The mixture was extracted with 8 mL of tert-butyl methyl ether by shaking for 5 min. After centrifugation, the organic layer was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 0.15 mL of methanol/water/formic acid (50:50:1 v/v/v) and sonicated, and then a 5-μL aliquot of the resulting solution was analyzed by LC-MS/MS. Separation of the analytes was achieved with a SunFire C18 column (50 mm × 2.1 mm i.d., 3.5 μm; Waters Corp., Milford, MA) using a Waters 600S HPLC system (Waters Corp.). The mobile phases were 1 mM ammonium formate/formic acid (1000:2, v/v) (solvent A) and methanol (solvent B). The column temperature was maintained at room temperature, and the HPLC system was set to operate at a flow rate of 0.25 mL/min under linear-gradient conditions. The HPLC eluate was introduced directly into a triple-quadrupole mass spectrometer, TSQ-7000 (Thermo Fisher Scientific, Inc., San Jose, CA), and the mass spectrometer was operated in the positive electrospray ionization selected reaction monitoring (SRM) mode. The SRM mode was used with the following transitions: delamanid, m/z 535.2 → 352; M1, m/z 466.2 → 352; M2, m/z 482.2 → 352; M3, m/z 480.2 → 352; M4, m/z 467.2 → 352; M5, m/z 484.2 → 352; M6 and M7, m/z 483.2 → 305; and M8, m/z 481.2 → 305. The other parameters were as follows: spray voltage, 4.5 kV; electron-multiplier voltage, 1.45 kV; nitrogen sheath gas pressure, 80 psi; nitrogen auxiliary gas pressure, 10 (arbitrary units); argon collision gas pressure, 2.0 mTorr; capillary temperature, 260°C. Data acquisition and processing were performed using Xcalibur software version 1.2 (Thermo Fisher Scientific Inc.). The calibration curve ranges of delamanid for mouse, rat, and dog plasma were 6–2000 ng/mL, 3–1000 ng/mL, and 3–1000 ng/mL, respectively, and those of the metabolites were 6–600 ng/mL, 3–300 ng/mL, and 3–1000 ng/mL, respectively. In humans, the concentrations of
delamanid and its metabolites were determined using previously published LC-MS/MS methods after extraction by protein precipitation (Gler et al., 2012).

The pharmacokinetic parameters, maximum plasma concentration (C<sub>max</sub>), time to maximum (peak) plasma concentration (t<sub>max</sub>), area under the plasma concentration–time curve from 0 to 24 h (AUC<sub>0-24h</sub>), area under the plasma concentration–time curve calculated to the last observable concentration at time t (AUC<sub>0-t</sub>), area under the concentration–time curve from time zero to infinity (AUC<sub>0-∞</sub>), mean residence time from time zero to infinity (MRT<sub>0-∞</sub>), and terminal-phase elimination half-life (t<sub>1/2,z</sub>) were calculated with the aid of WinNonlin software (version 5.0.1 or 5.2, noncompartmental model; Pharsight Corp., Mountain View, CA).

Identification of Human CYP Isoforms

For recombinant studies, the following recombinant microsomes were used in duplicate: CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5. Each incubation contained recombinant microsomes (0.7 mg/mL, 50 nM of CYP protein), phosphate buffer (100 mM, pH 7.4), metabolites (10 μM M1 or M2), NADPH (2.5 mM), and NADH (2.5 mM) in a total volume of 0.5 mL. Incubation mixtures were pre-incubated at 37°C for 5 min, and reactions were started by adding a mixture of NADPH and NADH. After 0, 10, and 30 min of incubation at 37°C, the reactions were terminated with 1 mL of acetonitrile, containing the internal standard (IS; in-house compound). After centrifugation, the supernatant was analyzed by LC-MS/MS.

For chemical inhibition studies, the following inhibitors were used in duplicate: furafylline (CYP1A2), ticlopidine (CYP2B6), sulfaphenazole (CYP2C9), benzylirivanol
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(CYP2C19), quinidine (CYP2D6), and ketoconazole (CYP3A4). Each incubation contained human liver microsomes (1.0 mg/mL), inhibitors (0, 1, and 10 μM), phosphate buffer (100 mM, pH 7.4), metabolites (10 μM M1 or M2), NADPH (2.5 mM), and NADH (2.5 mM) in a total volume of 0.5 mL. Incubation mixtures were preincubated at 37°C for 5 min, and reactions were started by adding a mixture of NADPH and NADH. For furafylline and ticlopidine, the prereaction was performed without delamanid derivative for 15 min. Reactions were performed at 37°C for a set time period within the time linearity limit (M2 from M1 for 40 min and M3 from M2 for 180 min). After the addition of 1 mL of acetonitrile containing IS, the solution was centrifuged, and the supernatant was analyzed by LC-MS/MS, referring to the animal methods.

**Binding of Metabolites to the Serum**

The in vitro binding of M1, M4, and M5 at the concentrations of 500 and 5000 ng/mL to animal and human sera was determined by equilibrium dialysis using a Spectra/Por2 molecular porous dialysis membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA). Because M1, M4 and M5, unlike delamanid, are stable in the serum at 37°C, equilibrium dialysis was conducted under the condition for 8 h at 37°C. After dialysis for protein binding, an aliquot of the dialyzed protein and dialysate in the 2 devices was sampled to determine the concentrations in the bound and unbound fractions. Cooled tert-butyl ether (mouse and rat) or diethyl ether (dog and human) containing IS was added to the sample and shaken for >5 min. The organic layer was evaporated, and the residue was dissolved in water/methanol/formic acid (50:50:1, v/v/v). The supernatant was analyzed using the modified LC-MS/MS method as described above.
Results

Investigation of Metabolites in Plasma

Initially we performed full-scan LC-MS analysis using the plasma extracts from mice, rats, and dogs following repeated oral doses of delamanid, but no peaks were detected. Delamanid formed the \([M + H]^+\) at \(m/z\) 535 in positive-ion scan mode, and the fragment ions of delamanid were mainly observed at \(m/z\) 352 in the product-ion scan at \(m/z\) 535 (Supplemental Fig. 2). Therefore, a precursor-ion scan at \(m/z\) 352 was performed, and 8 metabolites, M1 to M8, were detected in male dog plasma (Fig. 1A), as well as female one. Sex difference was not observed. The intensities of these peaks monitored at UV 254 nm were much lower than the intensity of delamanid, and no other remarkable peak was observed in the dogs (Fig. 1B). In addition, the mouse and rat plasma also showed no remarkable metabolites other than M1 to M8 (data not shown). The chemical structures of these metabolites were determined by matching retention times, parent \(m/z\) ions, and MS/MS fragmentation patterns with those of the authentic compounds (Table 1, Supplemental Fig. 2).

Single-dose Pharmacokinetics of Delamanid and Metabolites

Fig. 2 and Table 2 present information characterizing the pharmacokinetic behavior of an oral dose of delamanid in mice, rats, and dogs. After the administration of a single 3 mg/kg delamanid to mice, the maximum plasma concentration of delamanid (478.7 ng/mL) occurred at 2 h, followed by a decline in the plasma level with elimination \(t_{1/2,z}\) of 7.2 h. In the rats, delamanid (3 mg/kg) absorption had a \(t_{max}\) of 4 h and a \(C_{max}\) of 600.5 ng/mL. The \(t_{1/2,z}\) (5.1 h) was similar to that obtained in the mice. In the dogs treated with oral delamanid (10 mg/kg), the \(t_{max}\) and \(C_{max}\) were 8 h and 357.8 ng/mL, respectively.
The t_{1/2,z} in dogs was 18.4 h, which was longer than that in the rodents. In all animal species, the plasma concentrations of the delamanid metabolites were much less than the concentration of the parent compound. The metabolites appeared slowly in plasma, and had very long MRT and elimination half-lives in the dogs (Figs. 2C-1 and C-2), particularly M2, M3, and M8 (t_{max}: 156–456 h, MRT_{0-\infty}: 413.8–1488.0 h, t_{1/2,z}: 229.2–884.2 h).

**Multiple-dose Pharmacokinetics of Delamanid and Metabolites**

Table 3 lists the mouse, rat, dog, and human C_{max} and AUC values. The C_{max} of delamanid in male mouse plasma reached 2920.9 ng/mL at 6 h after repeated administration of 30 mg/kg/day. The C_{max} in the male rat plasma reached 1799.2 ng/mL at 4 h. The extent of delamanid absorption (C_{max} and AUC) did not alter significantly on multiple administrations in the mice and rats. The C_{max} of delamanid in male dog plasma reached 1400.7 ng/mL at 3 h after repeated administration at 30 mg/kg/day, and decreased with time. There was no remarkable difference between the ratios of delamanid between males and females in the rodents and dogs (data not shown). The C_{max} of delamanid in the human plasma reached 135 and 414 ng/mL after single and repeated administration at 100 mg BID, respectively. Approximately 3.7- and 3.1-fold delamanid accumulation was observed after repeated administration in dogs and humans, respectively (Table 3, Fig. 3). There was no difference among the C_{max} and AUC values for delamanid on days 14 to 56 in humans; therefore, a steady-state delamanid concentration was reached at \leq 14 days (Fig. 3).

Delamanid was metabolized into M1 to M8 in animals and humans. The metabolites accumulated in rodents and especially in dogs during repeated daily
administration. The AUC level of each metabolite was ≤10% of the total exposure in the rodents even after multiple doses. Following oral administration of multiple doses of delamanid to the dogs and humans, delamanid metabolites M1 and M3 appeared predominantly in the plasma and accounted for about 17% of the total exposure in dogs and about 13%–18% in humans.

**Identification of Human CYP Isoforms**

The major circulating metabolites of delamanid in humans were M1 and M3. The metabolite M1 has been shown to be produced from delamanid by albumin in plasma (Shimokawa et al., 2015) via extrahepatic metabolism. We further examined the types of CYP enzymes involved in the formation of M3 from M1 via M2 in humans using recombinant CYP enzymes and human liver microsomes with CYP inhibitors.

Among the 11 recombinant human CYP enzymes studied (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5), CYP1A1, CYP3A4, CYP2D6, and CYP2E1 time-dependently catalyzed the hydroxylation of M1 to M2 (Fig. 4A). No production of M2 was detected in the other CYP expression microsomes. Furthermore, CYP1A1 and CYP3A4 showed time-dependent metabolism of M2 to M3, and in the other CYPs, no production of M3 was detected (Fig. 4B). The metabolic activity of hydroxylation and dehydrogenation in recombinant human CYP1A1 was the highest among the 11 recombinant human CYP enzymes examined.

In the assay containing specific inhibitors for CYP (furafylline, ticlopidine, sulfaphenazole, benzylnirvanol, quinidine, and ketoconazole), only ketoconazole inhibited the metabolism of M1 to M2 and M2 to M3 in a dose-dependent manner,
whereas other inhibitors were ineffective (Fig. 5). These results indicated that CYP3A4 was mainly responsible for M2 and M3 formations in humans.

**Binding of Metabolites to Serum**

The *in vitro* protein binding of M1, M4, and M5 in mouse, rat, dog, and human sera is shown in Table 4. Calculation of protein binding for almost all metabolites at concentrations of 500 ng/mL was not performed because the metabolite levels in the buffer side were extremely low, which indicated high protein binding. At 5000 ng/mL, M1, M4, and M5 showed high protein binding (98.7%–99.8%). No differences in the metabolite intersubstrate and interspecies in addition to delamanid were observed.
Discussion

Eight compounds were detected as circulating metabolites after repeated oral administration of delamanid in mice, rats, and dogs (Fig. 1). Drug-related peaks, except those for delamanid and its 8 metabolites, were not observed on UV chromatograms (Fig. 1B), which indicated that there were no other significant metabolites in the plasma. All metabolites had a common product ion at $m/z$ 352 (Table 1), which is produced by the loss of the nitro-dihydro-imidazooxazole moiety of delamanid (Supplemental Fig. 2). Delamanid, M1, M2, M4, M5, M6, M7, and M8 gave the characteristic and intense product ions at $m/z$ 357, 288, 302, 289, 306, 305, 305, and 303, respectively (Table 1), which were generated by the loss of the trifluoromethoxy phenol moiety (Supplemental Fig. 2). These results suggest that the nitro-dihydro-imidazooxazole moiety is the main metabolic target site of delamanid. An analog of delamanid, PA-824, which currently is being developed in a clinical trial for TB therapy, has a 2-nitro-imidazooxazine as well. PA-824 is metabolized mainly at the nitro-imidazole moiety in the liver (Dogra et al., 2011); hence, delamanid and PA-824 have a common metabolic target site, which suggests that this moiety has a position with high metabolic reactivity.

After consideration of our findings, we propose metabolic pathways of delamanid. In general, metabolic reactions, such as a monooxygenase reaction and hydrogen abstraction reaction, are simple. In contrast, metabolic reactions of delamanid and M1 are complicated. Our most recent evidence indicates that delamanid is rapidly degraded to M1 by incubation in human plasma at 37°C with a half-life of 0.64 h via a plasma albumin-mediated reaction (Shimokawa et al., 2015). NADPH-dependent metabolites have been hardly detected in human liver microsomes (Matsumoto et al., 2006).
Therefore, albumin is considered to mainly produce M1 from delamanid. Moreover, all metabolites (M1 to M8) were detected in the animals following M1 administration (data not shown), which suggested that M1 is a crucial starting point of the metabolic pathway.

In this study, following oral administration of delamanid to animals and humans, M1 to M8 were detected and identified in plasma. After considering these findings, delamanid is thought to be primarily formed by hydrolytic cleavage of the hydroimidazo-oxazole moiety to (R)-2 amino-4,5-dihydrooxazole derivative (M1) and further catalyzed by 3 pathways (Fig. 6A). The first metabolic pathway (pathway 1) is hydroxylation of the oxazole moiety (M2) followed by oxidation of hydroxyl group and tautomerization of oxazole to a imino-ketone metabolite (M3, Fig. 6B-1). The second metabolic pathway (pathway 2) is hydrolysis and deamination of the oxazole amine (M4, Fig. 6B-2) followed by hydroxylation to M6 and M7 and oxidation of oxazole to another ketone metabolite (M8). The third metabolic pathway (pathway 3) is hydrolytic cleavage of the oxazole ring (M5, Fig. 6B-3).

After repeated administration of delamanid, the highest exposure in human subjects was to the parent compound (40% of the total exposure) followed by the metabolites M1 (18%) and M3 (13%) (Table 3). M1 showed the highest exposure among the 8 metabolites after repeated oral dosing in humans, which indicated that M1 is the predominant metabolite. The proposed major metabolic pathway of delamanid in humans is considered to be pathway 1. The exposures of M3 (17%), M1 (17%), M8 (13%), and M2 (12%) were high in the male dog plasma after repeated administration, which suggested that pathway 1 is the most important in dogs, followed by pathway 2. Conversely, exposure to M7 (4%–10%) was high in mice and rats, which suggested that
pathway 2 is the most important in rodents. We consider that the metabolic pathways in dogs were similar to those in humans but dissimilar to those in rodents.

Qualitatively, the 8 metabolites were commonly observed in the animals and humans evaluated. However, there was a quantitative difference among the species (Table 3). The exposure ratios of the AUCs of all metabolites (M1–M8) to the total AUC_{0-24h} were much higher in dogs (71%) and humans (60%) than in rodents (15%–18%), which indicated that dog metabolism is quantitatively similar to that of humans (Table 3). Regarding the species differences, a larger amount of M1, which is a crucial starting point in the metabolic pathway, was generated in dogs and humans than in rodents (Table 3). An interesting finding was that the formation rates of M1 in dogs and humans were much higher than those in rodents \textit{in vitro} via the plasma albumin-mediated reaction (Shimokawa et al., 2015). This difference in M1 formation is likely to have a significant impact on the subsequent metabolism of delamanid.

Protein binding is an important factor regarding interspecies comparison of systemic exposure. Therefore, we determined plasma protein bindings of M1, M4, and M5 which are leading metabolites in each pathway. We found that there were no differences in the protein bindings of delamanid (Shimokawa et al., 2015) and these metabolites among mice, rats, dogs, and humans (Table 4). The presence or absence of correction for protein binding has limited effectiveness as long as interspecies comparison of systemic exposure is concern. The metabolites in humans were observed commonly in experimental animals; hence, the experimental animals could receive sufficient exposure to the metabolites by increasing the dose of delamanid. The interspecies pharmacokinetic profiles suggest that the animals were appropriately
selected for the safety assessment of delamanid and its metabolites.

In humans, systemic exposure to delamanid after multiple oral dosing was 3.1 times higher than that after single administration, and a steady-state exposure was reached at 14 days (Fig. 3). In dogs like human, plasma levels of delamanid and also the metabolites increased during multiple dosing. In particular, the increases in M1, M2, M3, and M8 were extraordinarily large (Table 3). The accumulation is because of the extended half-lives ($t_{1/2,z}$: 108.0–884.2 h) of M1, M2, M3, and M8 (Fig. 2, Table 2). A possible explanation is that M1 distributes rapidly and becomes highly bound to many tissues. Because of the high affinity, moving back to the plasma compartment would be slow. This rapid tissue distribution and slow return to the plasma compartment may result in the extended half-lives in plasma. In fact, radioactive concentration in almost all tissues was higher than that in plasma following the administration of radiolabeled delamanid to rats (Miyamoto et al., 2005). Representative organs such as lung (target organ), liver and kidney showed high M1 ratio to total radioactivity, whereas M1 was seen at a very low level in the plasma. Therefore, many tissues exhibited an extremely high distribution of M1 compared with the plasma. Moreover, M1 is likely oxidized after liver uptake, and the oxidized metabolites are returned more slowly to the plasma compartment.

We also carefully examined the activities of CYP isoforms involved in pathway 1, the major metabolic pathway in humans. The *in vitro* metabolism of M1 and its oxide (M2) was investigated using human recombinant CYP isoforms. Recombinant CYP1A1 and CYP3A4 converted these compounds to the oxidized metabolite, but other recombinant CYPs had little metabolic activity (Fig. 4). Furthermore, we investigated the inhibition of formation of metabolites using CYP chemical inhibitors in human liver.
microsomes. Ketoconazole, a CYP3A4 inhibitor, decreased hydroxylation of M1 to M2 and oxidation of M2 to M3 in human liver microsomes in a concentration-dependent manner, whereas the other chemical inhibitors for other CYPs did not show any appreciable degree of inhibition (Fig. 5). On the basis of these studies, CYP3A4 is considered to be the major CYP isoform responsible for M2 and M3 formations. In addition, CYP3A4 was responsible for the reactions that formed M7 from M4 and M8 from M6 in the liver (data not shown), which involve similar hydroxylation and oxidation of the oxazole moiety. Conversely, CYP1A1 is not presumed to be principally involved in the metabolism because of the extremely low amounts of CYP1A1 in the human liver (Schweikl et al., 1993; Imaoka et al., 1996). Therefore, CYP3A4 was thought to play an important role in the overall metabolism from M1. Nevertheless, we consider that delamanid and the key metabolite, M1, are less affected by inhibitor CYPs, probably because the metabolism of delamanid to M1 is due to the high contribution of albumin, a nonhepatic process, and M1 has multiple metabolic pathways. No significant changes in delamanid and M1 exposure occurred when delamanid was co-administered with lopinavir/ritonavir, a CYP3A4 inhibitor (Paccaly et al., 2012). Furthermore, delamanid is not affected by efavirenz, a CYP3A4 inducer (Petersen et al., 2012). On the other hand, PA-824 exposures are substantially reduced by concomitant efavirenz while lopinavir/ritonavir had minimal effect on PA-824 exposures (Dooley et al., 2014). These findings suggest that even though albumin metabolism may occur with PA-824, the contribution of albumin on PA-824 metabolism is lower than that for delamanid. These metabolic features of delamanid are a key point of differentiation from many other drugs, which are catalyzed mainly by CYPs.
DMD#064527

In conclusion, delamanid is primarily degraded to M1 by albumin in plasma and further catalyzed by 3 metabolic pathways, which indicates that M1 is a crucial starting point. M1 had the highest exposure among the 8 metabolites detected after repeated oral dosing in humans. M1 was subsequently oxidized to M3 via M2 mainly by CYP3A4. The pharmacokinetics and overall metabolism of delamanid show species differences, which are probably caused by the metabolic activity of albumin on delamanid. We concluded that M1 formation caused by albumin is the most important contributor to the pharmacokinetics and metabolism of delamanid. Clinically significant drug–drug interactions of delamanid and M1 with other drugs are considered to be limited.
Acknowledgments

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Authorship Contributions

Participated in research design: Sasahara, Shimokawa, Hirao, Koyama, Kitano, and Umehara

Conducted experiments: Sasahara, Shimokawa, Hirao, Koyama, and Kitano

Contributed new reagents or analytic tools: Sasahara, Shimokawa, Hirao, Koyama, and Kitano

Performed data analysis: Sasahara, Shimokawa, Hirao, Koyama, Kitano, and Shibata

Wrote or contributed to the writing of the manuscript: Sasahara, Shimokawa, Hirao, Koyama, and Kitano
References


Drug PA-824 with Concomitant Lopinavir-Ritonavir, Efavirenz, or Rifampin.


Sasaki H, Haraguchi Y, Itotani M, Kuroda H, Hashizume H, Tomishige T, Kawasaki M,


Legends for Figures

Fig. 1. Chromatograms of the precursor-ion scan at $m/z$ 352 (A) and LC-UV at 254 nm (B) obtained from extracted dog plasma.

Plasma was collected at 24 h after daily oral dosing of delamanid at 100 mg/kg/day for 13 weeks.

Fig. 2. Plasma concentration–time profiles of delamanid and its metabolites in mice (A), rats (B), and dogs (C).

Delamanid was administered orally at single doses of 3 mg/kg (mice and rats, $n=3$) and 10 mg/kg (dogs, $n=4$). The horizontal axis represents time after administration from 0 to 48 h (A, B, C-1) and to 768 h (C-2). Each data point represents the mean + standard deviation.

Fig. 3. Changes in $C_{\text{max}}$ and AUC$_{0-24\text{h}}$ of delamanid in humans during multiple administrations for 56 days.

Delamanid was administered orally at 100 mg BID. Each data point represents the mean + standard deviation ($n=144$). AUC$_{0-24\text{h}}$ was calculated from the first dosing of BID on each day to 24 h.

Fig. 4. Hydroxylation of M1 to M2 (A) and dehydrogenation of M2 to M3 (B) catalyzed by human CYP isozymes.

Recombinant CYP enzyme (50 nM) was assayed with 10 μM delamanid metabolite at 37°C for 30 min. The product was monitored by LC-MS/MS (mean peak area ratio of
product and internal standard peak area, \( n = 2 \)).

Fig. 5. Effects of various CYP inhibitors on hydroxylation of M1 (A) and dehydrogenation of M2 (B) in human liver microsomes.

Human liver microsomes (1 mg/mL) were assayed with 10 \( \mu \)M delamanid metabolite in the presence of a chemical inhibitor at 37°C. The product was measured by LC-MS/MS (mean, \( n = 2 \)).

Fig. 6. Proposed metabolic pathways of delamanid (A) and metabolic mechanism of M2 to M3 (B-1), M1 to M4 (B-2), and M1 to M5 (B-3) in human.

Main reactions are symbolized by bold arrows. M1 to M3, M1 to M8, and M1 to M5 were defined as pathway 1, pathway 2, and pathway 3, respectively.
Table 1. Identified metabolites of delamanid.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention Time (min)</th>
<th>Chemical formula</th>
<th>Molecular weight</th>
<th>Structure and MS/MS product ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delamanid</td>
<td>22.8</td>
<td>( \text{C}<em>{23}\text{H}</em>{25}\text{F}_3\text{N}_4\text{O}_6 )</td>
<td>534.48</td>
<td>![Structure of Delamanid]</td>
</tr>
<tr>
<td>M1 (DM-6705)</td>
<td>17.3</td>
<td>( \text{C}<em>{23}\text{H}</em>{26}\text{F}_3\text{N}_3\text{O}_4 )</td>
<td>465.47</td>
<td>![Structure of M1]</td>
</tr>
<tr>
<td>M2 ((4R,5S)-DM-6720)</td>
<td>16.7</td>
<td>( \text{C}<em>{23}\text{H}</em>{26}\text{F}_3\text{N}_3\text{O}_5 )</td>
<td>481.46</td>
<td>![Structure of M2]</td>
</tr>
<tr>
<td>M3 ((S)-DM-6718)</td>
<td>17.8</td>
<td>( \text{C}<em>{23}\text{H}</em>{24}\text{F}_3\text{N}_3\text{O}_5 )</td>
<td>479.45</td>
<td>![Structure of M3]</td>
</tr>
<tr>
<td>M4 (DM-6704)</td>
<td>19.8</td>
<td>( \text{C}<em>{23}\text{H}</em>{25}\text{F}_3\text{N}_2\text{O}_5 )</td>
<td>466.45</td>
<td>![Structure of M4]</td>
</tr>
<tr>
<td>M5 (DM-6706)</td>
<td>16.7</td>
<td>( \text{C}<em>{23}\text{H}</em>{28}\text{F}_3\text{N}_3\text{O}_5 )</td>
<td>483.48</td>
<td>![Structure of M5]</td>
</tr>
<tr>
<td>M6 and M7 ((4R,5S)-DM-6721 and (4S,5S)-DM-6722)</td>
<td>18.6</td>
<td>( \text{C}<em>{23}\text{H}</em>{25}\text{F}_3\text{N}_2\text{O}_6 )</td>
<td>482.45</td>
<td>![Structure of M6 and M7]</td>
</tr>
<tr>
<td>M8 ((S)-DM-6717)</td>
<td>21.6</td>
<td>( \text{C}<em>{23}\text{H}</em>{25}\text{F}_3\text{N}_3\text{O}_6 )</td>
<td>480.43</td>
<td>![Structure of M8]</td>
</tr>
</tbody>
</table>
Table 2. Pharmacokinetic parameters of delamanid and its metabolites after single oral administration in the mouse, rat, and dog

<table>
<thead>
<tr>
<th>Species</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</th>
<th>t&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-t&lt;/sub&gt; (ng·h/mL)</th>
<th>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (ng·h/mL)</th>
<th>MRT&lt;sub&gt;0-∞&lt;/sub&gt; (h)</th>
<th>t&lt;sub&gt;1/2,z&lt;/sub&gt; (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Delamanid</td>
<td>478.7</td>
<td>2</td>
<td>5536.0</td>
<td>6150.8</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>8.5</td>
<td>12</td>
<td>113.0</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Rat</td>
<td>Delamanid</td>
<td>600.5</td>
<td>4</td>
<td>7941.8</td>
<td>7969.8</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>4.1</td>
<td>6</td>
<td>25.6</td>
<td>44.6</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>M7</td>
<td>4.5</td>
<td>12</td>
<td>57.0</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Dog</td>
<td>Delamanid</td>
<td>357.8</td>
<td>8</td>
<td>10628.0</td>
<td>10927.5</td>
<td>27.6</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>10.9</td>
<td>36</td>
<td>1158.9</td>
<td>1850.1</td>
<td>174.9</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>6.0</td>
<td>156</td>
<td>1155.6</td>
<td>2433.9</td>
<td>413.8</td>
</tr>
<tr>
<td></td>
<td>M3</td>
<td>16.3</td>
<td>240</td>
<td>6966.1</td>
<td>8272.3</td>
<td>491.4</td>
</tr>
<tr>
<td></td>
<td>M4</td>
<td>6.6</td>
<td>27</td>
<td>214.3</td>
<td>495.6</td>
<td>63.4</td>
</tr>
<tr>
<td></td>
<td>M5</td>
<td>2.6</td>
<td>84</td>
<td>205.6</td>
<td>1286.0</td>
<td>229.5</td>
</tr>
<tr>
<td></td>
<td>M7</td>
<td>2.1</td>
<td>32</td>
<td>64.2</td>
<td>501.6</td>
<td>122.4</td>
</tr>
<tr>
<td></td>
<td>M8</td>
<td>5.2</td>
<td>456</td>
<td>1582.8</td>
<td>6940.3</td>
<td>1488.0</td>
</tr>
</tbody>
</table>

NC: not calculated

Values are the mean of n = 3 (mouse and rat) and n = 4 (dog).

Data for quantified metabolites are summarized.
Table 3. Species differences in the systemic exposure in the mouse, rat, dog, and human after initial or multiple oral administrations

<table>
<thead>
<tr>
<th>Species</th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg/day)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>100 mg BID</td>
</tr>
<tr>
<td>Study duration</td>
<td>Initial</td>
<td>13 Weeks</td>
<td>Initial</td>
<td>26 Weeks</td>
</tr>
<tr>
<td>Number of subject</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

**C<sub>max</sub> (ng/mL)<sup>a</sup>**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delamanid</td>
<td>2314.1 (95)</td>
<td>2920.9 (90)</td>
<td>2695.3 (97)</td>
<td>1799.2 (82)</td>
</tr>
<tr>
<td>M1</td>
<td>66.6 (3)</td>
<td>135.6 (5)</td>
<td>26.2 (1)</td>
<td>40.7 (2)</td>
</tr>
<tr>
<td>M2</td>
<td>2.4 (0)</td>
<td>25.7 (1)</td>
<td>ND (0)</td>
<td>4.1 (0)</td>
</tr>
<tr>
<td>M3</td>
<td>ND (0)</td>
<td>2.0 (0)</td>
<td>ND (0)</td>
<td>ND (0)</td>
</tr>
<tr>
<td>M4</td>
<td>2.8 (0)</td>
<td>10.3 (0)</td>
<td>2.8 (0)</td>
<td>13.0 (1)</td>
</tr>
<tr>
<td>M5</td>
<td>10.6 (0)</td>
<td>46.8 (2)</td>
<td>6.1 (0)</td>
<td>33.6 (2)</td>
</tr>
<tr>
<td>M6</td>
<td>ND (0)</td>
<td>2.5 (0)</td>
<td>ND (0)</td>
<td>18.7 (1)</td>
</tr>
<tr>
<td>M7</td>
<td>23.8 (1)</td>
<td>74.4 (3)</td>
<td>39.8 (2)</td>
<td>215.3 (11)</td>
</tr>
<tr>
<td>M8</td>
<td>ND (0)</td>
<td>4.6 (0)</td>
<td>ND (0)</td>
<td>32.5 (2)</td>
</tr>
</tbody>
</table>

**AUC<sub>0-24h</sub> (ng·h/mL)<sup>a</sup>**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mouse</th>
<th>Rat</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delamanid</td>
<td>35840.3 (95)</td>
<td>36509.4 (85)</td>
<td>36639.7 (98)</td>
<td>34237.9 (82)</td>
</tr>
<tr>
<td>M1</td>
<td>1148.2 (3)</td>
<td>2512.8 (7)</td>
<td>409.2 (1)</td>
<td>792.7 (2)</td>
</tr>
<tr>
<td>M2</td>
<td>19.2 (0)</td>
<td>496.4 (1)</td>
<td>NC (0)</td>
<td>76.8 (0)</td>
</tr>
<tr>
<td>M3</td>
<td>NC (0)</td>
<td>4.0 (0)</td>
<td>NC (0)</td>
<td>NC (0)</td>
</tr>
<tr>
<td>M4</td>
<td>29.4 (0)</td>
<td>111.5 (0)</td>
<td>25.2 (0)</td>
<td>256.7 (1)</td>
</tr>
<tr>
<td>M5</td>
<td>109.1 (0)</td>
<td>957.1 (2)</td>
<td>48.8 (0)</td>
<td>645.2 (2)</td>
</tr>
<tr>
<td>M6</td>
<td>NC (0)</td>
<td>30.8 (0)</td>
<td>NC (0)</td>
<td>329.2 (1)</td>
</tr>
<tr>
<td>M7</td>
<td>403.0 (1)</td>
<td>1443.7 (4)</td>
<td>380.1 (1)</td>
<td>3954.0 (10)</td>
</tr>
<tr>
<td>M8</td>
<td>NC (0)</td>
<td>54.4 (0)</td>
<td>NC (0)</td>
<td>682.6 (2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The molar ratio of each analyte to the total exposure (%) is shown in parentheses.

<sup>b</sup> These values were calculated using higher concentration between two sampling points (2 or 6 h).

<sup>c</sup> Data were reported by Gler et al. (2012).

<sup>d</sup> AUC was not calculated because of an insufficient number of sampling points (2 or 6 h).

ND, not detected; NM, not measured; NC, not calculated.
Table 4. Protein binding of delamanid and metabolites in the mouse, rat, dog, and human

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration (ng/mL)</th>
<th>Protein binding (%)</th>
<th>Delamanid</th>
<th>M1</th>
<th>M4</th>
<th>M5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>500</td>
<td>99.5</td>
<td>NC</td>
<td>NC</td>
<td>98.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>99.6</td>
<td>99.7</td>
<td>99.6</td>
<td>98.8</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>500</td>
<td>99.6</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>99.6</td>
<td>99.4</td>
<td>99.7</td>
<td>98.9</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>500</td>
<td>99.5</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>99.3</td>
<td>99.6</td>
<td>99.8</td>
<td>99.3</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>500</td>
<td>99.5</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>99.6</td>
<td>99.7</td>
<td>99.6</td>
<td>99.2</td>
<td></td>
</tr>
</tbody>
</table>

* Data were reported by Shimokawa et al. (2015).
NC: not calculated (the concentration of the buffer side was below 6 ng/mL)
Values are the mean of n = 3.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure in Table 1