Metabolic Mechanism of Delamanid, a New Anti-Tuberculosis Drug, in Human Plasma

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AGP, α₁-acid glycoprotein; DSA, dog serum albumin; HPLC, high performance liquid chromatography; HSA, human serum albumin; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MS, mass spectrometry; RSA, rat serum albumin; TB, tuberculosis
Abstract

The metabolism of delamanid (OPC-67683, Deltyba<sup>TM</sup>), a novel treatment for multidrug-resistant tuberculosis, was investigated in vitro using plasma and purified protein preparations from humans and animals. Delamanid was rapidly degraded by incubation in the plasma of all species tested at 37°C, with half-life values (hours) of 0.64 (human), 0.84 (dog), 0.87 (rabbit), 1.90 (mouse), and 3.54 (rat). A major metabolite, M1, was formed in plasma by cleavage of the 6-nitro-2,3-dihydroimidazo[2,1-b]oxazole moiety of delamanid. Rate of M1 formation increased with temperature (0−37°C) and pH (6.0−8.0). Delamanid was not converted to M1 in plasma filtrate with a molecular mass cutoff of 30 kDa, suggesting that bioconversion is mediated by plasma proteins of higher molecular weight. When delamanid was incubated in plasma protein fractions separated by gel filtration chromatography, M1 was observed in the fraction consisting of albumin, γ-globulin, and α<sub>1</sub>-acid glycoprotein. In pure preparations of these proteins, only human serum albumin (HSA) metabolized delamanid to M1. The formation of M1 followed Michaelis–Menten kinetics in both human plasma and HSA solution with similar K<sub>m</sub> values, 67.8 µM in plasma and 51.5 µM in HSA. The maximum velocity and intrinsic clearance values for M1 were also comparable in plasma and HSA. These results strongly suggest that albumin is predominantly responsible for metabolizing delamanid to M1. We propose that delamanid degradation by albumin begins with nucleophilic attack of amino acid residues on the electron-poor carbon at 5 position of nitro-dihydro-imidazooxazole, followed by cleavage of the imidazooxazole moiety to form M1.
Introduction

Delamanid (OPC-67683, Deltyba™, nitro-dihydro-imidazooxazole derivative) is a new anti-tuberculosis drug for the treatment of multidrug-resistant tuberculosis. The in vitro metabolism of delamanid using human and animal liver microsomes has already been evaluated (Matsumoto et al., 2006). When delamanid was incubated with liver microsomes in the presence of reduced nicotinamide adenine dinucleotide phosphate, metabolites were nearly undetectable in the incubation mixture, suggesting that delamanid was not metabolized by cytochrome P450 enzymes. However, eight metabolites, including the abundant metabolite (R)-2-amino-4,5-dihydrooxazole derivative [DM-6705 (M1)], in human and animal plasma were detected and identified in the investigation for the in vivo pharmacokinetics and metabolism of delamanid as described in the companion manuscript (Sasahara et al., 2015) in this issue of Drug Metabolism and Disposition. The maximum plasma concentration of M1 was nearly half that of delamanid (0.32 μM vs. 0.78 μM) following twice daily administration of 100 mg delamanid for 56 days (Gler et al., 2012; Sasahara et al., 2015; Shimokawa et al., 2014), suggesting that M1 is the major metabolite. On the basis of the chemical structure of M1, it is proposed that delamanid is cleaved directly at its 6-nitro-2,3-dihydroimidazo[2,1-b]oxazole moiety by some extrahepatic mechanism (Matsumoto et al., 2006).

According to World Health Organization (WHO) guidelines, a novel drug for drug-resistant tuberculosis (TB) should be used for long-term administration as an add-on therapy to at least 3 or more other anti-TB drugs to prevent the development of resistance (WHO, 2011). It is important to identify the enzymes responsible for the metabolism of delamanid in humans. In the current study, biotransformation was first examined in animal and human plasma, and then the metabolic byproduct was identified by detection of radioactivity and simultaneous mass spectrometry (MS). Effects of temperature and pH on the formation of M1...
and the rates of delamanid metabolism by various plasma protein fractions isolated by ultrafiltration and gel filtration were also investigated to identify the responsible enzymes. Finally, the kinetic parameters of M1 production were compared between human plasma and specific plasma proteins, which identified albumin as a major mediator of delamanid degradation. This is the first report describing the in vitro mechanism of delamanid metabolism in plasma.

**Materials and Methods**

**Materials**

$^{14}$C-Delamanid, delamanid, and its metabolite M1 were obtained from Otsuka Pharmaceutical Co., Ltd. The chemical structure and labeled position of $^{14}$C-delamanid are shown in Fig. 1. The specific radioactivity of $^{14}$C-delamanid was 4.14 MBq/mg, and the radiochemical purity was 99.2% as determined by high-performance liquid chromatography (HPLC). A detailed synthesis and spectral characterization of M1 are shown in Supplemental Methods. Human plasma using heparin as anticoagulant and human serum were prepared from three healthy males with approval of the Institutional Ethics Committee. Heparin plasma and serum from male mouse (Institute of Cancer Research), rat (Sprague Dawley), rabbit (New Zealand White), and dog (beagle) were supplied by Kitayama Labes Co. (Nagano, Japan). These animals were used as the preclinical species in pharmacology, pharmacokinetics, and toxicology studies for delamanid. Purified human serum albumin (HSA), essentially fatty acid free prepared from serum Fraction V (product no A1887), rat serum albumin from Fraction V (RSA, A6272), canine serum albumin from Fraction V [dog serum albumin (DSA), A9263], human $\gamma$-globulin (G4386), and $\alpha_1$-acid glycoprotein [(AGP), G9885] were purchased from Sigma-Aldrich Co. (St. Louis, MO). Other reagents were commercially available and of analytical grade.
Metabolism of Delamanid in Plasma

$^{14}$C-Delamanid was dissolved in methanol at 2.07 MBq/0.5 mg/mL. The reaction mixture consisted of $^{14}$C-delamanid (5 µg/mL, 9.3 µM) and mouse, rat, rabbit, dog, or human plasma. The final solvent concentration was 1% (v/v). After preincubation of plasma at 37°C for 3 min, the reaction was started by adding $^{14}$C-delamanid (5 µg/mL). Incubation at 37°C was continued for 0, 0.5, 1, 2, and 4 h. Non-labeled delamanid (50 µg/mL) was also incubated at 37°C for 24 h in mouse plasma to investigate the molecular structure of metabolites by MS.

Effects of Temperature and pH on Metabolite Formation in Plasma

$^{14}$C-Delamanid (5 µg/mL) in human plasma was incubated at 25°C and 0°C for 0, 1, 2, and 4 h (in addition to at 37°C for 0, 0.5, 1, 2, and 4 h). Further, $^{14}$C-delamanid (5 µg/mL) was incubated at 37°C for 0, 0.5, 1, 2, and 4 h in 50 mM phosphate buffer (pH 6.0, 7.0, 7.5, and 8.0) containing 10% human plasma.

Metabolite Formation in Fractionated Plasma

Human plasma was centrifuged at 3000 g for 30 min using a Centricon YM-30 (molecular mass cutoff of 30kDa, Millipore Co., Billerica, MA). The plasma filtrate was incubated with $^{14}$C-delamanid (5 µg/mL) at 37°C for 0, 0.5, 1, 2, and 4 h.

To obtain plasma protein fractions, high-performance gel filtration chromatography of human plasma was performed at room temperature using the columns TSK-gel G4000SWXL (7.8 mm ID × 300 mm, 8 µm particle size, Tosoh Co., Tokyo, Japan) and TSK-gel G3000SWXL (7.8 mm ID × 300 mm, 5 µm particle size, Tosoh Co.) in combination, 50 mM phosphate buffer (pH 7.0) as the mobile phase at 1 mL/min, and ultraviolet (UV) detection at 280 nm. After injection of 200 µL human plasma, the effluent was fractionated every 1 min. Pure HSA (40 mg/mL), $\gamma$-globulin (12 mg/mL), and AGP (1 mg/mL) were also analyzed to confirm retention times. The eluate was adjusted to pH 7.5 with 1N sodium hydroxide and
incubated with 5 µg/mL of ¹⁴C-delamanid at 37°C for 8 h. Delamanid was also incubated with HSA (40 mg/mL), γ-globulin (12 mg/mL), or AGP (1 mg/mL) in place of the eluate fraction containing all three proteins.

**Kinetic Analysis on Metabolite Formation in Plasma and HSA**

¹⁴C-Delamanid [10, 25, 50, 100, 250, and 500 µM in 2% dimethyl sulfoxide (DMSO)] was incubated at 37°C for 0.25 h in human plasma or 40 mg/mL HSA (both in 50 mM phosphate buffer, pH 7.4). Total plasma protein concentration was determined using a Bio-Rad DC protein assay kit (Hercules, CA).

**Metabolite Profiling in Plasma and Albumin**

¹⁴C-Delamanid (5 µg/mL) was incubated in 50 mM phosphate buffer (pH 7.4) containing either 50% plasma or 20 mg/mL albumin from rat, dog, and human at 37°C for 0, 0.5, 1, and 2 h.

**Binding of Delamanid to Serum and HSA**

Degradation of delamanid is temperature-dependent. To avoid the degradation of delamanid, the protein binding studies were conducted at 20°C. The *in vitro* binding of ¹⁴C-delamanid (0.05, 0.5, and 5 µg/mL) to animal and human serum was determined by equilibrium dialysis for 4 h (rabbit and dog serum) or 8 h (rat, mouse, and human serum) using Spectra/Por2 molecular porous dialysis membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA). The binding of ¹⁴C-delamanid (0.05, 0.5, and 5 µg/mL) to RSA, DSA, and HSA solutions (all at 40 mg/mL) was also determined. Further, the binding of ¹⁴C-delamanid (3 µM) to HSA (15 µM, 1 mg/mL) was determined in the absence and presence of the site-specific HSA binding probes warfarin (Site I), diazepam (Site II), and digitoxin (Site III), all at 15, 75, and 150 µM. DMSO content was always ≤1% (v/v). The dialyzed protein and dialysate were analyzed to determine the delamanid concentrations in bound and unbound fractions. After a scintillator cocktail was added to the sample, the radioactivity was
determined by a liquid scintillation counter (LSC-3500, Aloka Co., Tokyo, Japan).

Sample Preparation for Radioactivity Counting and Mass Spectrometry

The reaction was terminated by mixing with 2 volumes of acetonitrile-formic acid (90:10, v/v). Following centrifugation at 21800 g for 5 min, 30 µL of the supernatant was analyzed by HPLC with simultaneous radioactive detection. Further, a scintillator cocktail (ACS II, Amersham Co., UK) was added to 30 µL of the supernatant, and the radioactivity determined by liquid scintillation (LSC-3500) to evaluate extraction and column recovery.

For measuring the metabolism of unlabeled delamanid in plasma, the reaction was terminated by mixing in an equal amount of acetonitrile, followed by centrifugation at 21800 g for 5 min and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

HPLC and LC-MS/MS Procedures

To investigate the metabolism of labeled delamanid in plasma, two HPLC protocols were used. HPLC Method 1 utilized a LC-10A HPLC system (Shimadzu Co., Kyoto, Japan) equipped with a TSK-gel ODS-80Ts QA C18 column (4.6 mm ID × 150 mm, 5 µm particle size, Tosoh Co.) for sample analysis. The analyte was separated using a binary solvent linear gradient from 0% B to 60% B [A, water-acetic acid (100:1, v/v); B, acetonitrile-acetic acid (100:1, v/v)] from minute (min) 0−35, 60% B to 90% B from min 35−40 at a flow rate of 1 mL/min, isocratic elution at 90% B from min 40−45, and 0% B from min 45−60 min. Before entering the radioactive flow detector, the column effluent was mixed in-flow with 1:2 scintillation cocktail (Ultima-Flo AP, PerkinElmer, Inc., Billerica, MA) pumped at a rate of 2 mL/min. The radioactivity in the effluent was monitored using a Radiomatic 525TR flow scintillation analyzer (PerkinElmer, Inc.). In HPLC Method 2 used to investigate the effects of temperature and pH, metabolite formation, kinetic analysis, and metabolite profiling in plasma and albumin were conducted using a model 2695 Alliance HPLC system (Waters Co., Milford, MA) equipped with a TSK-gel ODS-80Ts QA C18 column. The elution was
performed using a binary solvent linear gradient from 30% B to 90% B from min 0−15 at a flow rate of 1 mL/min, 30% B from min 15−20 at 1.2 mL/min, and 30% B from min 20−25 at a flow rate of 1 mL/min. The radioactivity in the effluent was monitored using a flow scintillation analyzer.

To investigate the metabolism of unlabeled delamanid in plasma by LC-MS/MS, delamanid and metabolite were analyzed using a TSK-gel ODS-80Ts QA C18 column in a binary solvent linear gradient of water-acetic acid and acetonitrile-acetic acid with UV detection at 254 nm. The LC eluate was introduced directly into a triple-quadrupole mass spectrometer (API3000, 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; curtain gas, 8; and collision gas, 8 (product ion scan only). Nitrogen was used in the ion source and the collision cell. Full scan and product ion scan were recorded within the mass range of m/z 100 to 600.

Data Processing

Data processing was performed using FLO-ONE version 3.65 (PerkinElmer, Inc.) in the flow scintillation analyzer. The radioactivity of delamanid metabolite in the sample was determined on the radiochromatogram, and the radioactivity was converted to equivalents of delamanid. The residual content of delamanid, metabolite formation, and other calculations were conducted with Microsoft Excel version 2003. The half-life and Michaelis–Menten parameters [Michaelis–Menten constant (K_m) and maximum velocity (V_max)] were calculated using a nonlinear least squares method by WinNonlin version 5.2 (Pharsight Co., St. Louis, MO). The intrinsic clearance (CL_int) was obtained from V_max/K_m. Analysis of LC-MS/MS was performed using Analyst version 1.4.2 (AB SCIEX).
Results

Metabolism of Delamanid in Plasma

The degradation of $^{14}$C-delamanid during incubation in plasma at 37°C is shown in Fig. 2 and Table 1. Delamanid was rapidly degraded by incubation in human, dog, rabbit, mouse, or rat plasma at 37°C, with shortest half-life in human plasma (0.64 h), followed by dog (0.84 h), rabbit (0.87 h), mouse (1.90 h), and rat (3.54 h). Typical HPLC radiochromatograms of delamanid metabolites in plasma are shown in Supplemental Fig. 1. The major delamanid byproduct, M1, increased as substrate concentration decreased in plasma samples from all species.

The chemical structure of M1 was further investigated by LC-MS/MS analysis of mouse plasma containing non-labeled delamanid. The mass spectra of the parent compound and the metabolite revealed protonated molecules ([M+H]$^+$) at $m/z$ 535 and 466, respectively, and a characteristic and intense fragment ion at $m/z$ 352 in both positive product ion spectra (Fig. 3). The peak profile of the metabolite indicated the existence of a 4-[4-(4-trifluoromethoxyphenoxy)piperidin-1-yl]phenoxy moiety without imidazooxazole. Additional fragment ions were observed at $m/z$ 357 in spectra of the parent drug and at $m/z$ 113, 288, and 449 in spectra of the metabolite. The metabolite M1 was identified as (R)-2-amino-4,5-dihydrooxazole derivative by comparing the mass spectra and retention time of the product in the plasma sample to those of the authentic standard (Supplemental Fig. 2).

Effects of Temperature and pH on Metabolite Formation in Plasma

The rates of M1 formation in human plasma incubated at various temperatures are shown in Fig. 4. The biotransformation to M1 after 4 h was 51.7% at 37°C and 36.3% at 25°C, whereas no M1 was detected after 4 h at 0°C.

Metabolism was also highly pH-dependent (Fig. 5). After 4 h at 37°C in 10% human plasma, M1 formation was 0.0% at pH 6.0, 4.8% at pH 7.0, 12.7% at pH 7.5, and 20.1% at pH 8.0.
pH 8.0. In contrast, M1 was not formed during incubation for 4 h in 50 mM phosphate buffer at any pH in the absence of plasma (data not shown).

**Metabolite Formation in Fractionated Plasma**

Delamanid was not converted to M1 in the filtrate of human plasma obtained with a molecular mass cutoff of 30 kDa, indicating that metabolism required the presence of plasma proteins of molecular mass ≥30 kDa. When delamanid degradation was examined in plasma fractions separated by gel chromatography, M1 was observed in the fraction containing albumin, γ-globulin, and AGP (Fig. 6). In the presence of HSA, delamanid was metabolized to M1, whereas no M1 was detected following delamanid incubation with γ-globulin or AGP. Thus, metabolism requires HSA.

**Kinetic Analysis on Metabolite Formation in Plasma and HSA**

The total protein concentration in human plasma samples was approximately 80 mg/mL. Several concentrations of delamanid were incubated in human plasma or 40 mg/mL HSA. The formation of M1 followed Michaelis–Menten kinetics in both human plasma and HSA (Fig. 7). The Eadie–Hofstee plot for the formation of M1 in plasma showed a monophasic profile. The $K_m$, $V_{max}$, and $CL_{int}$ values for plasma were 67.8 µM, 7.55 pmol/min/mg, and 0.111 µL/min/mg, respectively, and the values found in HSA alone, which were 51.5 µM, 11.7 pmol/min/mg, and 0.227 µL/min/mg (Table 2).

**Metabolite Profiling in Plasma and Albumin**

In addition to the kinetic profile, the metabolic pattern of delamanid in 50% plasma was similar to that in 20 mg/mL albumin (Fig. 8). The degradation rates of delamanid were highest in human plasma and HSA, followed by dog and rat plasma and albumin. The residual content of delamanid after 1 h at 37°C in 50% human plasma was 52.8%, substantially higher than in dog (82.6%) and rat (93.5%), whereas the corresponding biotransformation rate to M1 was highest in 50% human plasma (21.0%), followed by dog.
(6.5%) and rat (3.1%). Similarly, the residual delamanid content was lower after incubation (1 h at 37°C) in 20 mg/mL HSA (50.1%) compared with that in DSA (77%) and RSA (92.0%), and corresponding M1 formation was highest in HSA (19.9%), followed by DSA (9.7%) and RSA (5.2%).

**Binding of Delamanid to Serum and HSA**

The *in vitro* protein binding ratio was ≥99.3% in all serum samples at all 14C-delamanid concentrations tested (Table 3), while binding was ≥97.4% to RSA, DSA, and HSA. The binding of 14C-delamanid to HSA was not changed in the presence of the Site I-specific binding probe warfarin, the Site II-specific probe diazepam, or the Site III-specific probe digitoxin (Table 4). The radiochemical purity of delamanid incubated at 20°C in human serum and HSA was more than 86%, suggesting that delamanid was stable in the protein binding studies.

**Discussion**

When delamanid was incubated in human plasma at appropriate temperature and pH, the metabolite M1 increased in parallel with a decrease in the substrate concentration, indicating that M1 is a primary produce of plasma-mediated degradation. M1 was also the most abundant primary metabolite detected during incubation in human plasma, as well as in plasma from other species. Degradation of delamanid was temperature-dependent, pH-dependent, saturable, and followed Michaelis–Menten kinetics. The Eadie–Hofstee plot for M1 formation was monophasic (Fig. 7), suggesting that the formation is catalyzed by one enzyme. Further, the formation of M1 in HSA followed Michaelis–Menten kinetics, with a $K_m$ value similar to that in human plasma (Table 2), suggesting that plasma albumin, which constitutes about half of total plasma protein (40 mg/mL of 80 mg/mL), is likely responsible for the metabolism of delamanid. The $V_{\text{max}}$ and $CL_{\text{int}}$ values for delamanid in plasma (7.55
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pmol/min/mg and 0.111 μL/min/mg), were comparable to those in 40 mg/mL HSA (11.7 pmol/min/mg and 0.227 μL/min/mg of albumin). In contrast, no delamanid metabolism was observed following incubation with the other two high molecular weight proteins in the original HSA fraction, γ-globulin and AGP (Fig. 6). The purity of the commercial albumin employed in the in vitro studies was >96%, with the remainder being mostly globulins (Sigma, quality A-1887). Though hydrolase (mainly pseudo-cholinesterase) contamination of the purified HSA preparation cannot be completely excluded, metabolism was also observed by recombinant human albumin (product no A7223, Sigma-Aldrich Co.). This result and the similarity in kinetics between HSA and plasma strongly suggest that delamanid is metabolized predominantly by albumin in plasma.

The in vitro biotransformation of delamanid increased in a pH-dependent manner (Fig. 5), and delamanid did not degrade at pH 6.0. Considering that pKa of delamanid is approximately 4.3, the pH sensitivity suggests that the pKa value of the catalytic amino acid residue(s) in plasma albumin may be important for the reaction. The metabolic patterns of delamanid in dog and rat plasma were also similar to those in dog and rat albumin (Fig. 8), suggesting that plasma albumin is predominantly responsible for delamanid metabolism in rat and dog as well. Protein binding to delamanid was also similar to that in humans (Table 3). Nonetheless, the degradation rate of delamanid was highest in human plasma and albumin solution, followed by dog and rat. It was reported that the hydrolytic degradation of Boc5 in plasma was mediated by serum albumin, and that species differences in hydrolysis could be attributed to variations in albumin sequence and high-order structure across species (Ge et al., 2013). The species differences in the degradation rate of delamanid may thus also stem from species variation in the sequence of albumin.

Though extraction recovery and HPLC column recovery were favorable, the rate of delamanid degradation was higher than the rate of M1 formation in both plasma and albumin,
suggesting that M1 is the major but not the only metabolic byproduct. These other byproducts may include the minor metabolites observed at retention times from 24 to 30 min (Supplemental Fig. 1), which remain to be identified and characterized.

In a novel biotransformation, M1 was uniquely formed by cleavage of the 6-nitro-2,3-dihydroimidazo[2,1-b]oxazole moiety of delamanid in plasma albumin. On the basis of the fact that the authentic standard M1 is directly synthesized from delamanid and alkaline reagents such as 25% ammonia solution or alkylamines (Supplemental Fig. 2), basic amino acid residues such as lysine or arginine in albumin may be important for the metabolism of delamanid. The proposed degradation mechanism of delamanid by albumin is illustrated in Fig. 9. Because of the electron withdrawing property of the neighboring nitro group, the electron-poor C-5 of the delamanid 6-nitro-2,3-dihydroimidazo[2,1-b]oxazole moiety can react easily with a nucleophile. When amino acid residues in HSA attack this carbon, an albumin–delamanid adduct is produced. The delamanid adduct is further hydrolyzed in the presence of water, resulting in the primary metabolite M1. However, further work is necessary to resolve the details of the mechanism.

Albumin, the most abundant protein in plasma (Theodore, 1996), displays pseudo-enzymatic properties, and has been found to catalyze the hydrolysis of numerous compounds, such as cinnamoyl imidazole (Ohta et al., 1983), p-nitrophenyl esters (Kurono et al., 1979; Lockridge et al., 2008; Means et al., 1975; Sakurai et al., 2004; Watanabe et al., 2000), olmesartan medoxomil (Ma et al., 2005), carbaryl (Sogorb et al., 2004), aspirin (Liyasova et al., 2010; Rainsford et al., 1980), organophosphate insecticides (Sultatos et al., 1984), and long- and short-chain fatty acid esters (Wolfbeis et al., 1987). Among previous studies, the most relevant example of albumin-catalyzed metabolism to this study is that of N-trans-cinnamoyl imidazoles (Ohta et al., 1983). It appears that this interaction involves fast acylation of albumin to form cinnamoyl albumin, followed by a slow deacylation of
cinnamoyl albumin. The electron-withdrawing substituent, the carbonyl group (C=O) of N-trans-cinnamoyl imidazole, facilitates the acylation.

The electron-poor carbon at the C-5 position of the delamanid imidazooxazole structure is also able to react with a nucleophile. Considering the fact that a delamanid analog without the nitro group was not metabolized by HSA (data not shown), an electron-withdrawing nitro group of delamanid is suggested to be important for the propensity toward ring scission by albumin. Ohta et al. (1983) proposed that acylation by albumin occurs at a reactive residue of the R site (Tyr-411), which corresponds to Sudlow’s Site II (Ozeki et al., 1980; Salvi et al., 1997; Sudlow et al., 1976). The nucleophilic character of Tyr-411 for the esterase-like activity toward p-nitrophenyl esters was suggested that nucleophilic attack by albumin on the substrate results in an acylated albumin derivative that is then deacylated by general acid or base catalysis (Sakurai et al., 2004). Accordingly, a study using p-nitrophenyl acetate as a substrate showed that the enzymatic activity of HSA was dependent on the presence of Tyr-411 (Watanabe et al., 2000). For the protein bindings, at least three binding sites, Site I, Site II, and Site III, are reported to be present on HSA. The saturation of the protein binding capacity of delamanid to HSA (15 µM) was not observed at high concentrations (up to 30 µM; data not shown). Further, the protein binding of delamanid in HSA was not affected by varying the concentrations of Site I–III specific probes (warfarin, diazepam, and digitoxin, respectively; Table 4). These results suggest that delamanid may bind non-specifically to HSA. The effects of inhibitory protein binding ligands on delamanid metabolism require further study to clarify the molecular mechanisms of albumin-mediated metabolism.

Esterase-like activity of HSA on olmesartan medoxomil hydrolysis has also been reported (Ma et al., 2005). Chemically modified HSA derivatives (Tyr-, Lys-, His-, and Trp-modifications) and the mutant HSAs K199A, W214A, and Y411A exhibited significantly
lower reactivity, suggesting that (wild type) Lys-199, Trp-214, and Tyr-411 play important roles in hydrolysis. Moreover, using selective amino acid reagents, these authors concluded that Cys, Trp, Arg, and Tyr participate in the carbarylase activity of HSA (Sogorb et al., 2004). Finally, it was reported that the bioconversion of aspirin by albumin is a pseudo-esterase reaction in which aspirin stably acetylates lysines on albumin and releases salicylate (Liyasova et al., 2010). These reports collectively suggest that amino acid residues such as lysine, tryptophan and arginine, phenolic hydroxyl groups such as tyrosine, and thiol groups such as cysteine in HSA may be involved in the first step of delamanid metabolism by albumin.

The overall in vivo metabolism of delamanid was qualitatively similar across species, including humans and the predominant preclinical study species. However, quantitative differences were observed among species (Sasahara et al., 2015). For instance, M1 concentration after repeated administration was much higher in human and dog than in rodents, consistent with the more rapid formation of M1 in human and dog plasma in vitro (Fig. 2 and Table 1). As M1 formation appears to be the primary metabolic reaction of delamanid, it is the determinant of the interspecies differences in delamanid biotransformation.

In conclusion, the new anti-TB drug delamanid is metabolized to (R)-2-amino-4,5-dihydrooxazole derivative (M1) by albumin in plasma. The degradation of delamanid by albumin is proposed to begin with attack by amino acid residues of albumin on the electron-poor carbon at the 5 position of nitro-dihydro-imidazooxazole, followed by cleavage of the imidazooxazole moiety to M1.
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References


Figure Legends

Fig. 1. Chemical structure of $^{14}$C-delamanid.
The asterisk denotes the position of $^{14}$C-radiolabel.

Fig. 2. Stability of delamanid in animal and human plasma in vitro.
$^{14}$C-Delamanid (5 μg/mL, 9.3 μM) was incubated in rat, mouse, rabbit, dog, or human plasma at 37°C. Data points are the means of duplicate determinations.

Fig. 3. Product ion spectra of delamanid at m/z 535 (A) and M1 at m/z 466 (B).
The product was investigated by LC-MS/MS following incubation of delamanid in mouse plasma at 37°C.

Fig. 4. Temperature dependence of M1 formation from delamanid in human plasma.
$^{14}$C-Delamanid (5 μg/mL) was incubated in human plasma.
Data points are the means of duplicate determinations.

Fig. 5. The pH dependence of M1 formation from delamanid in human plasma.
$^{14}$C-Delamanid (5 μg/mL) was incubated in a solution of 10% plasma and 50 mM phosphate buffer (balance to the indicated pH) at 37°C. Data points are the means of duplicate determinations.

Fig. 6. M1 formation from delamanid in human plasma fractions separated by gel filtration chromatography.
(A): After 200 μL of human plasma was injected into the high-performance gel filtration chromatography system, the effluent was fractionated every 1 min. Authentic human albumin, γ-globulin, and AGP were also run.

(B): ¹⁴C-Delamanid (5 μg/mL) was incubated in the human plasma fractions at 37°C for 8 h.

Fig. 7. Michaelis–Menten and Eadie–Hofstee plots for M1 formation in human plasma and HSA.

¹⁴C-Delamanid (10 to 500 μM) was incubated in a solution of human plasma (A) or 40 mg/mL HSA (B) with 50 mM phosphate buffer (pH 7.4) at 37°C for 0.25 h. S is the substrate concentration of delamanid and V is the velocity of M1 formation. Insets are the corresponding Eadie–Hofstee plots. Data points are the means of triplicate determinations.

Fig. 8. Degradation of delamanid and M1 production in diluted plasma (A) and albumin (B) from rat, dog, and human.

¹⁴C-Delamanid (5 μg/mL) was incubated in 50% plasma or 20 mg/mL albumin (both in 50 mM phosphate buffer, pH 7.4) at 37°C. Data points are the means of duplicate determinations.

Fig. 9. Proposed degradation mechanism of delamanid by albumin.
Table 1. *In vitro* disappearance of delamanid in plasma of different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>3.54</td>
</tr>
<tr>
<td>Mouse</td>
<td>1.90</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.87</td>
</tr>
<tr>
<td>Dog</td>
<td>0.84</td>
</tr>
<tr>
<td>Human</td>
<td>0.64</td>
</tr>
</tbody>
</table>

14C-Delamanid (5 μg/mL) was incubated with plasma at 37°C in duplicate determinations.
Table 2. Kinetic parameters for M1 formation in human plasma and HSA

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
<th>$CL_{int}$ (μL/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human plasma</td>
<td>67.8</td>
<td>7.55</td>
<td>0.111</td>
</tr>
<tr>
<td>HSA</td>
<td>51.5</td>
<td>11.7</td>
<td>0.227</td>
</tr>
</tbody>
</table>

$^{14}$C-Delamanid (10 to 500 μM) was incubated in human plasma or 40 mg/mL HSA at 37°C for 0.25 h. M1 formation was determined by radio-HPLC analysis. Each value was calculated using the mean formation data of triplicate determinations.
Table 3. *In vitro* binding of delamanid to serum and albumin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 μg/mL</td>
</tr>
<tr>
<td>Rat serum</td>
<td>NT</td>
</tr>
<tr>
<td>Mouse serum</td>
<td>NT</td>
</tr>
<tr>
<td>Rabbit serum</td>
<td>NT</td>
</tr>
<tr>
<td>Dog serum</td>
<td>NT</td>
</tr>
<tr>
<td>Human serum</td>
<td>NT</td>
</tr>
<tr>
<td>RSA</td>
<td>98.5 ± 0.1</td>
</tr>
<tr>
<td>DSA</td>
<td>97.6 ± 0.1</td>
</tr>
<tr>
<td>HSA</td>
<td>97.4 ± 0.3</td>
</tr>
</tbody>
</table>

Equilibrium dialysis was performed at 20°C in serum or 40 mg/mL albumin (both spiked with $^{14}$C-delamanid). NT = Not Determined. Data are mean ± S.D. of triplicate determinations.
Table 4. Effects of warfarin, diazepam, and digitoxin on delamanid binding to HSA

<table>
<thead>
<tr>
<th>Probe</th>
<th>Probe concentration (μM)</th>
<th>Delamanid protein binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>98.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>98.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>98.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>98.5 ± 0.2</td>
</tr>
<tr>
<td>Warfarin</td>
<td>15</td>
<td>98.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>98.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>98.5 ± 0.2</td>
</tr>
<tr>
<td>Diazepam</td>
<td>15</td>
<td>98.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>98.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>98.7 ± 0.1</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>15</td>
<td>98.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>97.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>98.0 ± 0.4</td>
</tr>
</tbody>
</table>

Equilibrium dialysis was performed for 6 h at 20°C in 15 μM (1 mg/mL) HSA spiked with \(^{14}\)C-delamanid (3 μM) in the absence or presence of the protein binding probe. Data are mean ± S.D. of four determinations.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Supplemental Data for Drug Metabolism and Disposition

Metabolic Mechanism of Delamanid, a New Anti-Tuberculosis Drug, in Human Plasma

Yoshihiko Shimokawa, Katsunori Sasahara, Noriyuki Koyama, Kazuyoshi Kitano, Masakazu Shibata, Noriaki Yoda, and Ken Umehara
Supplemental Methods

Synthesis of M1 from delamanid with a single step reaction.

Method A. To a solution of delamanid (4.0 g, 7.5 mmol) in THF (40 mL) was added 25% aq. NH₃ solution (20 mL) at room temperature. The reaction mixture was refluxed for 20 min at 100 °C in sealed tube apparatus (ca. 0.8 MPa). The mixture was concentrated in vacuo. Purification of the residue by column chromatography (surface-modified silica gel with aminopropyl group; eluent, CH₂Cl₂/ MeOH=100/1) provided M1 (1.6 g, 47%) as an amorphous solid. ¹H NMR (500 MHz, DMSO- d₆) δ: 1.38 (3H, s), 1.74 (2H, dddd, \(J = 12.2, 9.2, 3.7\) Hz), 2.00–2.07 (2H, m), 2.93 (2H, ddd, \(J = 12.2, 9.2, 3.1\) Hz), 3.30 (1H, d, \(J = 12.2\) Hz), 3.36 (2H, ddd, \(J = 12.2, 5.8, 3.7\) Hz), 3.53 (1H, d, \(J = 12.2\) Hz), 3.85 (1H, d, \(J = 10.4\) Hz), 3.88 (1H, d, \(J = 10.4\) Hz), 4.54 (1H, tt, \(J = 8.2, 3.7\) Hz), 5.80 (2H, brs), 6.84 (2H, d, \(J = 9.2\) Hz), 6.91 (2H, d, \(J = 9.2\) Hz), 7.08 (2H, d, \(J = 9.2\) Hz), 7.28 (2H, dq, \(J = 9.2, 0.6\) Hz).

Method B. To a solution of delamanid (1.0 g, 1.9 mmol) in THF (20 mL) was added 1,3-diaminopropane (1.6 mL, 19 mmol) at room temperature. The reaction mixture was stirred for 16 h. The solvent was removed in vacuo. After addition of water, the mixture was extracted with CH₂Cl₂. The combined organic layers were dried over sodium sulfate. Removal of the solvent and purification of the crude product by column chromatography (surface-modified silica gel with aminopropyl group; eluent, CH₂Cl₂/MeOH=100/1) provided
**Synthesis of M1 with a stepwise procedure.** (Kitano et al., 2009)

**(R)-1-Azido-2-methyl-3-(4-(4-(4-trifluoromethoxy-phenoxy)-piperidin-1-yl)-phenoxy)-propan-2-ol (2).** To a suspension of (R)-1-(4-((2-methyloxiran-2-yl)-methoxy)phenyl)-4-(4-(trifluoro-methoxy)phenoxy)piperidine (1, Tsubouchi et al., 2008) (1.7 g, 4.0 mmol) in MeOH (40 mL) and water (5 mL) were added NH₄Cl (0.48 g, 8.9 mmol) and sodium azide (1.5 g, 20 mmol) and the mixture was refluxed for 16 h. The reaction mixture was allowed to cool to room temperature and the solvent was removed *in vacuo*. After addition of water, the mixture was extracted with AcOEt. The extract was washed with water, and then with a saturated aqueous solution of NaCl. The combined organic layers were dried over sodium sulfate. Removal of the solvent and purification of the crude product by column chromatography (silica gel; eluent, hexane/ AcOEt =65/35) provided 2 (1.8 g, 98%) as a colorless oil. ¹H NMR (250 MHz, CDCl₃) δ: 1.33 (3H, s), 1.80–2.25 (4H, m), 2.85–3.10 (2H, m), 3.25–3.50 (4H, m), 3.80 (1H, d, J = 9.1 Hz), 3.86 (1H, d, J = 9.1 Hz), 4.25–4.50 (1H, m), 6.50–7.00 (6H, m), 7.14 (2H, d, J = 9.6 Hz).

**(R)-1-Amino-2-methyl-3-(4-(4-(4-trifluoromethoxy-phenoxy)-piperidin-1-yl)-phenoxy)-propan-2-ol (3).** A mixture of azide 2 (2.7 g, 5.7 mmol) and palladium on activated carbon
(10 wt % Pd, 0.27 g) in EtOH (50 mL) was stirred at room temperature under a hydrogen atmosphere at normal pressure. The reaction mixture was filtered over Celite. Removal of the solvent from the combined filtrates afforded 3 (2.1 g, 83%) as a gray amorphous solid.

\(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta\): 1.26 (3H, s), 1.80–2.25 (4H, m), 2.64 (1H, d, \(J = 12.9\) Hz), 2.80–3.15 (2H, m), 2.98 (1H, d, \(J = 12.9\) Hz), 3.25–3.40 (2H, m), 3.79 (2H, s), 4.30–4.50 (1H, m), 6.75–7.00 (6H, m), 7.13 (2H, d, \(J = 9.1\) Hz).

\((R)\)-5-Methyl-5-(4-(4-(4-trifluoromethoxy-phenoxy)-piperidin-1-yl)-phenoxymethyl)-4,5-di-hydro-oxazol-2-ylamine (M1). To a solution of 2-aminoalcohol (3) (4.9 g, 11 mmol) in anhydrous MeOH (50 mL) were added sodium acetate (2.0 g, 24 mmol) and cyanogen bromide (1.5 g, 13 mmol) at room temperature. The reaction mixture was stirred for 2.5 h at room temperature and then refluxed for 3.5 h. The reaction mixture was allowed to cool to room temperature and the solvent was removed \textit{in vacuo}. After addition of a saturated aqueous solution of NaHCO\(_3\), the mixture was extracted with AcOEt. The combined organic layers were dried over sodium sulfate. Removal of the solvent and purification of the crude product by column chromatography (surface-modified silica gel with aminopropyl group; eluent, CH\(_2\)Cl\(_2\)/ MeOH =100/1) and recrystallization from hexane–AcOEt provided M1 (3.5 g, 67%) as a white powder, mp 109–111 °C. \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\): 1.38 (3H, s), 1.74 (2H, dddd, \(J = 12.2, 9.2, 8.2, 3.7\) Hz), 2.00–2.07 (2H,
m), 2.93 (2H, ddd, $J = 12.2$, 9.2, 3.1 Hz), 3.30 (1H, d, $J = 12.2$ Hz), 3.36 (2H, ddd, $J = 12.2$, 5.8, 3.7 Hz), 3.53 (1H, d, $J = 12.2$ Hz), 3.85 (1H, d, $J = 10.4$ Hz), 3.88 (1H, d, $J = 10.4$ Hz), 4.54 (1H, tt, $J = 8.2$, 3.7 Hz), 5.80 (2H, brs), 6.84 (2H, d, $J = 9.2$ Hz), 6.91 (2H, d, $J = 9.2$ Hz), 7.08 (2H, d, $J = 9.2$ Hz), 7.28 (2H, dq, $J = 9.2$, 0.6 Hz).
Supplemental Fig. 1. HPLC radio chromatograms of delamanid metabolites in human plasma.

$^{14}$C-Delamanid (5 μg/mL) was incubated at 37°C for 0 h (A), 0.5 h (B), 1 h (C), 2 h (D), and 4 h (E) in human plasma.
Supplemental Fig. 2. Synthesis scheme of authentic standard M1.

(A) is a single step reaction, and (B) is a stepwise procedure.
Supplemental References
