COMPARTMENTAL AND ENZYME KINETIC MODELING
TO ELUCIDATE THE BIOTRANSFORMATION PATHWAY
OF A CENTRALLY-ACTING ANTITRYPANOSOMAL PRODRUG

Claudia N. Generaux, Garrett R. Ainslie, Arlene S. Bridges, Mohamed A. Ismail,
David W. Boykin, Richard R. Tidwell, Dhiren R. Thakker, and Mary F. Paine

Divisions of Molecular Pharmaceutics (C.N.G.) and Pharmacotherapy and Experimental
Therapeutics (D.R.T., M.F.P.), UNC Eshelman School of Pharmacy and Curriculum in
Toxicology (G.R.A., M.F.P.) and Department of Pathology and Laboratory Medicine, School of
Medicine (A.S.B., R.R.T.), The University of North Carolina at Chapel Hill, Chapel Hill, NC and
Department of Chemistry, Georgia State University, Atlanta, GA (M.A.I., D.W.B.).
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Corresponding author:

Mary F. Paine, RPh, PhD
2320 Kerr Hall, CB #7569
Eshelman School of Pharmacy
University of North Carolina Chapel Hill
Chapel Hill, NC 27599-7569
Telephone: 919-966-9984
FAX: 919-962-0644
E-mail: mpaine@unc.edu

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Abbreviations: ABT, 1-aminobenzotriazole; Cl_{app}, apparent clearance; Cl_{int}, intrinsic clearance; CNS, central nervous system; DB829, 2,5-bis (5-amidino-2-pyridyl) furan; DB868, 2,5-bis [5-(N-methoxyamidino)-2-pyridyl] furan; P450, cytochrome P450; d_6-DB829, deuterated DB829; d_6-DB868, deuterated DB868; HLM, human liver microsomes; HPLC, high performance liquid chromatography; MS, mass spectrometry; MS/MS or MS^n, tandem mass spectrometry; RLM, rat liver microsomes; SCHH, sandwich-cultured human hepatocytes; SCRH, sandwich-cultured rat hepatocytes.
Abstract

DB868 [2,5-bis [5-(N-methoxyamidino)-2-pyridyl] furan], a prodrug of the diamidine DB829 [2,5-bis(5-amidino-2-pyridyl) furan], has demonstrated efficacy in murine models of human African trypanosomiasis. A cross-species evaluation of prodrug bioconversion to the active drug is required to predict the disposition of prodrug, metabolites, and active drug in humans. The phase I biotransformation of DB868 was elucidated using liver microsomes and sandwich-cultured hepatocytes from humans and rats. All systems produced four NADPH-dependent metabolites via O-demethylation (M1, M2) and N-dehydroxylation (M3, M4). Compartmental kinetic modeling of the DB868 metabolic pathway suggested an unusual N-demethoxylation reaction that was supported experimentally. A unienzyme Michaelis-Menten model described the kinetics of M1 formation by human liver microsomes (HLM) (K_m, 11 \mu M; V_{max}, 340 pmol/min/mg), whereas a two-enzyme model described the kinetics of M1 formation by rat liver microsomes (RLM) (K_{m1}, 0.5 \mu M; V_{max1}, 12 pmol/min/mg; K_{m2}, 27 \mu M; V_{max2}, 70 pmol/min/mg). Human recombinant CYP1A2, CYP3A4, and CYP4F2, rat recombinant Cyp1a2 and Cyp2d2, and rat purified Cyp4f1 catalyzed M1 formation. M2 formation by HLM exhibited allosteric kinetics (S_{50}, 18 \mu M; V_{max}, 180 pmol/min/mg), whereas M2 formation by RLM was negligible. Recombinant CYP1A2/Cyp1a2 catalyzed M2 formation. DB829 was detected in trace amounts in HLM at the end of the 180-min incubation and was detected readily in sandwich-cultured hepatocytes from both species throughout the 24-h incubation. These studies demonstrated that DB868 biotransformation to DB829 is conserved between humans and rats. An improved understanding of species differences in the kinetics of DB829 formation would facilitate preclinical development of a promising antitrypanosomal prodrug.
Introduction

Human African trypanosomiasis (HAT) is a neglected parasitic disease that afflicts exclusively the world’s poorest populations (Barrett et al., 2007; Paine et al., 2010). HAT is caused by the flagellated protozoan *Trypanosoma brucei*, specifically *T. b. rhodesiense* and *T. b. gambiense*. The disease is characterized by two defined stages. During first stage infection, trypanosomes are confined to the hemolymphatic system. Symptoms include headache, malaise, fever, and joint pain (Sternberg, 2004). Second stage infection is triggered once parasites enter the central nervous system (CNS), causing deterioration of neurologic function and disruptions in sleep/wake patterns (Paine et al., 2010), hence the term “sleeping sickness” (Sternberg, 2004). Without treatment, progressive CNS damage leads to coma and ultimately death.

Trypanosomes elude the immune system by switching surface glycoprotein coats, precluding development of effective vaccines (McCulloch, 2004). As such, HAT treatment necessitates chemotherapeutic agents. Current agents approved as monotherapy [pentamidine, suramin (first stage), melarsoprol, eflornithine (second stage)] are unsatisfactory due to toxicities and/or impractical administration regimens, increasing clinical failure, high costs, and poor distribution (Paine et al., 2010). All treatments require parenteral administration over a course of at least seven days (pentamidine) to four weeks (suramin) (Barrett et al., 2007). Toxicities range from severe dysglycemia, hypotension, and liver injury (pentamidine) to reactive, sometimes fatal, encephalopathy (melarsoprol) (Legros et al., 2002). Eflornithine, albeit not life-threatening, must be administered as four infusions daily for 14 days (Barrett et al., 2007). Although Nifurtimox-Eflornithine Combination Therapy (Priotto et al., 2009) decreases the frequency of eflornithine infusions (2 daily x 7 days), 24-month survival rates appear to be only moderately improved compared to eflornithine alone (Balasegaram et al., 2009). These shortcomings have spurred development of orally effective and safer treatments, particularly for
second stage infection, to alleviate the cost and personnel burden associated with current HAT treatments.

An analog of pentamidine, 2,5-bis(5-amidino-2-pyridyl) furan (DB829), has demonstrated excellent in vitro activity against different sub-species of trypanosomes, including *T. b. gambiense*, which is associated most often with second stage HAT. The in vitro potency of DB829 against three *T. b. gambiense* isolates, specifically STIB930, ITMAP141267, and K03048, was comparable to that of melarsoprol, as assessed by IC$_{50}$ values <0.13 $\mu$M (Wenzler et al., 2009). DB829 (20 mg/kg i.p. daily x 10 days) was 100% curative in the GVR35 CNS murine model of second stage HAT; a single dose (10 mg/kg i.p.) was 100% curative in the STIB900 murine model of first stage HAT (Wenzler et al., 2009).

The two positive charges on diamidines (calculated pK$_a$s range from 9-11) render these scaffolds impermeant to biologic membranes via passive diffusion, resulting in poor oral availability and low efficacy (Boykin et al., 1995). To improve oral availability of DB829, a prodrug strategy was applied by masking each amidino group with $N$-methoxy functional groups, producing 2,5-bis [5-($N$-methoxyamidino)-2-pyridyl] furan (DB868) (Fig. 1) (Ismail et al., 2003). DB868 was 100% curative when administered orally to both the GVR35 CNS (100 mg/kg daily x 5 days) and STIB900 (50 mg/kg daily x 4 days) murine models (Wenzler et al., 2009). These observations indicated that the prodrug strategy was effective in improving oral availability and efficacy of DB829.

Prior to committing the prodrug DB868 as a development candidate, an understanding of DB868 biotransformation across species is essential to ensure that the active drug, DB829, will be available in sufficient concentrations to achieve efficacy in humans, as well as to facilitate selection of the most appropriate species for toxicity testing. In the present study, established in vitro systems derived from humans and rats (recombinant/purified enzymes, liver microsomes, sandwich-cultured hepatocytes) were used in conjunction with compartmental and enzyme kinetic modeling to elucidate the phase I biotransformation of DB868 to the active drug, DB829,
and to screen candidate oxidative enzymes involved in the biotransformation pathway. Results will help advance preclinical evaluation of DB868.
Materials and Methods

Chemicals and Materials. DB868 lot D (tetrahydrochloride salt), $d_6$-DB868, the intermediate metabolites of DB868 (M1, M2, M3), DB829 (trihydrochloride salt), and $d_6$-DB829 (dihydrochloride salt) were synthesized as described previously (Ismail et al., 2003; Ismail and Boykin, 2006). Pooled mixed gender human liver and male rat liver microsomes (HLM and RLM, respectively) were purchased from XenoTech, LLC (Lenexa, KS). Purified rat Cyp4f1, Cyp4f4, Cyp4f5, and Cyp4f6 were gifts from Dr. Deanna Kroetz (Department of Bioengineering and Therapeutic Sciences, University of California at San Francisco, San Francisco, CA). All human and rat recombinant enzymes, derived from insect cells (Supersomes™), were purchased from BD Gentest (Woburn, MA). Human CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and rat Cyp1a2, Cyp2d1, and Cyp2d2 were co-expressed with NADPH-cytochrome P450 reductase. Human CYP4F2 and rat Cyp2a1, Cyp2a2, Cyp2b1, Cyp2c6, Cyp2c11, Cyp2c13, Cyp2e1, and Cyp3a1 were co-expressed with NADPH cytochrome P450 reductase and cytochrome b$_5$. Dubelcco’s modified Eagle’s Medium (DMEM, without phenol red) and purified recombinant human NADPH-cytochrome P450 reductase were purchased from Invitrogen (Carlsbad, CA). Male human and rat sandwich-cultured hepatocytes (SCHH and SCRH, respectively) were purchased from CellzDirect (Durham, NC); the human donor was a 61 year-old Caucasian man who weighed 98 kg and had a history of smoking. 1-Aminobenzotriazole (ABT), ammonium formate, catalase, dilauroylphosphatidylcholine, dimethyl sulfoxide (DMSO), formic acid, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, methanol, β-NADPH, sodium cholate, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile and water were purchased from Fisher Scientific (Pittsburgh, PA).

Identification and Quantification of DB868 Phase I Metabolites. Identification. DB868 was dissolved in DMSO to yield a 3 mM solution. Duplicate incubation mixtures consisted of HLM or RLM (0.5 mg/mL), potassium phosphate buffer (100 mM, pH 7.4), MgCl$_2$ (10 mM), and DB868 (15 μM). After equilibrating at 37°C for 3 min, reactions were initiated by addition of
NADPH to yield a final volume of 500 μL (0.5% DMSO) and final NADPH concentration of 2 mM. Reactions were terminated at 0, 5, 15, 30, 60, 90, 120, and 180 min by removing 50-μL aliquots and adding to an equal volume of ice-cold acetonitrile. Proteins were precipitated by centrifugation (1500 x g for 10 min), and the supernatant was analyzed for prodrug, intermediate metabolites, and DB829 by HPLC/MS/MS (described below). Quantification. DB868 was dissolved in DMSO to yield a 2 mM solution. Triplicate incubation mixtures consisted of HLM or RLM (0.5 mg/mL), potassium phosphate buffer (100 mM, pH 7.4), MgCl$_2$ (10 mM), NADP$^+$ (1 mM), glucose 6-phosphate (5 mM), glucose 6-phosphate dehydrogenase (1 unit/ml), and DB868 (10 µM). The NADPH-regenerating system was used to ensure that cofactor was not depleted during the incubation period; similar results were obtained using NADPH (2 mM) in a repeat experiment. Reactions were initiated at 10-sec intervals by addition of the NADPH-regenerating system to yield a final volume of 250 μL (0.5% DMSO). Reactions were terminated at 0, 2.5, 5, 10, 15, 30, 45, 60, 90, 120, and 180 min by addition of ice-cold acetonitrile (125 µL). Proteins were precipitated by centrifugation, and the supernatant was analyzed for prodrug, intermediate metabolites, and DB829 by HPLC-UV (described below).

Mechanism-based Inactivation of Cytochrome P450 Enzymes. To evaluate whether M3 was an N-demethoxylation product of DB868, the non-specific mechanism-based P450 inhibitor, ABT (Ortiz de Montellano and Mathews, 1981), was used to inhibit formation of M1, a precursor of M3. ABT was dissolved in water to yield a 20 mM solution. Primary incubation mixtures consisted of HLM or RLM (5 mg/mL), ABT (1 mM), potassium phosphate buffer (100 mM, pH 7.4), and MgCl$_2$ (10 mM). Reactions were initiated with NADPH (2 mM) or water (control) to yield a final volume of 200 μL (0.5% DMSO). After 30 min, 20-μL aliquots were removed and diluted 10-fold into secondary incubation mixtures consisting of HLM or RLM (0.5 mg/mL), DB868 (10 µM), potassium phosphate buffer (100 mM, pH 7.4), MgCl$_2$ (10 mM), and NADPH (2 mM). After 5 (HLM) or 10 (RLM) min, secondary reactions were terminated by addition of ice-
cold acetonitrile (100 µL). Proteins were precipitated by centrifugation, and the supernatant was analyzed for M1 and M3 by HPLC-UV.

**Enzyme Kinetics and Enzyme Screening.** Kinetics. DB868 and M1 were dissolved in DMSO to yield solutions ranging from 0.02-20 mM and 0.02-5 mM, respectively. Triplicate incubation mixtures consisted of HLM or RLM (0.5 mg/mL), potassium phosphate buffer (100 mM, pH 7.4), MgCl₂ (10 mM), and DB868 (0.1-100 µM) or the metabolite M1 (0.1-25 µM). Reactions were initiated by addition of NADPH (2 mM), yielding a final volume of 250 µL (0.5% DMSO). Reactions were terminated after 5 (HLM) or 10 (RLM) min by addition of acetonitrile (125 µL) containing the internal standard, d₆-DB868 (0.2 µM, 0.01% DMSO). Proteins were precipitated by centrifugation, and the supernatant was analyzed by HPLC/MS/MS to quantify metabolites M1 and M3 generated from DB868 and metabolites M2 and M3 generated from M1. All reactions were evaluated under initial rate conditions such that substrate consumption was <20% (not shown). Recombinant Enzyme Incubations. Incubation mixtures consisted of recombinant enzyme (0.1 pmol/µL), potassium phosphate buffer (100 mM, pH 7.4), MgCl₂ (10 mM), and DB868 or M1 (5 µM). Reactions were initiated by addition of NADPH (2 mM), yielding a final volume of 200 µL (0.5% DMSO), and were terminated after 15 min by addition of ice-cold acetonitrile (100 µL). Proteins were precipitated by centrifugation, and the supernatant was analyzed by HPLC/UV for M1 formed from DB868 or for M2 formed from M1. Purified Rat Cyp4f Incubations. Incubations with purified rat Cyp4fs were carried out according to the method of Xu et al. (2004) with minor modifications. Briefly, mixtures containing Cyp4f (0.1 pmol/µL), cytochrome b₅ (0.1 pmol/µL), NADPH-cytochrome P450 reductase (1 pmol/µL), catalase (10 µg/mL), dilauroylphosphatidylcholine (20 µg/mL), and sodium cholate (0.2 mg/mL) were incubated for 10 min at 37°C prior to addition of potassium phosphate buffer (100 mM, pH 7.4), MgCl₂ (10 mM), and DB868 (5 µM). Reactions were initiated by addition of NADPH (2 mM), yielding a final volume of 100 µL (0.5% DMSO), and were terminated after 30 min by addition of...
ice-cold acetonitrile (50 µL). Proteins were precipitated by centrifugation, and the supernatant was analyzed for metabolite M1 by HPLC-UV.

**Phase I Biotransformation of DB868 in Sandwich-Cultured Hepatocytes.** To assess whether the negligible formation of DB829 from DB868 by HLM and RLM was a limitation of the microsomal system, DB868 was incubated with SCHH and SCRH, in which metabolic activities of the major P450 and phase II enzymes are maintained for a longer time period compared to these activities in suspended or plated hepatocytes (Kern et al., 1997). Unlike suspended hepatocytes, sandwich-cultured hepatocytes have the advantage of expressing exteriorized efflux transporters on both the basolateral and apical membranes (Chandra and Brouwer, 2004), permitting delineation of trafficking of the prodrug and metabolites between extracellular and intracellular compartments across cell membranes. SCHH and SCRH were cultured in 24- and 12-well plates, respectively, and experiments commenced on the fifth (human) or fourth (rat) day post-seeding. On the day of the experiment, culture medium was removed from wells, and hepatocytes were rinsed three times with warm DMEM. Reactions were initiated by adding DMEM (1 or 0.5 mL for 12- or 24-well plates, respectively) containing DB868 (10 µM; 0.1-0.2% DMSO) to the wells. After 0.5, 2, 4, and 24 h at 37°C, medium (0.3 or 0.75 mL) was collected and stored in microcentrifuge tubes at -80°C until further processing. The remaining medium was aspirated from wells, and the wells were rinsed three times with ice-cold DMEM to stop enzyme and transporter activity. A chilled 7:1 methanol:water (0.1% TFA) solution (v/v) containing $d_6$-DB868 (0.03 µM) and $d_6$-DB829 (0.1 µM) was added to lyse the cells, and the contents were scraped into microcentrifuge tubes. The tubes were vortex-mixed (10 min) and stored at -80°C until further processing. Medium and cell lysates were analyzed for DB868 and phase I metabolites by HPLC/MS/MS. Total protein content was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA).

**Analytic Methods.** *HPLC-UV Chromatography.* Chromatographic analysis of DB868 and phase I metabolites was achieved with an Agilent 1100 Series HPLC system (Palo Alto, CA).
using methods described previously (Wang et al., 2006) with minor modifications. Briefly, DB868 and metabolites were separated using an Agilent (Palo Alto, CA) Zorbax SB-CN column (5 µm, 2.1 x 150 mm) equipped with a Zorbax SB-CN guard column (5 µm, 4.6 x 12.5 mm). Quantification of DB868 and metabolites was achieved using an external calibration curve and comparison of peak areas to those of authentic standards (with the exception of the metabolite M4, for which no authentic standard was available). Calibration curves for all analytes ranged from 50-15,000 nM (lower limit of quantification (LLOQ) was 50 nM) and were linear within this range ($r^2 \geq 0.98$). External standard CVs were ≤15% from nominal concentrations. **HPLC-Mass Spectrometry. Metabolite Identification.** The HPLC/MS system was an Agilent 1100 system composed of an autosampler, binary pumps, column heater, diode array ultraviolet detector, fluorescence detector, and ion trap mass spectrometer. The HPLC component was controlled by Chemstation (v.A.9; Agilent, Wilmington, DE), and the ion trap MS was controlled by Trap (v.4.1; Bruker Daltonics, Bremen, Germany). The sample (40 μL) was injected onto an Agilent Zorbax SB-CN column (5 µm, 2.1 x 150 mm) equipped with a Zorbax SB-CN guard column (5 µm, 4.6 x 12.5 mm) and eluted with a mobile phase (flow rate, 0.35 mL/min) consisting of 35 mM formic acid and 15 mM ammonium formate in 100% water (A) and 80:20 acetonitrile:water (B). The gradient began with 5% B and increased linearly to 60% over 22 min. A sharp increase to 100% B occurred over 30 sec and was maintained for the next 2.5 min. The column (25°C) was equilibrated with 5% B for 4 min. UV absorbance was monitored at 359 nm, and fluorescence was monitored at 359 nm (excitation) and 462 nm (emission). Data from the ion trap were collected in positive ion mode as either MS only or as targeted MS$^n$ total ion chromatograms. MS only detection was used to determine the m/z to be included in MS$^n$ analysis in subsequent injections. Based on MS data and known metabolism of analogous compounds (Zhou et al., 2002; Zhou et al., 2004; Ansed et al., 2005), proposed intermediate metabolites were synthesized. Confirmation of the proposed metabolites involved matching both the retention times and fragmentation patterns of analytes detected in metabolic incubations.
with those of purified analytical standards. **Metabolite Quantification.** Phase I metabolites generated from SCHH and SCRH were quantified using a triple quadrupole mass spectrometer (API4000) with Turbolonspray® source (Applied Biosystems, Foster City, CA). Injection volume typically was 4 µL (Leap CTC thermostatted autosampler, Carrboro, NC). Wash solvents for the syringe and injection loop were 50:50 (A) and 80:20 (B) methanol:water with 0.1% formic acid. Analytes were eluted from an Aquasil C18 column (d_p = 5 µm, 2.1 x 50 mm; Thermo Electron Corporation, San Jose, CA) by a Shimadzu solvent delivery system (Columbia, MD) using a mobile phase gradient. Mobile phases consisted of 0.1% formic acid in 100% water (A) and 0.1% formic acid in 100% methanol (B). The gradient began with a 0-0.5 min hold at 10% B; 0.5-4.0 min linear gradient to 90% B; 4.0-5.0 min hold at 90% B; 5.0-5.5 min linear gradient to 10% B; and a 5.5-6.0 min hold at 10% B. The flow rate was 0.5 mL/min, with the exception of the 90% B wash (1.2 mL/min). Eluent from 0-0.8 min was diverted to waste; eluent from 0.8-4.2 min was directed to the mass spectrometer. Total runtime, including equilibration, was 6 min per injection. The mass spectrometer was operated in positive ion mode using multiple reaction monitoring. With the exception of metabolite M4, operator-controlled parameters were optimized by direct infusion of analytical standards. Tuning, operation, integration, and data analysis used Analyst® (v.1.4.1; Applied Biosystems). Calibration curves, ranging from 5-15,000 nM (LLOQ, 5 nM), were prepared in the appropriate matrix and were linear within this range (r^2 ≥ 0.98). Accuracies were within 15% of nominal concentrations. d_6-DB829 was used as the internal standard for DB829, and d_6-DB868 was used as the internal standard for all other analytes. The wide concentration range of DB868 (0.1-100 µM), used in the enzyme kinetic experiments, made quantification of the M1 metabolite difficult by HPLC/MS/MS due to co-elution and ion-suppression of d_6-DB868 by DB868 (at concentrations >50 nM). This difficulty was overcome by cross-validating the M1 metabolite calibration curve between the HPLC-UV and HPLC/MS/MS platforms (range of overlapping concentrations, 50-10,000 nM).
**Data Analysis.** Model fits to concentration-time profiles from HLM and RLM, as well as enzyme kinetic parameter estimates, were obtained by nonlinear least-squares regression analysis using WinNonlin (v5.0.1; Pharsight, Mountain View, CA). Compartmental model selection/goodness-of-fit was based on standard criteria, including visual comparison of observed data with predicted concentration-time profiles/velocities, residual analysis, Akaike's Information Criterion, and precision of parameter estimates (CV%).

**Compartmental Kinetic Modeling.** A compartmental kinetic modeling approach was used to substantiate the proposed biotransformation pathway of DB868; this approach provided insight into a rare \( N \)-demethoxylation metabolic reaction. The base model (Fig. 2) was structured according to the proposed pathway described for the analog, \([2,5\text{bis}(4\text{amidinophenyl})\text{furan-bis-O-methylamidoxime}]\) (pafuramidine) (Zhou et al., 2004). M4 was omitted, as this metabolite was detected in trace amounts compared to other intermediates and could not be quantified due to lack of an authentic standard. The rate constant, \(k_1\), represented conversion of DB868 to M1; \(k_2\) and \(k_3\) represented conversion of M1 to M2 and M3, respectively. The base model was modified to incorporate \(k_4\), which represented direct conversion of DB868 to M3. Each model was fit with DB868, M1, M2, and M3 data from RLM simultaneously. The final rat model, which incorporated \(k_4\), was fit with corresponding data from HLM. All processes were assumed to be first-order and unidirectional, with no product inhibition. Initial estimates were obtained from pilot substrate depletion experiments with DB868 and M1 (data not shown).

**Enzyme Kinetic Parameter Determination.** M1 formation in HLM was described by the Michaelis-Menten equation for a unienzyme system,

\[
v = \frac{V_{\text{max}} \cdot S}{K_m + S}
\]

(Eq. 1)

where \(v\) is observed velocity and \(S\) is substrate concentration. M1 formation in RLM was described by a two-enzyme system,
M2 formation in HLM exhibited allosteric kinetics and was described by the Hill equation,

\[ v = \frac{v_{\text{max}1} \cdot S}{K_{m1} + S} + \frac{v_{\text{max}2} \cdot S}{K_{m2} + S} \]  

(Eq. 2)

where \( S_{50} \) is analogous to \( K_m \) and represents the concentration required to achieve half \( V_{\text{max}} \), and \( n \) is the Hill coefficient.

**Statistical Analysis.** Statistical analysis was carried out using GraphPad InStat (v.3.06; San Diego, CA). Data are presented as means ± SDs of triplicate determinations unless noted otherwise. First-order rate constants and enzyme kinetic parameters are presented as mean estimates and coefficients of variation (%CVs).
Results

Identification of intermediate metabolites preceding DB829 formation.

Biotransformation of the prodrug, DB868, was NADPH-dependent in both HLM and RLM (Fig. 3). After 180-min incubation, four intermediate metabolites were produced. In both the UV (Fig. 3) and total ion current (not shown) chromatograms, prodrug and intermediate metabolites eluted at 18.5 (DB868) 14.5 (M1), 12.3 (M3), 10.5 (M2), and 8.3 (M4) min. DB829 was formed in trace amounts by HLM, with a retention time of 5.8 min, and was not detected in reaction mixtures containing RLM. Full scan analysis of incubations of DB868 with HLM or RLM revealed the presence of compounds with molecular ions ([M+H]+) at m/z 367 (DB868), 353 (M1), 337 (M3), 339 (M2), and 323 (M4). Molecular ions of these compounds were fragmented into product ions by sequential MS^n to determine the chemical structures. This analysis suggested the presence of two sequential O-demethylation products (m/z 353 and 339) and two sequential N-dehydroxylation products (m/z 337 and 323) (Table 1). The structures of M1-M3 metabolites were confirmed further by comparison of their retention times and mass spectra with those of authentic standards. MS^n analysis of the active drug, DB829, was not possible due to low signal; however, the retention time matched that of authentic standard in UV chromatograms. The biotransformation pattern observed in a previous study with an analog [2,5-bis(4-amidinophenyl)furan-bis-O-methylamidoxime] (pafuramidine) (Zhou et al., 2004) further assisted in the identification of the metabolites of DB868, as well as the proposed biotransformation of DB868 to DB829 (Fig. 4).

Differential metabolism of DB868 by human and rat liver microsomes. Incubations of DB868 (10 µM) with HLM and RLM showed differential extents and rates of metabolite formation (Fig. 5). DB868 was depleted by nearly 100% (Fig. 5A) and by 43% (Fig. 5B) in HLM and RLM, respectively, at the end of the 180-min incubation. M1 was the major metabolite formed by liver microsomes from both species at the end of the incubation, accounting for approximately 45% of the initial prodrug concentration. M2 was detected readily in incubations
with HLM but was just above the LLOQ in incubations with RLM at the end of the incubation period, accounting for ~41% and <3%, respectively, of initial DB868 concentration. M3 accounted for ~12% (HLM) and 9% (RLM) of initial DB868 concentration at the end of the incubation period. M4 was detected in incubations with liver microsomes from both species, but quantification was not possible due to lack of authentic standard. A near quantitative mass balance (~100%) achieved by quantifying M1-M3 at 180 min indicated that M4 accounted for a small percentage of total prodrug-related material in both species. DB829 was detected in UV chromatograms at concentrations below the LLOQ in 180-min incubations with HLM (Fig. 3).

Compartmental kinetic modeling was used to substantiate the proposed biotransformation pathway of DB868 and to determine relative rates of metabolite formation. The base model (Fig. 2) adequately described the data representing the depletion of DB868 and formation of M1 and M2 by RLM, but the early time points for M3 formation were largely underestimated (Fig. 5B). Incorporation of a process representing direct formation of M3 from DB868 ($k_4$) improved the model fit to the M3 data, suggesting an additional metabolic process was involved in M3 formation. The rate constant associated with M1 formation ($k_1$) by RLM was eightfold higher than that for M2 ($k_2$) and M3 ($k_3$ and $k_4$) formation, as reflected by the apparent clearance ($Cl_{app}$) (Table 2). The rate constants associated with $O$-demethylation of M1 ($k_3$) and $N$-demethoxylation of DB868 ($k_4$) to M3 were similar in reactions involving RLM. The modified rat model, which incorporated $N$-demethoxylation of DB868 (Fig. 2), was fit to the metabolism data obtained with HLM. The model described DB868, M1, and M3 disposition adequately; however, although precision for $k_2$ (M2 formation) was acceptable (Table 2), discrepancies were evident between the predicted and observed profiles for M2 (Fig. 5A). Thus, additional experiments were conducted to define the kinetics of formation of M1 from DB868 and M2 from M1 by HLM as a function of substrate concentration.

M1 formation from DB868 was described adequately by a unienzyme model with HLM (Fig. 6A) but by a two-enzyme model with RLM (Fig. 6B) (Table 3). Formation of M2 from M1 by HLM
was described adequately by a sigmoidal model (Fig. 6C, Table 3). Because saturation was not achieved for M2 formation in reactions involving RLM (Fig. 6D), K_m and V_max were not recoverable. Similarly, saturation was not achieved for formation of M3 from M1 by either HLM or RLM (data not shown).

**Evidence for conversion of DB868 to M3 without obligate intermediate formation of M1.** Compartmental kinetic modeling of data from HLM and RLM suggested that at least a portion of M3 was formed directly from DB868 without the intermediate formation of M1. This process implied that P450-mediated oxidative demethylation of DB868 to M1 is not required for formation of M3. To test this hypothesis, P450 enzymes in liver microsomes were inactivated by incubation with the mechanism-based inhibitor ABT in the presence of NADPH, and M3 was measured. As expected, inactivation of P450 by ABT significantly reduced M1 formation (>60%) by HLM (Fig. 7A) and RLM (Fig. 7B); M3 formation was reduced modestly (<15%).

**Human and rat P450s involved in the O-demethylation reactions of the DB868 to DB829 biotransformation pathway.** Incubations of DB868 with human recombinant enzymes showed that CYP1A2, CYP3A4, and CYP4F2 catalyzed M1 formation; CYP1A2 catalyzed M2 formation (Fig. 8A). Incubations of DB868 with rat recombinant enzymes and purified rat Cyp4fs showed that Cyp1a2, Cyp2d2, and Cyp4f1 catalyzed M1 formation (Fig. 8B), and Cyp1a2 catalyzed M2 formation (Fig. 8B). Formation of M1 and M2 with the other human and rat recombinant enzymes tested (CYP2C9, CYP2C19, Cyp2a1, Cyp2a2, Cyp2b1, Cyp2c6, Cyp2c11, Cyp2c13, Cyp2d1, Cyp2e1) was below the LLOQ (data not shown).

**Biotransformation of DB868 in human and rat hepatocytes.** Biotransformation of DB868 in SCHH and SCRH was examined to assess whether or not (1) the trace formation of active drug, DB829, by liver microsomes was a limitation of the microsomal system and (2) pharmacologically relevant amounts of active drug could be formed. Incubations of DB868 (10 µM) with SCHH showed nearly complete depletion of the prodrug from the medium after 4 h (Fig. 9A), whereas roughly half the initial amount remained at 4 h in incubations with SCRH (Fig.
9B). As with HLM and RLM, M1 was detected readily in the medium of SCHH and SCRH, peaking at 4 h. M2 in the medium of SCHH was markedly higher than that of SCRH (below LLOQ) throughout the 24-h period. M3 was detected in the medium from both species during the course of the incubation, with the highest amounts detected at 24 h. DB829 in the medium of SCHH was quantifiable throughout the incubation period and was quantifiable in SCRH only at 24 h. DB868 in cell lysates from both species was quantifiable up to 4 h (Fig. 9C and 9D). M1 and M3 in cell lysates from both species peaked at 4 h and declined by 24 h. M2 was quantifiable in human cell lysates but was below the LLOQ in rat cell lysates throughout the 24-h period. DB829 in cell lysates from both species increased steadily to 24 h, by nearly 13-fold (human) and 23-fold (rat) compared to the 2-h time point. DB829 in lysates from SCHH at 24 h was ~7800-fold higher than that in lysates from SCRH.
Discussion

A comprehensive understanding of the biotransformation of a prodrug to the active drug in preclinical species and humans is critical for assessment of the value of the prodrug strategy for safe and efficacious delivery of the active drug, as well as for selection of the most appropriate species for toxicity testing (Wu and Farrelly, 2007; Wu, 2009). The antitrypanosomal prodrug, DB868, was 100% curative when administered orally to the murine model of second stage HAT (Wenzler et al., 2009), indicating sufficient bioconversion to active drug (DB829) in vivo. These encouraging observations prompted the present study, which was to compare the biotransformation pathway of DB868 between humans and rats using established in vitro systems.

The design of DB868 as a prodrug of the diamidine drug DB829 to improve oral delivery was based on the design of the structural analog pafuramidine (Zhou et al., 2004). DB868 was expected to be O-demethylated oxidatively by P450 enzymes to M1 and M2, which were expected to be reduced by one or more enzymes to the corresponding amidines M3, M4, and DB829. NADPH-dependent oxidative and reductive intermediate metabolites were formed by both HLM and RLM. The expected intermediate phase I metabolites (M1-M4) were identified (Table 1). M1 and M2 were produced via O-demethylation of DB868 and M1, respectively, whereas M3 and M4 were produced via N-dehydroxylation of M1 and M2, respectively. Because the active drug, DB829, was detected only in incubations involving HLM, and in trace amounts, sandwich-cultured hepatocytes were selected as a more complete in vitro system to evaluate DB829 formation. As anticipated, DB829 and M1-M4 were detected readily in sandwich-cultured hepatocytes from both species. Based on observations in microsomes, hepatocytes, and previous reports describing the identification of metabolites of structural analogs, the phase I biotransformation pathway of DB868 leading to formation of DB829 was proposed (Fig. 4).

DB868 was metabolized extensively by liver microsomes and sandwich-cultured hepatocytes from both species, indicating that hepatic metabolism is essential for DB829
formation and DB868 clearance. As assessed by the formation of M1, the O-demethylated product of DB868, disappearance of DB868 by HLM and RLM was comparable, with Cl_{int} values (calculated as the ratio of V_{max} to K_{m}) of 31 and 24 µL/min/mg protein, respectively (Table 3). However, based on K_{m} values of 11 and 0.5 µM for HLM and RLM, respectively, saturation of metabolism in rats would be expected to occur at much lower concentrations compared to humans. This discrepancy is supported by the change in magnitude of Cl_{app} (calculated as the product of the first-order rate constant and incubation volume) when DB868 was incubated at a concentration of 10 µM. At this concentration, Cl_{app} in HLM and RLM was 15 and 2.3 µL/min/mg protein, respectively (Table 2), suggesting that the enzymes were operating at approximately one-half and one-tenth of respective Cl_{int} values (Table 3). These observations suggested that, in vivo, hepatic metabolism of DB868 in rats will be more easily saturable compared to that in humans.

DB868 O-demethylation (M1 formation) was catalyzed most efficiently by human recombinant CYP4F2, followed by CYP1A2 and CYP3A4 (Fig. 8A). These results are consistent with those reported by Wang and colleagues (2006), who showed that O-demethylation of a DB868 analog (pafuramidine) also is catalyzed most efficiently by CYP4F2. In contrast to observations with human enzymes, DB868 O-demethylation was catalyzed most efficiently by rat Cyp2d2, followed by Cyp1a2 and Cyp4f1 (Fig. 8B). It should be noted that incubations with recombinant CYP3A4 did not contain cytochrome b_{5}, which has been reported to enhance CYP3A4 catalytic activity when added to co-expressed P450/NADPH P450 reductase systems (Yamazaki et al., 2002). Accordingly, the rates of M1 formation by CYP3A4 in the current work may have been lower than actual rates, and M2 formation by CYP3A4 may have been masked by the potentially low CYP3A4 activity. A marked species difference was observed in M2 formation by microsomes. The amount of M2 formed by HLM (Fig. 5A) was 25-fold greater than that formed by RLM (Fig. 5B). This difference could be explained by the distinct difference in the kinetics driving M2 formation (Table 3). That is, allostERIC kinetics governed M2 formation by
HLM (Fig. 6C), whereas linear (nonsaturable) kinetics governed M2 formation in RLM (Fig. 6D). M2 formation in both species was catalyzed by CYP1A2/Cyp1a2 (Fig. 8), which has demonstrated allosteric behavior with other substrates (Sohl et al., 2008). More detailed studies are needed to phenotype the enzymes involved in the oxidation of DB868 and were beyond the scope of this work.

Compartmental kinetic modeling of DB868 metabolism by microsomes suggested that M3 formation occurred not only via N-dehydroxylation of M1, but also via N-demethoxylation of DB868 (Fig. 4). This observation implied that M3 could be produced from DB868 without the involvement of any P450 enzymes. Accordingly, the mechanism-based P450 inactivator ABT was used to eliminate P450 enzymes from HLM and RLM to determine if M3 is produced under conditions when M1 is not formed from DB868. Results showed that inhibition of P450-mediated M1 formation did not reduce M3 formation appreciably (<15%) by either HLM or RLM (Fig. 7), providing evidence that M3 can be formed independent of M1. To the authors’ knowledge, N-demethoxylation reactions are rare with mammalian enzymes, although such reactions have been described in environmental research, where soil bacteria and fungi were shown to degrade the N-methoxy-containing phenylurea herbicides linuron and metabromuron (Berger, 1998; Badawi et al., 2009). Parallel clearance pathways are a desirable characteristic for drug development, as they reduce the impact of drug-drug interactions (DDIs) (Ito et al., 2005). Assuming DB868 biotransformation occurs predominately in the liver, discovery of a parallel pathway for DB868 not only reduces the potential impact of DDIs, but also provides an additional route of clearance leading to active drug formation.

The phase I intermediate metabolites generated by SCHH and SCRH were identical to those generated by liver microsomes, consistent with a previous report (Yan et al., 2011). Unlike with liver microsomes, DB829 was detected readily in sandwich-cultured hepatocytes, largely in cell lysates (Fig. 9). DB868 disappeared rapidly from culture medium, with a half-life of 1 and 3.6 h in SCHH and SCRH, respectively, and was paralleled by a steady rise in lysate DB829
concentrations. The negligible appearance of DB829 in the medium is consistent with the poor ability of this dication to cross biologic membranes. M1, M2, and M3 were detected in the medium from SCHH, whereas only M1 and M3 were detected in the medium from SCRH. The absence of M2 in the medium from SCRH, as well as in RLM, indicated that additional toxicity studies involving M2 may be necessary if rat is used as a toxicologic model for development of DB868. The higher extent of DB829 observed in hepatocytes compared with microsomes could correlate to precursors of N-dehydroxylated metabolites having access to cytosolic or mitochondrial reductases not present in microsomes. For example, benzamidoxime reductase in the outer membrane of mitochondria was shown to convert N-hydroxylated prodrugs to amidines (Clement et al., 2005; Havemeyer et al., 2006). The apparent time-dependent cellular accumulation of DB829 in both SCHH and SCRH, with a negligible appearance in medium, suggested that systemic availability of DB829 may be limited by inefficient or lack of sinusoidal and/or canalicular efflux processes, extensive binding to cellular components, and/or lysosomal trapping (Gong et al., 2007; Kaufmann and Krise, 2007). These results may have negative implications regarding efficacy for second stage HAT. Additional studies are needed to assess the exposure of DB829 after DB868 oral administration to animal models and to determine the dose and frequency needed to achieve efficacious DB829 concentrations in the human CNS.

In summary, the current work demonstrated that the prodrug, DB868, is biotransformed extensively by both human and rat hepatic enzymes to the active drug, DB829. The higher extent of formation of DB829 in SCHH compared to HLM suggests that non-microsomal reductive enzymes may be essential for generation of DB829 in vivo. Despite efficient bioconversion to DB829 in hepatocytes, extensive cellular accumulation of DB829 highlights the importance of intracellular processes and/or hepatic efflux transporters in influencing availability of DB829 to the systemic circulation. An improved understanding of the processes that govern DB829 disposition will progress development of an urgently needed and promising oral antitrypanosomal agent.
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Authorship Contributions

Participated in research design: Generaux, Thakker, Tidwell, and Paine.

Conducted experiments: Generaux and Ainslie.

Contributed new reagents or analytic tools: Boykin, Ismail, and Bridges.

Performed data analysis: Generaux.

Wrote or contributed to writing: Generaux, Ainslie, Thakker, and Paine.
References


Footnotes

a. This work was supported by the Consortium for Parasitic Drug Development (CPDD).

b. A portion of this work was presented originally at the Globalization of Pharmaceutics Education Network (GPEN) meeting, Leuven, Belgium (2008).

c. Reprint requests: Mary F. Paine, RPh, PhD

2320 Kerr Hall, CB #7569
Eshelman School of Pharmacy
University of North Carolina Chapel Hill
Chapel Hill NC 27599-7569
Telephone: (919) 966-9984
Fax: (919) 962-0644
Email: mpaine@unc.edu
Legends for Figures

Fig. 1. Chemical structures of the prodrug, DB868, and the active drug, DB829.

Fig. 2. Compartmental model scheme representing the biotransformation of DB868 by liver microsomes. The base model included first-order rate constants (k₁, k₂, k₃) denoting formation of intermediate metabolites M1, M2, and M3, respectively. The final model was modified to incorporate k₄, which represented direct conversion of DB868 to M3 (dashed line).

Fig. 3. Representative HPLC-UV chromatograms depicting detection of DB868 (18.5 min) and intermediate metabolites M1 (14.5 min), M2 (10.5 min), M3 (12.3 min), and M4 (8.3 min). The chromatograms show analysis of 180 min-incubations of DB868 (10 µM) with HLM or RLM (0.5 mg/mL) in the presence (upper and middle panels) or absence (lower panel) of NADPH. Trace amounts of DB829 (5.8 min) were detected in the UV chromatogram of incubations with HLM. The profile for HLM control was similar to that for the RLM control (not shown).

Fig. 4. Proposed scheme for the metabolic conversion of the prodrug, DB868, to the active diamidine drug, DB829.

Fig. 5. Concentration-time profile of DB868 (●) and intermediate metabolites M1 (♦), M2 (▲), and M3 (■) in HLM and RLM. HLM (A) or RLM (B) were incubated with DB868 (10 µM) for up to 180 min, and the intermediate metabolites were quantified by HPLC-UV. Quantification of M4 was not possible due to lack of an authentic standard. Concentrations of DB829 were below the lower limit of quantification (50 nM). Symbols and error bars denote means and SDs, respectively, of triplicate incubations. Solid lines denote model-generated fits of the final rat model (Fig. 2) to the data generated with HLM (A) or RLM (B). The dashed line depicts underprediction of M3 when the rate constant, k₄, was omitted from the model.

Fig. 6. Plots depicting rates of M1 and M2 formation by human liver microsomes (HLM) (A, C) and rat liver microsomes (RLM) (B, D). M1 formation data are from incubations of HLM or RLM (0.5 mg/mL) with DB868 (0.1-100 µM) for 5 or 10 min, respectively. M2 formation data are from incubations of HLM with M1 (0.1-25 µM) for 5 min. All reactions were initiated with NADPH (2
mM final concentration). M1 formation by HLM was described by a single-enzyme Michaelis-Menten model, whereas M1 formation by RLM was described by a two-enzyme model. M2 formation by HLM was described by the empiric Hill equation; M2 formation by RLM did not reach saturation at the concentrations examined. Insets show Eadie-Hofstee plots characteristic of a single-enzyme system (A), a biphasic system (B), and an allosteric system (C). Symbols and error bars denote means and SDs, respectively, of triplicate incubations. Lines denote model-generated fits to observed data.

**Fig. 7.** Inactivation of P450 enzymes by ABT show evidence of DB868 N-demethoxylation. Primary incubations containing HLM (A) or RLM (B) (5 mg/mL) were treated with the non-specific mechanism-based P450 inhibitor ABT (1 mM) in the absence or presence of NADPH (2 mM). Aliquots were removed from the primary incubations after 30 min and were added to secondary incubation mixtures (10-fold dilution) containing DB868 (10 μM) and NADPH (2 mM). Secondary incubations were terminated after 5 (HLM) or 10 (RLM) min by the addition of acetonitrile. Bars and error bars denote means and SDs, respectively, of triplicate incubations and represent the rate of formation of M1 (white) and M3 (black).

**Fig. 8.** Human and rat P450 enzymes catalyzing the formation of M1 (open bar) and M2 (diagonal stripes). Panel A depicts human recombinant enzymes incubated with DB868 or M1. Panel B depicts rat recombinant enzymes (left) incubated with DB868 or M1 and purified rat Cyp4fs (right) incubated with DB868. All reactions were initiated with NADPH (2 mM). Bars denote means of duplicate incubations. **BLQ**, below limit of quantification.

**Fig. 9.** DB868 (●), phase I intermediate metabolites [M1 (♦), M2 (▲), M3 (■)], and DB829 (○) formed by SCHH (upper panels) and SCRH (lower panels). DB868 (10 μM) was incubated with SCHH or SCRH at 37°C for 24 h. DB868 and metabolites were quantified in medium (A, B) and cell lysates (C, D) at selected time points from 0.5 to 24 h. Symbols and error bars denote
means and SDs, respectively, of 6 (human) or 4 (rat) replicates. Quantification of M4 was not possible due to lack of an authentic standard.
Table 1. Structural Information for DB868 and intermediate metabolites.

<table>
<thead>
<tr>
<th>ID</th>
<th>Known/Proposed Structure</th>
<th>Parent Ion [M+H]^+ (m/z)</th>
<th>Proposed MS^n Fragmentationsa</th>
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<td><img src="image" alt="DB868 Structure" /></td>
<td>367</td>
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<td>M4</td>
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<td>323</td>
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</table>

aArrows on structures represent proposed fragmentation sites.
Table 2. Apparent first-order rate constants and apparent clearances (Cl_{app}) associated with the biotransformation of DB868 (10 µM) by HLM and RLM.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reaction</th>
<th>Rate constant</th>
<th>Estimate (min^{-1}) [CV(%)][a]</th>
<th>Cl_{app} (µL/min/mg protein)[b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>DB868→M1</td>
<td>k_{1}</td>
<td>0.0300 (5.6)</td>
<td>15.0</td>
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<tr>
<td></td>
<td>M1→M2</td>
<td>k_{2}</td>
<td>0.0060 (7.6)</td>
<td>3.00</td>
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<tr>
<td></td>
<td>M1→M3</td>
<td>k_{3}</td>
<td>0.0002 (18)</td>
<td>0.10</td>
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<tr>
<td></td>
<td>DB868→M3</td>
<td>k_{4}</td>
<td>0.0057 (15)</td>
<td>2.86</td>
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<tr>
<td>Rat</td>
<td>DB868→M1</td>
<td>k_{1}</td>
<td>0.0046 (2.6)</td>
<td>2.32</td>
</tr>
<tr>
<td></td>
<td>M1→M2</td>
<td>k_{2}</td>
<td>0.0006 (36)</td>
<td>0.30</td>
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<tr>
<td></td>
<td>M1→M3</td>
<td>k_{3}</td>
<td>0.0007 (52)</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>DB868→M3</td>
<td>k_{4}</td>
<td>0.0005 (25)</td>
<td>0.28</td>
</tr>
</tbody>
</table>

[a] Values denote mean (CV) obtained by non-linear least squares regression using WinNonlin (v.5.0.1; Pharsight, Mountain View, CA).

[b] Apparent clearance, calculated as the product of the first-order rate constant and incubation volume.
Table 3. Enzyme kinetic parameter estimates for the formation of M1 and M2 by HLM and RLM.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reaction</th>
<th>Parameter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cl&lt;sub&gt;int&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (μL/min/mg protein)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td><strong>V&lt;sub&gt;max&lt;/sub&gt;</strong> (pmol/min/mg protein)</td>
<td><strong>K&lt;sub&gt;m&lt;/sub&gt;</strong> (μM)</td>
</tr>
<tr>
<td>Human</td>
<td>DB868→M1 (Eq. 1)</td>
<td>340 (4.5)</td>
<td>11 (6.7)</td>
</tr>
<tr>
<td></td>
<td>M1→M2 (Eq. 3)</td>
<td>180 (12)</td>
<td>18 (3)</td>
</tr>
<tr>
<td>Rat</td>
<td>DB868→M1 (Eq. 2)</td>
<td>V&lt;sub&gt;max1&lt;/sub&gt;: 12 (26)</td>
<td>K&lt;sub&gt;m1&lt;/sub&gt;: 0.5 (34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V&lt;sub&gt;max2&lt;/sub&gt;: 70 (14)</td>
<td>K&lt;sub&gt;m2&lt;/sub&gt;: 27 (36)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values denote mean (CV) obtained by non-linear least squares regression using WinNonlin (v.5.0.1; Pharsight, Mountain View, CA).

<sup>b</sup>Intrinsic clearance, calculated as the ratio of V<sub>max</sub> to K<sub>m</sub>. 
Appendix

COMPARTMENTAL KINETIC PARAMETER ABBREVIATIONS

\( C_{\text{DB868}} \) : concentration of DB868 in incubation

\( C_{\text{M1}} \) : concentration of M1 in incubation

\( C_{\text{M2}} \) : concentration of M2 in incubation

\( C_{\text{M3}} \) : concentration of M3 in incubation

\( k_1 \) : first-order rate constant representing metabolic conversion from DB868 to M1

\( k_2 \) : first-order rate constant representing metabolic conversion from M1 to M2

\( k_3 \) : first-order rate constant representing metabolic conversion from M1 to M3

\( k_4 \) : first-order rate constant representing metabolic conversion from DB868 to M3

COMPARTMENTAL KINETIC MODEL EQUATIONS

\[
\frac{dC_{\text{DB868}}}{dt} = -(k_1 + k_4) \cdot C_{\text{DB868}}
\]

\[
\frac{dC_{\text{M1}}}{dt} = k_1 \cdot C_{\text{DB868}} - (k_2 + k_3) \cdot C_{\text{M1}}
\]

\[
\frac{dC_{\text{M2}}}{dt} = k_2 \cdot C_{\text{M1}}
\]

\[
\frac{dC_{\text{M3}}}{dt} = k_3 \cdot C_{\text{M1}} + k_4 \cdot C_{\text{DB868}}
\]
Fig. 1

DB868 → DB829
Fig. 2

Diagram showing the relationships between DB868, M1, M2, and M3 with the following reactions:

- $k_1$ from DB868 to M1
- $k_2$ from M1 to M2
- $k_3$ from M1 to M3
- $k_4$ from DB868 to M3
Fig. 3

Retention Time (min)

Absorbance (mAU)

HLM

DB829  M4  M2  M3  M1  DB868

RLM

RLM (No NADPH)
A. Human

B. Rat