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TITLE PAGE

Unusual Dehydroxylation of Antimicrobial Amidoxime Prodrugs by Cytochrome b_5 and NADH Cytochrome b_5 Reductase[a]

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Running Title: Metabolism of Antimicrobial Amidoxime Prodrugs

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List of Non-standard Abbreviations:

ABT	1-Aminobenzotriazole
API	Atmospheric Pressure Ionization
b5	Expressed Cytochrome b_5
b5R	Expressed Cytochrome b_5 reductase
HLMs	Human Liver Microsomes
HMB	ρ-Hydroxymercuribenzoate
NAD	Reduced Nicotinamide Adenine Dinucleotide
NADP	Reduced Nicotine Adenine Dinucleotide Phosphate

Abstract

Furamidine is an effective antimicrobial agent; however, oral potency of furamidine is poor. A prodrug of furamidine, DB289, has greatly improved oral potency. DB289 is transformed to furamidine via O-demethylation and N-dehydroxylation reactions with four intermediate metabolites formed. The O-demethylation reactions have been shown to be catalyzed by CYP450. The enzymes catalyzing the reductive N-dehydroxylation reactions have not been determined. The objective of this study was to identify the enzymes that catalyze N-dehydroxylation of metabolites M1, a monoamidoxime, and M2, a diamidoxime, formed during generation of furamidine. M1 and M2 metabolism was investigated using human liver microsomes and human soluble cytochrome b_5 and NAD cytochrome b_5 reductase, expressed in *Escherichia coli*. Kinetics of M1 and M2 reduction by human liver microsomes exhibited high affinity and moderate capacity. Metabolism was significantly inhibited by antibodies to cytochrome b_5 and b_5 reductase and by chemical inhibitors of b_5 reductase. The amidoximes were efficiently metabolized by liver mitochondria, which contain cytochrome b_5/b_5 reductase, but not by liver cytosol, which contains minimal amounts of these proteins. Expressed cytochrome b_5/b_5 reductase, in the absence of any other proteins, efficiently catalyzed reduction of both amidoximes. K_m values were similar to those for microsomes and V_{max} values were 33-36-fold higher in the recombinant system, compared to microsomes. Minimal activity was seen with cytochrome b_5 or b_5 reductase alone, or with cytochrome P450 reductase alone or with cytochrome b_5. These results indicate that cytochrome b_5 and b_5 reductase play a direct role in metabolic activation of DB289 to furamidine.

2,5-Bis(4-amidinophenyl)furan-bis-O-methylamidoxime (DB289) is an antimicrobial prodrug developed for the treatment of a variety of microbial infections. DB289 has completed Phase II clinical trials for African trypanosomiasis in Angola and the Democratic Republic of Congo, and is currently enrolled in Phase II trials for malaria in Thailand and for *Pneumocystis* pneumonia in Peru. In the Phase II clinical trials involving patients with primary stage African trypanosomiasis, treatment with DB289 achieved cure rates of approximately 95%. Moreover, DB289 was found to be well tolerated, with no significant side effects (Allen et al., unpublished).

The study of drug metabolism is a key component in the drug discovery process. A compound's metabolic pathway can provide valuable information, including the identification of metabolites, the rate and extent of metabolism, the enzymes responsible for catalyzing metabolism and potentially dangerous drug-drug interactions. More importantly for DB289 is the role of drug metabolism for activation of this inactive prodrug. The phase I metabolic pathway for DB289 conversion to the active dicationic compound 2,5-bis(4-amidinophenyl)furan (furamidine; DB75) has been determined in vitro using freshly isolated rat hepatocytes (Zhou, 2001). DB289 uptake and metabolism by rat liver hepatocytes was rapid, with furamidine detectable inside the cells within 30 min. Further investigations resulted in the detection of four other intermediate phase I metabolites as shown in Fig. 1. The metabolic conversion of DB289 to furamidine is complex with two different metabolic routes that converge on M4. Efficient transformation and enzymatic activation through this metabolic pathway are required for sufficient quantities of furamidine to reach its target. Therefore, characterizing the enzymes involved in the metabolic conversion is important for evaluating the metabolic disposition of these compounds in vivo.

Preliminary results using rat hepatocytes with 1-aminobenzotriazole (ABT), a mechanism-based inhibitor of P450, indicated that the first step in the metabolic pathway is P450-mediated (Zhou, 2001). The metabolism of DB289 was inhibited approximately 70% in ABT-treated samples. Hence, it was hypothesized that the oxidative O-demethylations were catalyzed by specific P450 enzymes. In support of the latter, recent metabolism results have demonstrated that CYP1A and CYP3A4 are involved in the oxidative O-demethylations (Saulter et al., unpublished).

Previous studies with aromatic amidoximes have shown that these functional groups are reduced to amidines by an NAD-dependent liver microsomal pathway (Clement et al., 1997; Trepanier and Miller, 2000), which includes cytochrome b_5 and NAD cytochrome b_5 reductase (Clement et al., 1997; Clement, 2002; Kurian et al., 2004; Andersson et al., 2005). Cytochrome b_5, along with its reductase, is an electron transfer protein involved in reduction reactions with methemoglobin (Hultquist and Passon, 1971), oxidized ascorbate (Ito et al., 1981; Shirabe et al., 1995), fatty acid desaturases (Oshino et al., 1971) and some P450 enzymes (Hildebrandt and Estabrook, 1971). Controversy exists, however, over whether these two proteins can directly reduce xenobiotic amidoximes, (Kurian et al., 2004) or whether a third P450 or other protein is required for reduction (Clement et al., 1997; Clement and Lopian, 2003; Andersson et al., 2005).

Because other amidoximes and hydroxylated amines have been developed to increase the absorption of antihypertensive and antithrombotic drugs, the enzymes responsible for amidoxime reduction, and drug bioactivation, are clinically important and should be completely characterized (Weller et al., 1996; Clement and Lopian, 2003; Song et al., 2003). Therefore, these studies were directed towards examining the roles of cytochrome b_5 and

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cytochrome b_5 reductase in the reductive N-dehydroxylation reactions of the amidoximes M1 and M2, intermediate metabolites of DB289 activation to furamidine. Selective chemical and antibody inhibition of cytochrome b_5/b_5 reductase and reconstitution studies with expressed cytochrome b_5 and b_5 reductase were used to assess the role of cytochrome b_5/b_5 reductase in the metabolic pathway of DB289. Results presented here demonstrate that amidoximes can be efficiently metabolized by cytochrome b_5 and b_5 reductase without the involvement of a third P450 or other protein.

Methods

Chemicals and Reagents. The chemical synthesis of M1 (DB775-dihydrochloride salt; 2-(4-hydroxyamidinophenyl)-5-(4-methoxyamidinophenyl)furan) and M2 (DB290-dihydrochloride salt; 2,5-bis(4-amidinophenyl)furan-bis-*O*-amidoxime) and the stable isotopically labeled internal standard, d_8–DB289 (d_8-2,5-bis(4-amidinophenyl)furan-bis-*O*-methylamidoxime), were performed as previously described (Boykin et al., 1996; Stephens et al., 2001; Anbazhagan et al., 2003). Acetonitrile, propanol, methanol and HPLC grade water were obtained from Fisher Scientific (Fairlawn, NJ). Magnesium chloride, reduced NADP (NADP), reduced NAD (NAD), potassium dihydrogen phosphate salt, sodium hydrogen phosphate salt, ammonium acetate, ABT, 4-methylpyrazole (MP), allopurinol (ALP), control rabbit serum, 6-propylthiouracil (PTU) and *para*-hyrdroxymercuribenzoate (HMB) were obtained from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes (HLMs), human liver cytosol and human liver mitochondria were obtained from Xenotech LLC (Lenexa, KS). Expressed P450 reductase and expressed P450 reductase with cytochrome b_5 were obtained from Gentest Corp. (Bedford, MA). Purified human recombinant cytochrome

b_5 reductase (b5R), cytochrome b_5 (b5) and antisera to b5R and b5 were prepared as described (Kurian et al., 2004). Borosilicate glass vials and capmats were obtained from Q-Glass Corp. (Towaco, NJ). All other analytical grade chemicals were obtained from available commercial sources.

In Vitro Metabolism by Pooled Human Liver Microsomes. The standard incubation mixture (final volume, 0.5 mL) contained 0.5 mg/mL HLMs and 1 mM NAD in 100 mM phosphate buffer, pH 6.3. Pilot experiments were performed to determine optimum metabolic activity, and the cofactors NAD and NADP were incubated with substrate at varying pH (pH 5–8). Michaelis-Menten kinetics for the metabolism of M1 and M2 were determined using final concentrations of 0.01 – 50 μM (0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10, 50 μM). The substrate (M1 or M2) was added in methanol (final concentration, 0.2%) and all incubations were performed at 37˚C. After an initial pre-incubation of 3 min with substrate, reactions were initiated with the addition of NAD. Aliquots (50 μL) were removed at 0, 2, 5, 10, 15 and 30 min and quenched in 100 μL of acetonitrile containing 15 nM d_8-DB289 as internal standard. Controls included incubations without NAD.

In Vitro Metabolism by Expressed Cytochrome b_5 and NAD Cytochrome b_5 Reductase. Soluble human b5 and b5R were expressed and purified by methods described previously (Kurian et al., 2004). Typical incubation mixtures for the reconstitution system consisted of a 10:1 ratio of b5 to b5R and 1 mM NAD in 100 mM phosphate buffer (pH 6.3). The optimization of a 10:1 ratio of b5 to b5R for in vitro reduction has been described by Kurian et al., and this stoichiometry has been found in native liver microsomes (Yang and Cederbaum, 1996). Michaelis-Menten kinetics for the metabolism of M1 and M2 were

determined using final concentrations of 0.01 – 50 μM as described for HLMs. Incubations without NAD served as controls.

Chemical Inhibition Assays. Enzyme inhibitors were added to the standard incubation mixture in HLMs and in the expressed b5/b5R system. Chemical inhibitors were added in methanol (final methanol concentration, 0.4%) at the following final concentrations: 30 μM MP (alcohol dehydrogenase inhibitor), 20 μM ALP (xanthine oxidase inhibitor), 1 mM HMB (cytochrome b_5 reductase inhibitor) and 5 mM PTU (cytochrome b_5 reductase inhibitor). These competitive inhibitors were added just before the addition of substrate (M1 or M2; final concentration 1.0 μM selected on the basis of the calculated K_m values). Reactions were then initiated by the addition of NAD and carried out as described above. For incubations with the mechanism-based P450 inhibitor, ABT, the inhibitor was first pre-incubated in the presence of NADP with HLMs at 37˚C for 15 min before the reaction was initiated by addition of the respective substrate. Controls included incubations containing substrate without inhibitors (final methanol concentration, 0.4%).

Immunoinhibition Assays. Antisera to human cytochrome b_5 and b_5 reductase were generated in rabbits using standard protocols as described previously (Kurian et al., 2004). Cytochrome b_5 or b_5 reductase antisera (40 μL) was added to the standard incubation mixture in HLMs or in the expressed b5/b5R system. The antisera was pre-incubated with HLMs or expressed b5/b5R at room temperature for 30 min before addition of substrate (M1 or M2; final concentration, 1.0 μM). The reactions were then performed as described above using 1 mM NAD to initiate each reduction reaction. Controls included incubations with control rabbit serum.

Reductive Metabolism by Human Liver Cytosol and Mitochondrial Fractions. To further probe the involvement of cytochrome b_5 and b_5 reductase in amidoxime reduction, incubations were performed in the presence of human liver cytosol (0.5 mg/mL protein) or human liver mitochondrial fractions (0.5 mg/mL protein). The substrate (M1 or M2; final concentration, 1.0 μM) was added in methanol and reactions were performed as described above for HLMs, using 1 mM NAD to initiate each reduction reaction. Incubations without NAD served as controls. Immunoblots of cytochrome b_5 and b_5 reductase in human liver cytosol, human liver microsomes and S9 fraction were obtained from methods described by Kurian et al. (Kurian et al., 2004)

In Vitro Metabolism by Expressed Cytochrome P450 Reductase and Cytochrome b_5 Reductase. The standard incubation mixture (final volume, 0.5 mL) contained microsomes with expressed human cytochrome P450 reductase (0.5 mg/mL), or expressed human P450 reductase plus cytochrome b_5 (0.5 mg/mL) or purified expressed human soluble b5R (6.3 μg) or b5 (29.8 μg). 1 mM NAD in 100 mM phosphate buffer, pH 6.3 was used for incubations with b5R or b5. 1mM NADP in 100 mM phosphate buffer, pH 7.4 was used for the incubations containing P450 reductase only. The substrate (M1 or M2; final concentration, 1.0 μM) was added in methanol (final concentration, 0.2%) and all incubations were performed at 37˚C. After an initial pre-incubation of 3 min with substrate, reactions were initiated with the addition of NAD or NADP. Aliquots (50 μL) were removed at 0, 2, 5, 10, 15 and 30 min and quenched in 100 μL of acetonitrile containing 15 nM d_8-DB289 as internal standard.

HPLC/MS Analyses. Each amidoxime reduction reaction to amidine was measured using reversed-phase HPLC with tandem triple quadrupole mass spectrometry (HPLC/MS-

MS) methods. The analytical system consisted of an Agilent 1100 binary pump (Palo Alto, CA), a thermostatic CTC PAL Leap autosampler (Hamilton Co., Reno, NV), Valco solvent divert valve (Houston, TX), and an Applied Biosystems API 4000 triple quadrupole mass spectrometer equipped with a TurboIon Spray® interface (MDS Sciex, San Francisco, CA) and PEAK nitrogen generator (Punta Gorda, FL). All equipment was controlled using Analyst v. 1.3 software (Applied Biosystems, Foster City, CA). Elution of M1, M2 and d_8-DB289 through a Zorbax Bonus RP 2.1 x 50 mm, 3.5 µm analytical column required gradient HPLC, at room temperature, with a flow rate of 500 µL/min. Initial gradient conditions of 90% solvent A (10 mM ammonium acetate in HPLC grade water:propanol, 99:1 v/v) were held for 30 sec. The amount of solvent B (100% methanol) was increased linearly over 90 sec until it reached 90%. The mobile phase composition was held constant (10:90 v/v, solvent A:solvent B) for the next 30 sec. Initial conditions were reintroduced in a linear fashion over the next 30 sec and maintained for one min. Total run time was 4 min. Samples were kept chilled (6ºC) in covered 96-well plates containing borosilicate glass sample inserts. The typical injection volume was 5 µL. After each injection, the syringe, injector valve and loop were washed repeatedly with wash solvent 1 (60:40 v/v, methanol:water) and wash solvent 2 (50: 50 v/v, methanol:water). Mass spectrometric conditions (user-controlled voltages, gas pressures, and source temperature) were optimized for the maximum detection of M1, M2 or d_8-DB289 using direct infusion of each compound in the manual tuning mode of Analyst 1.3. Data acquisition was performed using multiple reaction monitoring. Post-acquisition quantitative analyses were performed using Analyst 1.3 software. Unknown substrate (M1 or M2) concentrations were calculated from the

weighted (1/x) quadratic curve determined by the least-squares regression constructed from the peak area ratios of analyte to d_8-DB289, versus analyte concentration.

Enzyme Kinetic Analyses. Substrate depletion data was analyzed using GraphPad Prism 4.0 (San Diego, CA). For substrate depletion analysis, the percentage remaining versus time at each substrate concentration was fitted to first order decay functions to determine substrate depletion rate constants (k). The rates of substrate depletion were calculated by expressing the velocity (v) in terms of half-life ($t_{1/2}$) and concentration of substrate at time = 0 [$(S)_0$] : $v = \ln 2\ (S)_0 / t_{1/2}$. Enzyme kinetic data was also transformed and plotted on Eadie-Hofstee plots to assess linearity. Substrate depletion rates versus substrate concentration were fit to a single-site Michaelis-Menten equation. K_m and V_{max} values were then determined by nonlinear regression of the reaction velocity versus substrate concentration data. The kinetic values were not corrected for protein binding. Data are presented as averages of duplicate experiments.

Results

Reduction of Amidoximes M1 and M2 by Human Liver Microsomes. The symmetric diamidoxime metabolite, M2, can be converted via sequential reduction reactions first to the amidine/amidoxime metabolite, M4, then to the diamidine, furamidine (Fig. 1). Both M4 and furamidine were detected at all time points examined when M2 was incubated with HLMs under standard reaction conditions (NAD, pH 6.3). Unfortunately, an authentic standard for M4 was not available. M2 reductive metabolism was thus monitored as rate of substrate depletion. M2 reduction by human liver microsomes was maximal at pH 6.5, with NAD as cofactor (Fig. 2). The rate of M2 metabolism was reduced approximately 2.5 fold at

pH 7.4, although NADP could serve as an alternative cofactor for M2 reduction, with activity equal to that for NAD at pH 7.4. Minimal activity, however, was observed at pH 6.5 with NADP (Fig. 2). All subsequent M2 reduction reactions were monitored at pH 6.3 with NAD as cofactor.

The amidoxime/methylamidoxime metabolite, M1, can be metabolized by competing oxidation and reduction reactions (Fig. 1). M1 can be reduced to the amidine/methylamidoxime metabolite, M3. Alternatively, M1 can be oxidatively O-demethylated to form the diamidoxime, M2. The demethylation reaction is catalyzed by human liver microsomal cytochromes P450, with maximal activity at pH 7.4 with NADP as cofactor (Saulter et al., unpublished). M1 reduction to M3 by HLMs, however, predominates at pH 6.3 with NAD as cofactor, with negligible quantities of M2 formed (Fig 3). Thus, M1 reduction was monitored as M1 substrate depletion at pH 6.3 with NAD. Kinetic parameters for metabolism of M1 and M2 were estimated by fitting substrate depletion rates versus substrate concentration to a one-site binding equation with use of nonlinear regression analysis (Fig. 4). Estimates of apparent K_m for M1 and M2 were 4.3 ± 0.4 and 5.9 ± 0.9 μM, respectively, which suggests high affinity for both metabolites. V_{max} values were 908.2 and 1099 pmol/min/mg protein for M1 and M2, respectively, which suggest moderate capacity. M1 and M2 were not metabolized in control reactions without NAD.

Reduction of Amidoximes M1 and M2 by Expressed Cytochrome b_5 and NAD Cytochrome b_5 Reductase. The expressed b5/b5R system efficiently catalyzed the reduction of both M1 and M2 (Fig. 4). Kinetic parameters were derived by fitting the data to a single-site binding equation, as described above using HLMs. Apparent K_m values obtained for the expressed b5/b5R system were comparable to values obtained with HLMs

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(7.1 ± 1.2 and 5.2 ± 0.7 μM for M1 and M2, respectively). V_{max} values were dramatically increased, as expected for the expressed enzymes. V_{max} values for M1 and M2 reduction by the expressed b5/b5R system were 33 and 36 times higher, respectively, than values for HLMs (Fig. 4).

Chemical Inhibition of M1 and M2 Metabolism. The effects of inhibitors on amidoxime reduction by HLMs and expressed b5/b5R are shown in Fig. 5. HMB and PTU, inhibitors of b_5R (Kariya et al., 1984; Trepanier and Miller, 2000), significantly inhibited M1 and M2 reduction by both HLMs and expressed b5/b5R. HMB suppressed liver microsomal M1 and M2 substrate depletion rates by 67 and 71%, respectively, compared to control microsomes with no inhibitors. HMB inhibited expressed b5/b5R catalyzed amidoxime metabolism even more completely, decreasing M1 and M2 substrate depletion rates by 97 and 95%, respectively, compared to uninhibited control enzymes. Amidoxime reduction was inhibited to a similar extent by PTU. PTU decreased liver microsomal M1 and M2 metabolism by 59 and 63%, respectively, and b5/b5R M1 and M2 metabolism by 96 and 99%, respectively. Reduction of M1 or M2 was not significantly inhibited by the alcohol dehydrogenase inhibitor, 4-methylpyrazole, or the xanthine oxidase inhibitor, allopurinol (Fig. 5). The P450 inhibitor, ABT, also had negligible effect on M1 and M2 reduction.

Immunoinhibition of M1 and M2 Metabolism. Antisera to cytochrome b_5 and cytochrome b_5 reductase inhibited M1 reduction by HLMs by 39 and 44%, respectively, compared to control serum (Fig. 6). This was comparable to the inhibition of M1 reduction by these antisera in the expressed b5/b5R system, with 46 and 55% inhibition, respectively. Similar results were obtained for M2 metabolism. Antisera to cytochrome b_5 and b_5

reductase inhibited M2 reduction catalyzed by HLMs by 49 and 56%, respectively, and M2 reduction catalyzed by the expressed b5/b5R system by 44 and 51%, respectively (Fig. 6).

M1 and M2 Reductive Activity in Human Liver Cytosol and Human Liver Mitochondrial Fractions. To further probe the involvement of cytochrome b_5 and b_5 reductase in amidoxime reduction, metabolism of M1 and M2 was examined using human liver cytosol and mitochondrial fractions. Cytochrome b_5 and b_5 reductase serve as electron donors in human liver mitochondria (Voet and Voet, 1995). These proteins, however, are present in negligible quantities in mammalian liver cytosol (Giordano and Steggles 1993). Efficient reduction was observed with human liver mitochondria (56.2 and 86.6 pmol/min/mg for M1 and M2, respectively). However, metabolic rates observed with human liver cytosol were 51- and 46-fold lower (1.1 and 1.9 pmol/min/mg for M1 and M2, respectively). Data shown in Fig. 7 also demonstrate that, unlike in HLM, immunoreactive cytochrome b_5 and b_5 reductase cannot be detected in human liver cytosol.

Reductive Metabolism of M1 and M2 by Expressed Cytochrome P450 Reductase

To further evaluate amidoxime reduction, experiments were performed with microsomes containing expressed human cytochrome P450 reductase, P450 reductase co-expressed with cytochrome b_5, or purified expressed b5R or b5 alone. Metabolic activities observed with these preparations were minimal (Fig. 8). The substrate depletion rates for M1 in incubations with P450 reductase, P450 reductase with cytochrome b_5, b5R and b5 were 6.8, 10.8, 15.8 and 25.7 pmol/min/mg. Similarly, depletion rates for M2 were 5.6, 12.2, 21.7 and 23.3 pmol/min/mg, respectively (Fig. 8). These results suggest that P450 reductase, alone or in combination with cytochrome b5, b5R and b5 alone cannot efficiently metabolize M1 or M2.

Discussion

Amidoxime reduction of M1 and M2 to their corresponding amidines was readily detected in several enzyme systems, including pooled human liver microsomes, mitochondria and an expressed b5/b5R system. Together, our data point to an important role for cytochrome b_5 and its reductase in the reductive N-dehydroxylations of amidoximes to amidines. More importantly, our data demonstrates that amidoximes can be successfully converted to amidines in human liver microsomes, providing additional support for the concept that amidoximes can serve as prodrug functionalities for amidino drugs (Clement, 2002). Strong basic nitrogen-containing functional groups, which are protonated under physiological conditions and are not absorbed as cations, can be made orally available by introducing a hydroxyl group, which lowers the pK_a values significantly (Clement, 2002; Saulter et al., unpublished). Therefore, amidoximes and hydroxylamines can be absorbed as free bases, and after subsequent reduction by the enzyme system described in this report, the corresponding active amidine can be produced. Other orally active drugs that utilize this prodrug approach include some antihypertensive and antithrombotic agents (Weller et al., 1996; Song et al., 2003; Clement and Lopian, 2003; Andersson et al., 2005).

Other investigations with cytochrome b_5 have shown that this enzyme can assume several intermediary functions in drug metabolism (Schenkman and Jansson, 2003), such as direct electron transfer to cytochrome P450. For the metabolism of amidoximes specifically, there is controversy regarding the direct role of cytochrome b_5 and its reductase in xenobiotic reduction. It has been suggested that a CYP2D-like third protein is required, in addition to cytochrome b_5/ b_5 reductase for the reduction of N-hydroxylated derivatives (Clement et al.,

1997; Clement, 2002). Other studies suggest that cytochrome b_5 and b_5 reductase have a direct role in reduction without the involvment of P450 (Trepanier and Miller, 2000; Kurian et al., 2004), or that a different third non –P450 protein may be required (Andersson et al., 2005). In support of the latter two hypotheses, none of the known human cytochrome P450 enzymes have been found to be involved in the reduction of the amidoxime prodrug, ximelagatran (Clement and Lopian, 2003). Furthermore, the lack of inhibition by azide, nitrogen, carbon monoxide and P450 antibody and chemical inhibitors, and a lack of correlation between N-reduction and P450 activity suggest that a cytochrome P450 is not involved (Cribb et al., 1995; Lin et al., 1996; Trepanier and Miller, 2000; Clement & Lopian 2003). Recent results from Kurian et al. (2004), suggest that cytochrome b_5 may directly reduce xenobiotics without an intermediary protein, in that hydroxylamines and amidoximes were efficiently reduced by purified cytochrome b_5 and b_5 reductase without the requirement for other proteins, and titration of microsomal protein into the recombinant system did not increase activity more than additively (Kurian et al., 2004).

The present report clearly demonstrates that cytochrome b_5 and b_5 reductase play an important role in DB289 bioactivation. Over a broad range of substrate concentrations, these enzymes efficiently catalyzed the reduction of the DB289 intermediates, M1 and M2. Reduction was active under incubation conditions that preferred NAD to NADP, and had greater activity at pH 6.3 than at physiological pH. The kinetic parameters obtained for both M1 and M2 in HLMs indicate that these compounds have high affinity and moderate capacity for the metabolizing enzymes. In fact, the apparent K_m values obtained in the expressed b5/b5R system were nearly indistinguishable to those obtained with HLMs, which support the hypothesis that additional microsomal proteins, at least those that would alter

substrate affinity, are not required for these reactions. Low metabolic activities were observed in incubations consisting of P450 reductase or P450 reductase with b_5, which suggests that NADP P450 reductase is not important for this bioactivation. Reduction activity was inhibited by chemical inhibitors of b5R and antibodies to either cytochrome b_5 or b_5 reductase. In HLMs and in the expressed b5/b5R system, the b_5 reductase inhibitors, HMB and PTU, significantly inhibited reductive metabolism of M1 and M2. Both inhibitors decreased metabolism of M1 and M2 by 59-71% in HLMs and by 95-99% in the expressed b5/b5R system. The greater inhibition activity observed in the expressed system by these inhibitors may be due to increased access of these hydrophilic inhibitors to the soluble forms of b5 and b5R in the b5/b5R system. The expressed system contained purified human soluble b5 and b5R, which constituted a less lipophilic incubation environment than HLMs. The fact that b_5 and b_5 reductase antisera demonstrated similar inhibitory activity in HLMs and in the expressed system, further support the above theory. Inhibitors of alcohol dehyrogenase and xanthine oxidase had no significant effect on metabolism, and importantly, the non-specific P450 inhibitor ABT, which inhibits CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 (Emoto, 2003), did not diminish microsomal reduction activity.

Cytochrome b_5 and b_5 reductase antisera inhibited reductive metabolism of M1 and M2 in HLMs and in the expressed b5/b5R system 39-55%. Because M1 and M2 are highly protein bound, the amount of antisera added could not exceed 40 μL, which is a sub-optimal concentration for this inhibition reaction according to Kurian et al. (2004), who suggest the addition of 150 μL. Thus, the degree of antibody inhibition could have perhaps been increased if more antisera were added without a protein binding effect. However, the degree

of inhibition was similar between the recombinant and HLM systems. Therefore, incomplete inhibition could have been a property of the antisera (e.g. non-inhibitory antibodies in excess that block access of inhibitory antibodies to critical epitopes; Kurian et al., 2004). Reduction activity was minimal in human liver cytosol, which contains insignificant levels of b_5 (Giordano and Steggles, 1993). In contrast, efficient reduction activity was observed in human liver mitochondria, which indirectly supports the role of cytochrome b_5 and b_5 reductase in amidoxime metabolism, as this cellular component contains both of these enzymes (Voet and Voet, 1995).

In conclusion, the results presented in this report characterize the amidoxime reduction reactions in the metabolic pathway of the antimicrobial prodrug DB289. Because efficient transformation and enzymatic activation through this metabolic pathway are required for sufficient quantities of the active compound, furamidine, to reach its target, the elucidation of these enzymes is important for predicting the metabolic disposition of these compounds in vivo. Moreover, identification of the enzymes responsible for metabolism can be used to help predict potentially dangerous drug-drug interactions and interindividual variability in response to drug administration. We have determined that cytochrome b_5 and b_5 reductase can efficiently catalyze the amidoxime reduction reactions without involvement of P450. Because other amidoximes and hydroxylated amines have been developed to increase the absorption of many antihypertensive and antithrombotic drugs (Weller et al., 1996; Clement and Lopian, 2003; Song et al., 2003), further studies of the direct role of cytochrome b_5 and b_5 reductase in the bioactivation of other amidoxime prodrugs are warranted.

References

Anbazhagan M, Saulter J, Hall J and Boykin D (2003) Synthesis of metabolites of the prodrug 2,5-bis(4-*O*-methoxyamidinophenyl) furan. *Heterocycles* **60**:1133-1145.

Andersson S, Hofmann Y, Nordling A, Li XQ, Nivelius S, Andersson TB, Ingelman-Sundberg M and Johansson I (2005) Characterization and partial purification of the rat and human enzyme systems active in the reduction of N-hydroxymelagatran and benzamidoxime. *Drug Metab Dispos* [Epub ahead of print]

Boykin, DW, Kumar A, et al. (1996) Anti-pneumocystis activity of bis-amidoximes and bis-o-alkylamidoximes prodrugs. *Bioorg Med Chem Lett* **6**:3017-3020.

Clement B (2002) Reduction of N-hydroxylated compounds: amidoximes (N-hydroxyamidines) as pro-drugs of amidines. *Drug Metab Rev* **34**(3):565-579.

Clement B and Lopian K (2003) Characterization of in vitro biotransformation of new, orally active, direct thrombin inhibitor ximelagatran, an amidoxime and ester prodrug. *Drug Metab Dispos* **31**(5):645-651.

Clement B, Lomb R and Moller W (1997) Isolation and characterization of the protein components of the liver microsomal O2-insensitive NAD-benzamidoxime reductase. *J Biol Chem* **272**(31):19615-19620.

Cribb A, Nuss C and Wang R (1995) Antipeptide antibodies against overlapping sequences differentially inhibit human CYP2D6. *Drug Metab Dispos* **23**(7):671-675.

Emoto C, Murase S, Sawada Y, Jones BC and Iwasaki K (2003) In Vitro Inhibitory Effect of 1-Aminobenzotriazole on Drug Oxidations Catalyzed by Human Cytochrome P450 Enzymes: A Comparison with SKF-525A and Ketoconazole. *Drug Metab Pharmacokinet* **18**(5):287-295.

Giordano SJ and Steggles AW (1993) Differential expression of the mRNAs for the soluble and membrane-bound forms of rabbit cytochrome b_5. *Biochimica et Biophysica Acta* **1172**:95-100.

Hildebrandt A and Estabrook RW (1971) Evidence for the participation of cytochrome b5 in hepatic microsomal mixed-function oxidation reactions. *Arch Biochem Biophys* **143**(1):66-79.

Hultquist DE and Passon PG (1971) Catalysis of methaemoglobin reduction by erythrocyte cytochrome B5 and cytochrome B5 reductase. *Nat New Biol* **229**(8):252-254.

Ito T, Ueda MJ, Okada TS and Ohnishi S (1981) Phagocytosis by macrophages. II. The dissociation of the attachment and ingestion steps. *J Cell Sci* **51**:189-201.

Kariya K, Lee E, Yamaoka M and Ishikawa H. (1984) Selective induction of cytochrome b5 and NAD cytochrome b5 reductase by propylthiouracil. *Life Sci* **35**(23):2327-2334.

Kurian JR, Bajad SU, Miller JL, Chin NA and Trepanier LA (2004) NAD cytochrome b5 reductase and cytochrome b5 catalyze the microsomal reduction of xenobiotic hydroxylamines and amidoximes in humans. *J Pharmacol Exp Ther* **311**(3):1171-1178.

Lin LY, Fujimoto M, Distefano EW, Schmitz DA, Jayasinghe A and Cho AK (1996) Selective mechanism-based inactivation of rat CYP2D by 4-allyloxymethamphetamine. *J Pharmacol Exp Ther* **277**(2):595-603.

Oshino N, Imai Y and Sato R (1971) A function of cytochrome b5 in fatty acid desaturation by rat liver microsomes. *J Biochem* (Tokyo) **69**(1):155-167.

Schenkman JB and Jansson I (2003) The many roles of cytochrome b5. *Pharmacol Ther* **97**(2):139-152.

Shirabe K, Landi MT, Takeshita M, Uziel G, Fedrizzi E and Borgese N (1995) A novel point mutation in a 3' splice site of the NAD-cytochrome b5 reductase gene results in immunologically undetectable enzyme and impaired NAD-dependent ascorbate regeneration in cultured fibroblasts of a patient with type II hereditary methemoglobinemia. *Am J Hum Genet* **57**(2):302-310.

Song Y, Clizbe L, Bhakta C, Teng W, Wong P, Huang B, Tran K, Sinha U, Park G, Reed A, Scarborough RM and Zhu BY (2003) Design and synthesis of factor Xa inhibitors and their prodrugs. *Bioorg Med Chem Lett* **13**(2):297-300.

Stephens CE, Patrick DA, Chen H, Tidwell RR and Boykin DW (2001) Synthesis of Deuterium Labelled 2,5-Bis(4-amidinophenyl)furan, 2,5-Bis(4-ethoxyamidinophenyl)furan, and 2,7-Diamidinocarbazole. *J. Labelled Cpd. Radiopharm.* **44**:197-208.

Trepanier LA and Miller JL (2000) NAD-dependent reduction of sulphamethoxazole hydroxylamine in dog and human liver microsomes. *Xenobiotica* **30**(12):1111-1121.

Voet D and JG Voet (1995) *Biochemistry*. 2nd ed. John Wiley and Sons, Inc, New York.

Weller T, Alig L, Beresini M, Blackburn B, Bunting S, Hadvary P, Muller MH, Knopp D, Levet-Trafit B, Lipari MT, Modi NB, Muller M, Refino CJ, Schmitt M, Schonholzer P, Weiss S and Steiner B (1996) Orally active fibrinogen receptor antagonists. 2. Amidoximes as prodrugs of amidines. *J Med Chem* **39**(16):3139-3147.

Yang MX and Cederbaum A (1996) Interaction of ferric complexes with NAD-cytochrome b5 reductase and cytochrome b5: lipid peroxidation, H_2O_2 generation and ferric reduction. *Arch Biochem Biophys* **331:** 69-78.

Zhou L (2001) Mechanisms for the absorption and metabolism of 2,5-bis(4-amidinophenyl)furan-bis-O-methylamidoxine, an orally active prodrug of the antimicrobial agent 2,5-bis(4-amidinophenyl)furan. Ph.D. Dissertation, University of North Carolina at Chapel Hill, Chapel Hill.

Footnotes

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Citation of meeting abstracts where the work was previously presented:

"Reductive N-dehydroxylation of amidoximes by cytochrome b_5 and b_5 reductase" Federation of American Societies for Experimental Biology **(FASEB)** – Washington D.C. April 2004

"Metabolism of DB289, An Antimicrobial Prodrug, by Cytochrome P450 and Cytochrome b_5 Enzymes" International Society for the Study of Xenobiotics (**ISSX)** – Vancouver, Canada August 2004

[b]Person to receive reprint requests: Janelle Y. Saulter, 15803 Kersten Ridge Court, Chesterfield, MO 63017, email: jsaulter@hotmail.com.

Legends for figures:

1. Metabolic pathway of DB289 conversion to Furamidine.

2. Rate of M2 metabolism by human liver microsomes as a function of pH and cofactor. Incubations included 1.0 μM M2 with 1 mM NAD or NADP in 100 mM phosphate buffer. Data is expressed as mean ± SE. Optimum reduction activity of M2 was achieved with NAD in phosphate buffer at pH 6.5.

3. M1 (1 μM) metabolism by human liver microsomes. M1 reduction to M3 by HLMs, predominates at pH 6.3 with NAD as cofactor, with negligible quantities of M2 formed. Data are expressed as average of duplicate experiments.

4. Upper panel: Kinetics of M1 and M2 metabolism by human liver microsomes. Incubations included varying concentrations of M1 or M2 with 1 mM NAD in 100 mM phosphate buffer at pH 6.3. Substrate depletion rates were fit to one-site binding equations as described under *Results.* Data represents two experiments performed in duplicate, and are given as means ± SE. Apparent K_m and V_{max} values were calculated from Michaelis-Menten equations of the data.

Lower panel: Kinetics of M1 and M2 metabolism by expressed cytochrome b_5 and NAD cytochrome b_5 reductase. Incubations included purified human recombinant cytochome b_5 and NAD cytochrome b_5 reductase (optimal 10:1 stoichiometry) in 100 mM phosphate buffer, pH 6.3, with 1 mM NAD. Substrate depletion rates were fit to one-site binding equations as described under *Results*. Data represents two experiments performed in

duplicate, and are given as means ± SE. Apparent K_m and V_{max} values were calculated from Michaelis-Menten equations of the data.

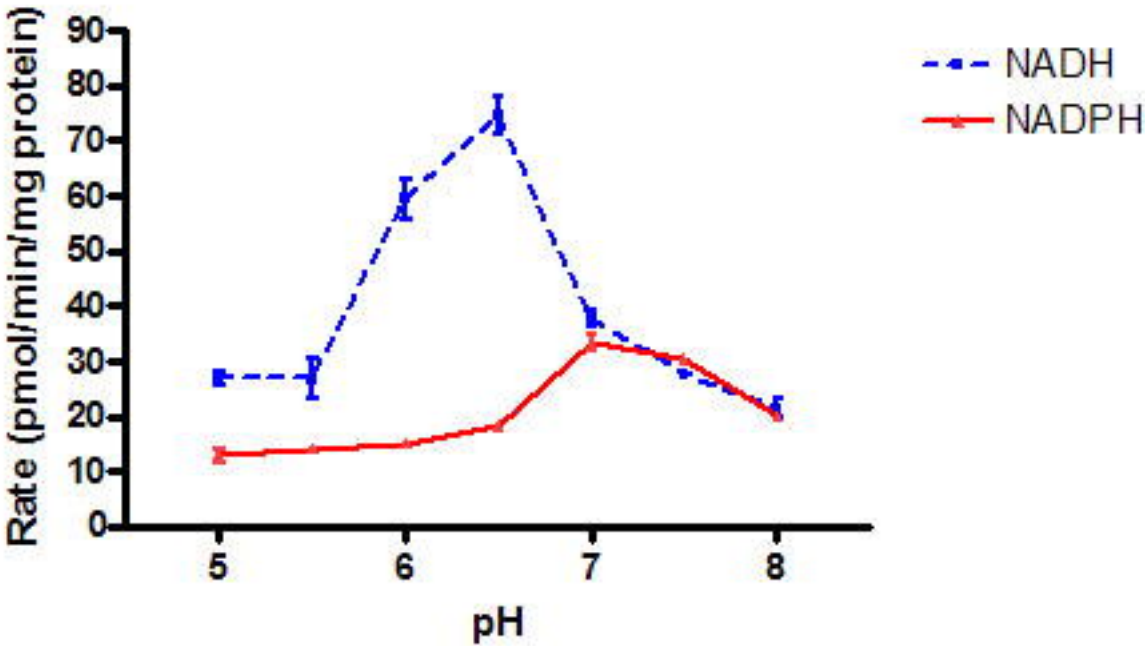
5. Inhibition of amidoxime metabolism by chemical inhibitors of NAD cytochrome b_5 reductase. Incubations included 1 μM substrate with 1 mM NAD in 100 mM phosphate buffer at pH 6.3. Reduction activity is expressed as percent of control activity ( ) with no inhibitors present. (A) M1 reduction with human liver microsomes; (B) M2 reduction with human liver microsomes; (C) M1 reduction with expressed cytochrome b_5 and NAD cytochrome b_5 reductase; (D) M2 reduction with expressed cytochrome b_5 and NAD cytochrome b_5 reductase. The following chemical inhibitors were used: 1 mM ρ-hydroxymercuribenzoate (HMB; b_5 reductase inhibitor); 5 mM 6-propylthiouracil (PTU; b_5 reductase inhibitor); 30 μM 4-methylpyrazole (MP; alcohol dehydrogenase inhibitor); 20 μM allopurinol (ALP; xanthine oxidase inhibitor). Data are presented as mean ± SE.

6. Antisera to cytochrome b_5 and to cytochrome b_5 reductase inhibited amidoxime metabolism catalyzed by human liver microsomes and expressed cytochrome b_5/b_5 reductase. Antiserum (40 μL) to cytochrome b_5 or cytochrome b_5 reductase was added to each incubation mixture. Substrate concentrations and incubation conditions were the same as in Fig. 5. Activities are presented as percent of control incubations containing pre-immune serum (40 μL). (A) M1 reduction catalyzed by human liver microsomes; (B) M2 reduction catalyzed by human liver microsomes; (C) M1 metabolism catalyzed by cytochrome b5/b5 reductase; (D) M2 metabolism catalyzed by cytochrome b5/b5 reductase. Data are presented as mean ± SE.

7. Immunoreactivity of subcellular fractions to cytochrome b_5 reductase (A) and cytochrome b_5 (B). Antisera in human liver micrsomes (lane 1), S9 (lane 2), human liver cytosol (lane 3), human serum albumin (lane 4) and recombinant protein (lane 5).

8. Amidoxime reduction is poorly catalyzed by expressed human cytochrome P450 reductase alone, expressed human cytochrome P450 reductase with cytochrome b_5, or purified expressed soluble human cytochrome b_5 reductase or cytochrome b_5 alone. Upper panel, M1 metabolism; lower panel, M2 metabolism. Reactions containing cytochrome P450 were performed with 1 μM substrate and 1 mM NADP in 100 mM phosphate buffer at pH 7.4. Reactions containing b5 or b5R were performed with 1 μM substrate and 1 mM NAD in 100 mM phosphate buffer at pH 6.3. Activity is normalized to mg of protein added to each reaction. Data are expressed as mean ± SE.

Figure 1.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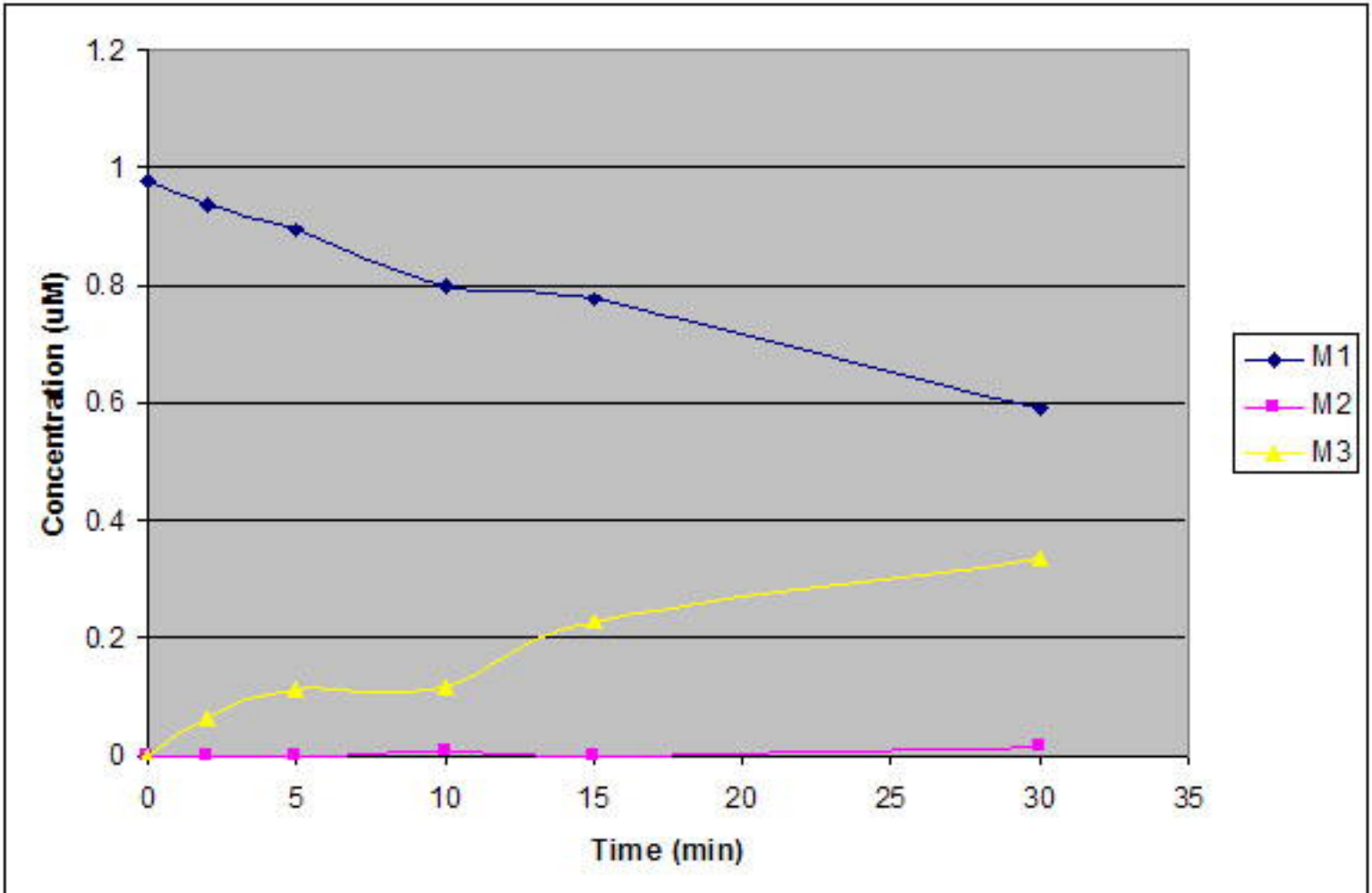
40
30
20
10
0
5
6
7
8
pH
NADH
NADPH

Figure 2.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0.8
0.6
0.4
0.2
0
Concentration (uM)
0
5
10
15
20
25
30
35
Time (min)
M1
M2
M3

Figure 3.

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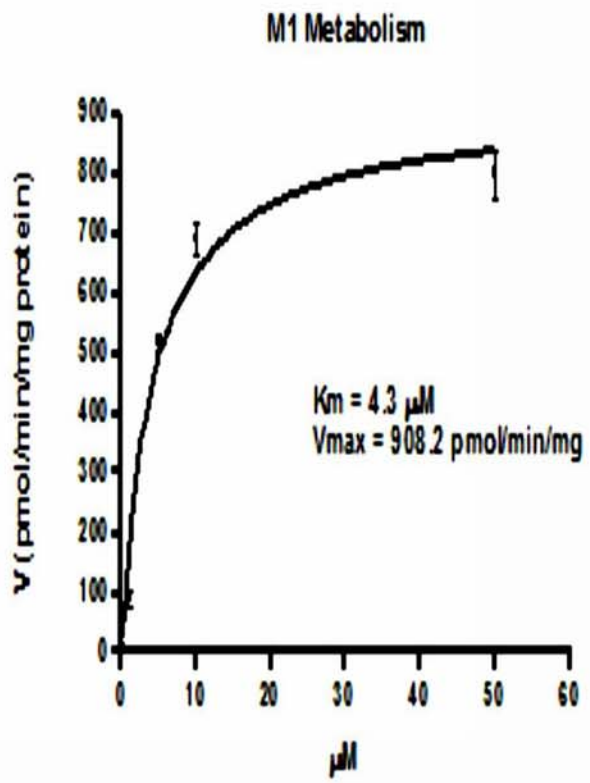

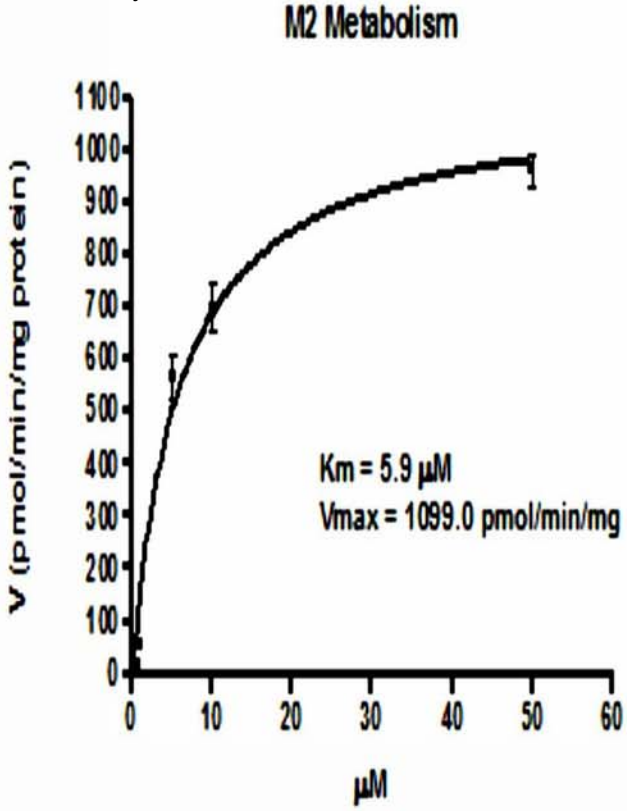

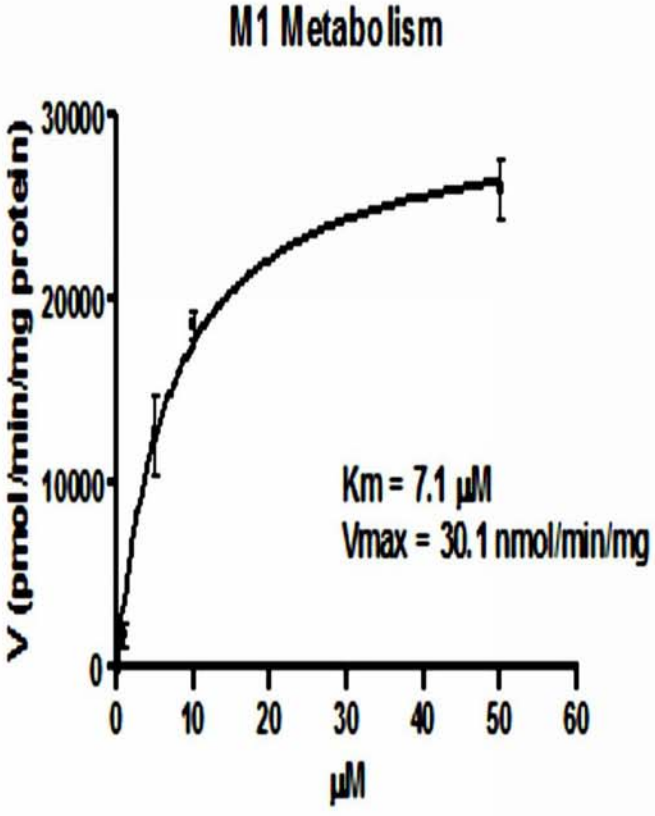

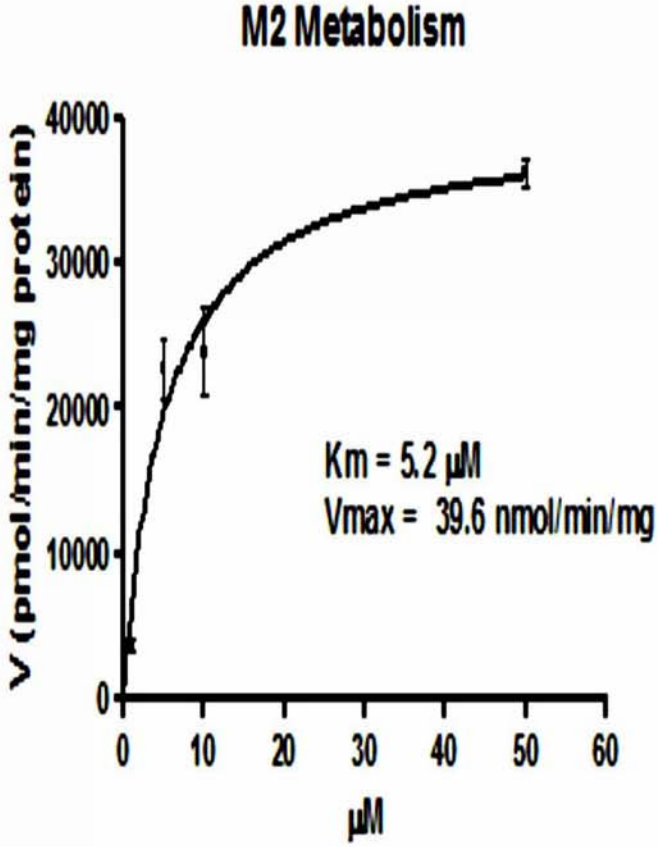

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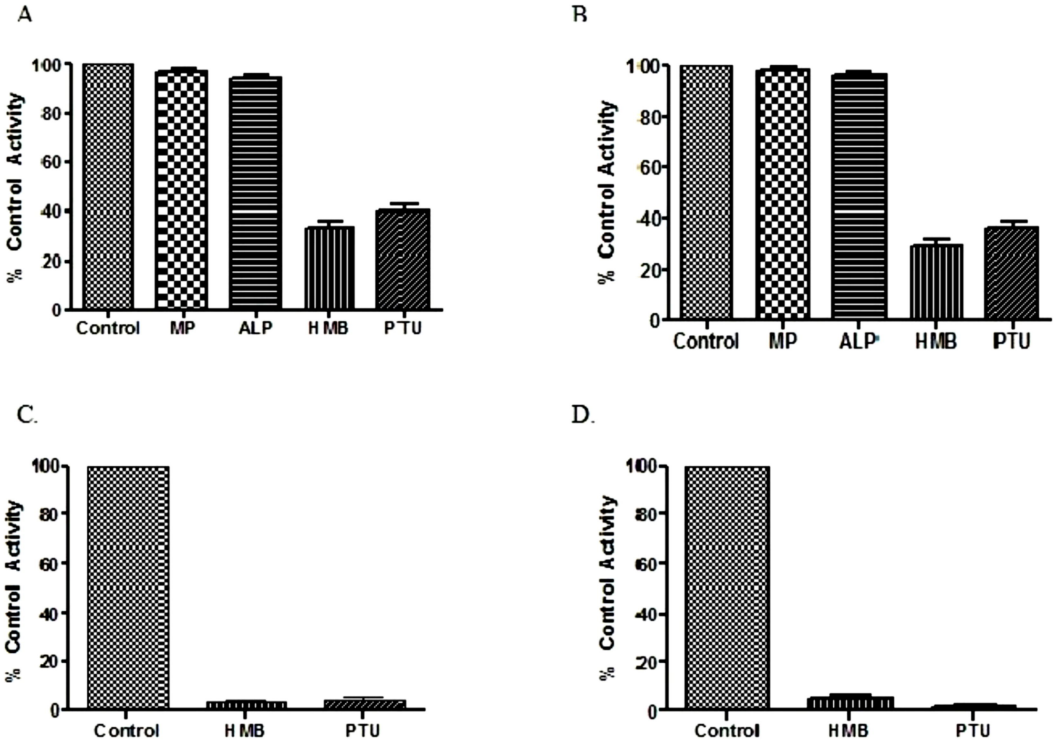
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80
60
40
20
0
Control
MP
ALP
HMB
PTU
B
% Control Activity
100
80
60
40
20
0
Control
MP
ALP
HMB
PTU
C.
% Control Activity
100
80
60
40
20
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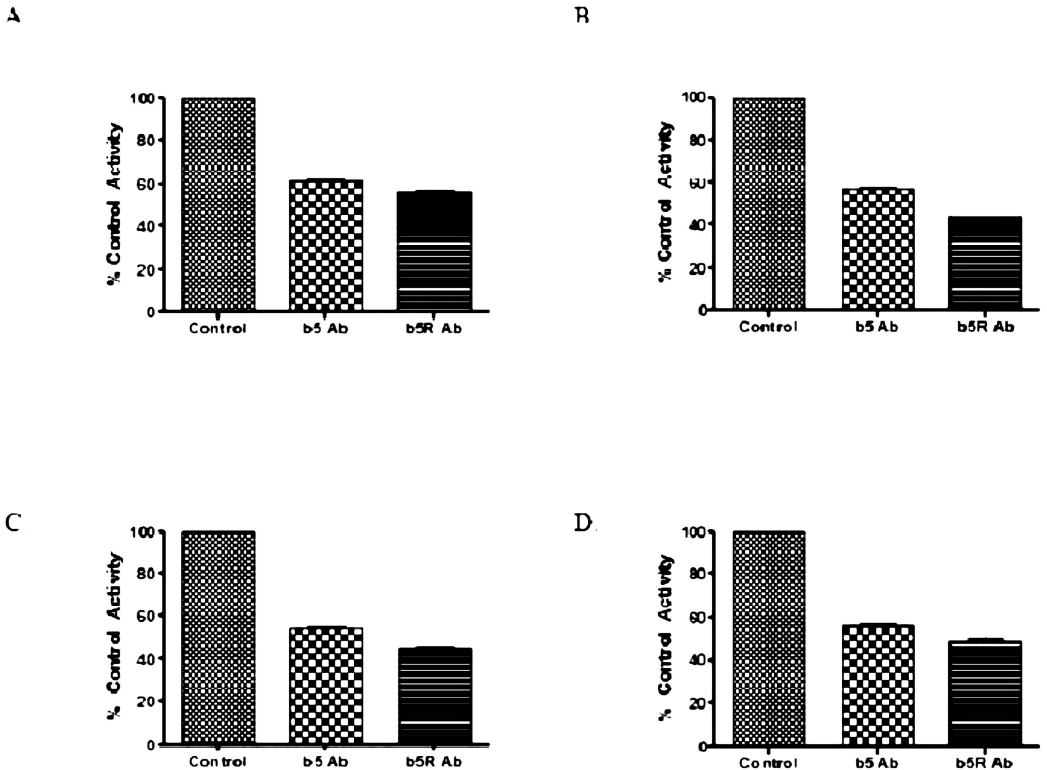
Control
HMB
PTU
D.
% Control Activity
100
80
60
40
20
0
Control
HMB
PTU

Figure 5.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0
Control
b5 Ab
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100
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20
0
Control
b5 Ab
b5R Ab
C
% Control Activity
100
80
60
40
20
0
Control
b5 Ab
b5R Ab
D
% Control Activity
100
80
60
40
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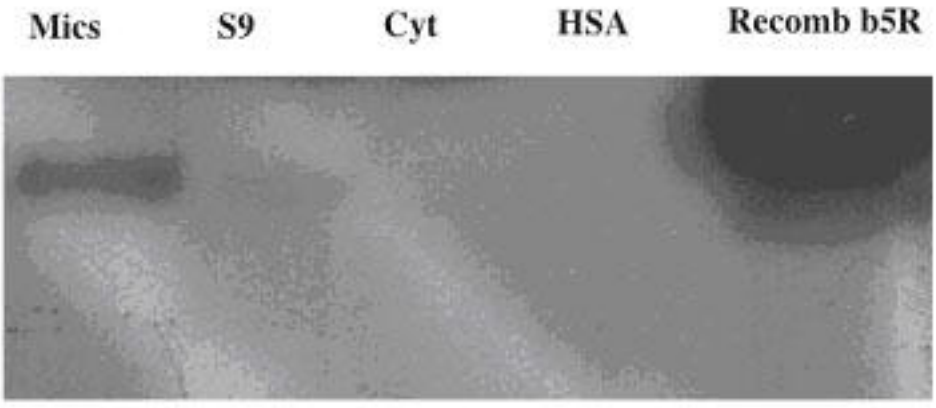
0
Control
b5 Ab
b5R Ab

Figure 6.

A.



B.

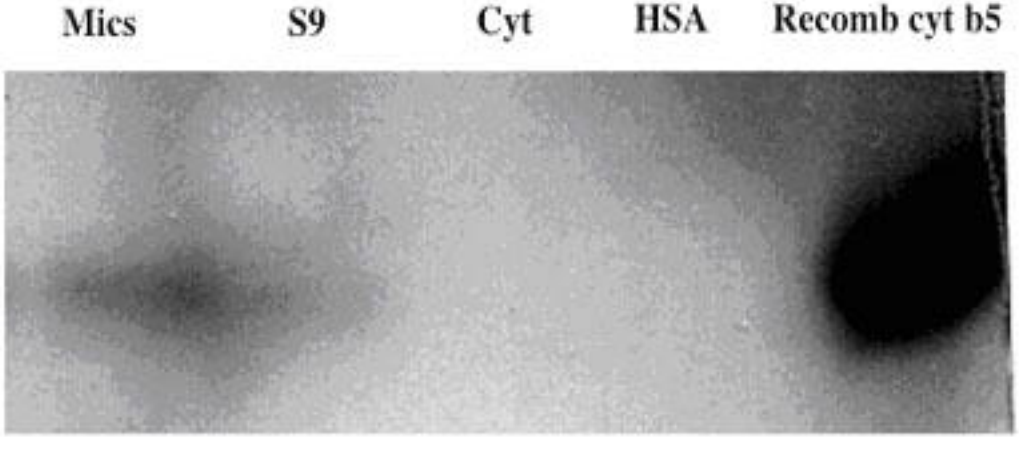
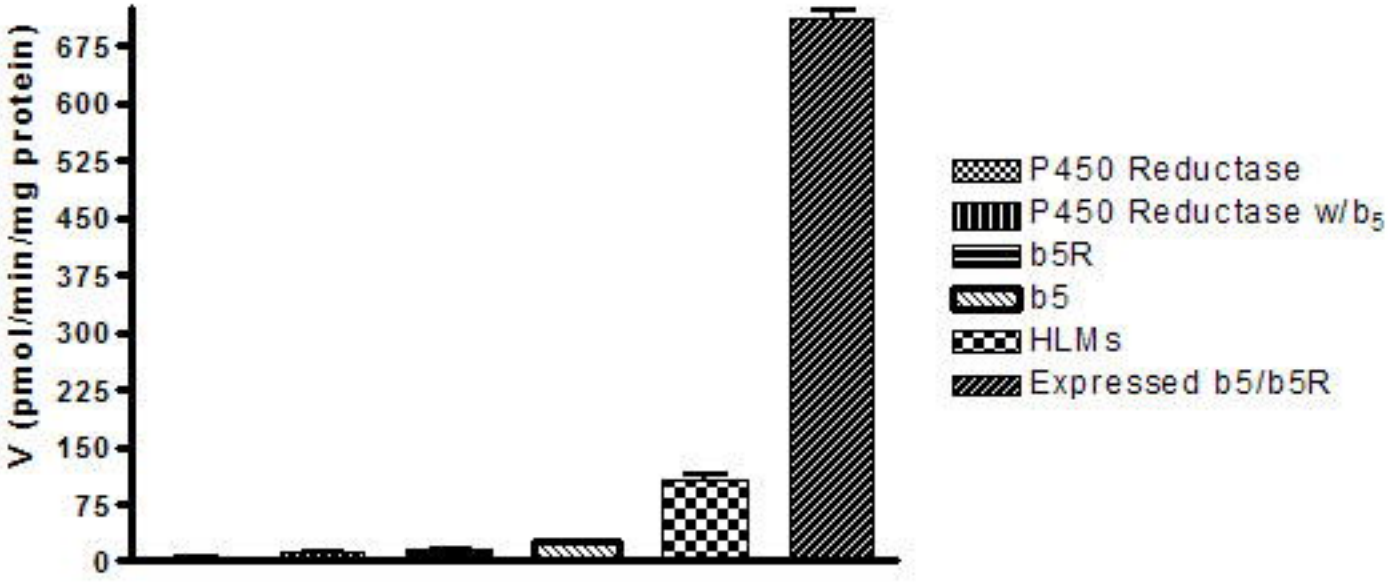


Figure 7.

A.


V (pmol/min/mg protein)
675
600
525
450
375
300
225
150
75
0
P450 Reductase
P450 Reductase w/b5
b5R
b5
HLMs
Expressed b5/b5R

B.

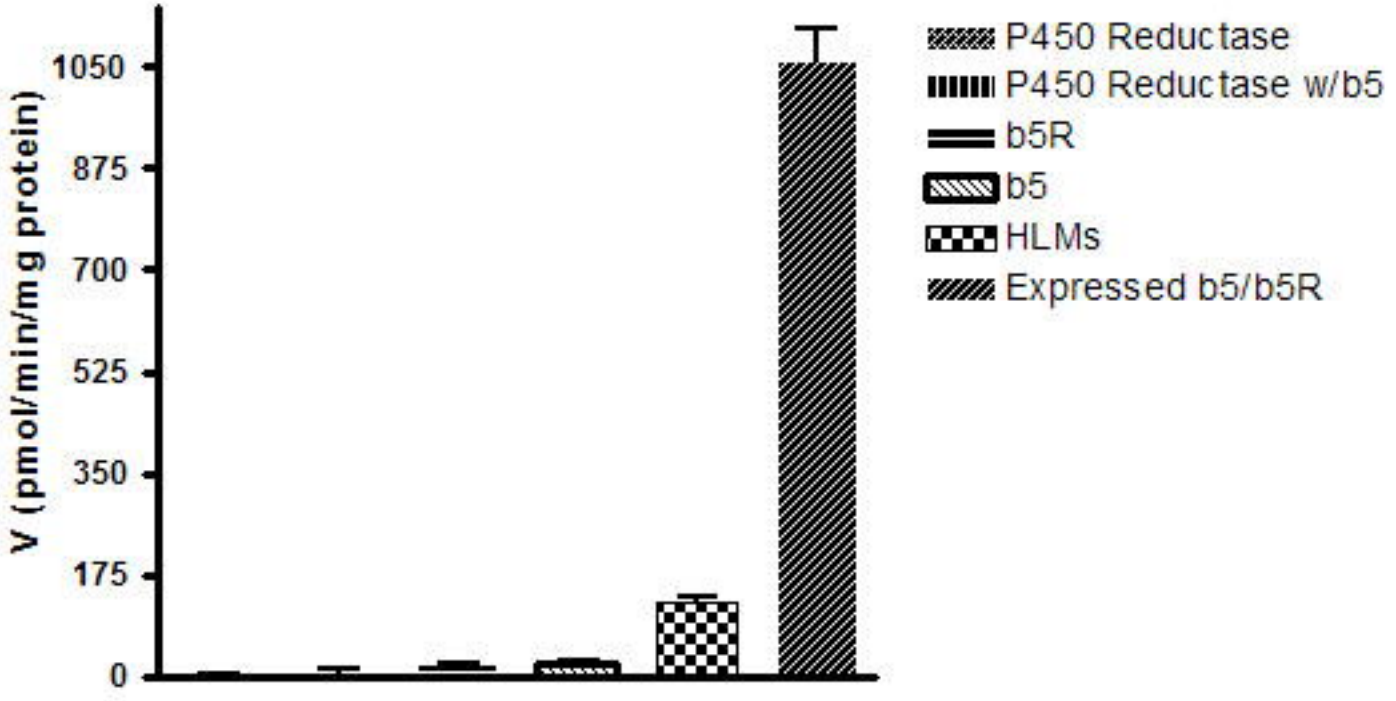

V (pmol/min/mg protein)
1050
875
700
525
350
175
0
P450 Reductase
P450 Reductase w/b5
b5R
b5
HLMs
Expressed b5/b5R

Figure 8.