# CYP4F ENZYMES ARE THE MAJOR ENZYMES IN HUMAN LIVER MICROSOMES THAT CATALYZE THE *O*-DEMETHYLATION OF THE ANTIPARASITIC PRODRUG DB289

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DB289 is a prodrug that is converted by several steps to the active metabolite DB75, as shown in Figure 1. DB289 exhibits enhanced oral efficacy and reduced acute toxicity over DB75, an aromatic dicationic compound that is effective against a broad range of pathogens in vitro including African trypanosomiasis (African sleeping sickness). The conversion of DB289 to DB75 involves several *O*-demethylation and reductive *N*-dehydroxylation reactions (**Figure 1**). The latter reactions were recently shown to be catalyzed by cytochrome b<sub>5</sub>/NADH b<sub>5</sub> reductase, (Saulter, et al., 2005). However, little is known about the oxidative O-demethylation pathways of DB289. Accordingly, we have performed a reaction phenotyping study to identify the enzymes responsible for the first step in the conversion of DB289 to DB75, i.e., oxidative O-demethylation (M1 formation). These data have been accepted for publication (Wang, et al., 2006).

## MATERIALS & METHODS \_

DB289 was synthesized by Medichem (Woodlake, IL, USA) using previously described methods (Das and Boykin, 1977; Boykin, et al., 1996). The intermediate Phase I metabolites (M1, M2, and M3), the active diamidine DB75, and deuterium-labeled DB289 (DB289-d<sub>8</sub>) (internal standard, IS) were synthesized as described previously (Stephens, et al., 2001; Anbazhagan, et al., 2003). 1-aminobenzotriazole (ABT), coumarin, diethyldithiocarbamate (DDC), fluvoxamine, furafylline, ketoconazole, lauric acid,  $\alpha$ -naphthoflavone, omeprazole, quinidine, sulfaphenazole, thio-TEPA, trimethoprim, troleandomycin (TAO) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 17-Octadecynoic acid (17-ODYA), HET0016 (*N*-hydroxy-*N*'-(4-*n*-butyl-2-methylphenyl)formamidine), and arachidonic acid (AA) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Ebastine was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). All other reagents and solvents were of analytical grade.

### Human Liver Microsomes, Antibodies and Recombinant Human P450s

Pooled human liver microsomes (n = 50; mixed gender) and pre-immune immuno-globulin (IgG) from rabbit were prepared by XenoTech, LLC (Lenexa, KS). Polyclonal antibody against CYP4F2, raised in rabbits, was purchased from Research Diagnostics, Inc. (Concord, MA) (Source A; 1 mg IgG/mL) or was kindly provided by Dr. Yoshihiko Funae (Osaka City University Medical School, Osaka, Japan) (Source B; 40 mg lgG/mL). Polyclonal antibody against CYP2J2 (40 mg lgG/mL) was also a gift from Dr. Funae (Hashizume et al., 2001; Hashizume, et al., 2002). Supersomes™ prepared from baculovirus-infected insect cells expressing human CYP enzymes and NADPH-cytochrome P450 reductase were purchased from BD Biosciences (Woburn, MA).

### **Standard Incubation Conditions**

Typically, incubation mixtures contained 50 or 100 mM phosphate buffer (pH 7.4), 3.0 or 3.3 mM MgCl<sub>2</sub>, 1 mM EDTA and 1 mM NADPH, unless indicated otherwise. Reactions were initiated with the addition of NADPH (or substrate for incubations with mechanism-based inhibitors) and were carried out at 37 °C. With the exception of ebastine (initially dissolved in chloroform and serially diluted with methanol) all chemical inhibitors were dissolved in methanol. Reactions were stopped with ice-cold acetonitrile containing 0.1% formic acid (v/v) and DB289-d<sub>8</sub> as internal standard. Precipitated protein was removed by centrifugation and the supernatant fractions were analyzed by LC/MS/MS.

### LC/MS/MS Assays

The quantification of DB289 and M1 by LC/MS/MS was performed on an Applied Biosystems API 4000 triple quadrupole mass spectrometer equipped with a Turbo IonSpray® interface (MDS Sciex, San Francisco, CA). Samples (4 µL) were introduced to the mass spectrometer with a thermostatted (6 °C) CTC PAL LEAP autosampler (Carrboro, NC), a Shimadzu pumping system (Kyoto, Japan) and a Valco solvent divert valve (Houston, TX). All equipment was controlled with Analyst software (Version 1.3, Applied Biosystems, Foster City, CA). DB289 and M1 were separated on an Aquasil C18 HPLC column (2.1 mm  $\times$  50 mm, 5  $\mu$ M) (Thermo Electron, Waltham, MA). HPLC mobile phases were HPLC-grade water containing 0.1% formic acid and methanol containing 0.1% formic acid. The characteristic MRM (multiple reaction monitoring) transitions for DB289, M1 and DB289-d<sub>8</sub> were m/z 365.10 > 334.10, m/z 351.10 >320.10 and m/z 373.00 > 342.00, respectively. The range of the calibration curve was 25 nM – 5000 nM for DB289 and 1 nM – 2500 nM for M1.

Figure 1 shows the oxidative O-demethylation of DB289 to M1, the first step in the biotransformation of DB289 to its active diamidine DB75.

Human liver microsomes convert DB289 to M1 with high affinity and high capacity (which is desirable for a prodrug). In incubations of DB289 ranging from 0.05 µM to 15 µM with pooled human liver microsomes (0.02 mg/mL) at 37 °C for 3 min, the kinetic constants ( $K_m$  and  $V_{max}$ ) for the formation of M1 were 0.5  $\mu$ M and 3770 pmol/min/mg protein, respectively.

Figure 2 shows that DB289 was rapidly metabolized by three of the recombinant human CYP enzymes typically screened in reaction phenotyping studies: CYP1A1 1A2 and 1B1. CYP1A1 and 1B1 are not expressed in human liver microsomes (Wrighton and Stevens, 1992), so it was hypothesized that CYP1A2 was responsible for the high rate of conversion of DB289 to M1 by human liver microsomes. Chemical inhibition, antibody inhibition and correlation analysis experiments were performed to test this hypothesis. Apparent  $K_m$  ( $\mu M$ ) and  $V_{max}$  (nmol/min/nmol CYP) values for rCYP1A1 and 1A2 were 0.5 and 26 and 0.4 and 13, respectively (data not shown).

Figure 3 shows that, contrary to expectation, the CYP1A2 inhibitors  $\alpha$ -naphthoflavone, fluvoxamine and furafylline (a mechanism-based inhibitor of CYP1A2) did not inhibit the conversion of DB289 (3 µM) to M1 by human liver microsomes. Likewise, antibody against CYP1A2 did not inhibit M1 formation (results not shown).

The non-selective cytochrome P450 inhibitor, ABT, significantly inhibited M1 formation (86%), indicating that this O-demethylation reaction is indeed mediated by

cytochrome P450. Ketoconazole partially inhibited M1 formation, even though recombinant CYP3A4 had little capacity to *O*-demethylate DB289.

Contrary to expectation, the sample-to-sample variation in the rate of conversion of DB289 (3 µM) to M1 by a panel of individual samples of human liver microsomes (n = 16) did not correlate with CYP1A2 [7-ethoxyresorufin O-dealkylation (EROD)] activity (as shown in Figure 4), nor did it correlate with CYP2A6, 2B6, 2C8 2C9, 2C19, 2D6, 2E1, 3A4, 4A11 or FMO activity (results not shown).

Although the results of the experiment with recombinant enzymes implicated CYP1A2 in the conversion of DB289 to M1, this interpretation was contradicted by the results of chemical inhibition, antibody inhibition and correlation analysis experiments. Therefore, additional recombinant human enzymes were examined for their ability to convert DB289 to M1, and the results are shown in Figure 5. Recombinant CYP2J2, 4F2 and 4F3B all metabolized DB289, hence, these enzyments were investigated for their contribution to M1 formation in human liver microsomes. Apparent  $K_m$  ( $\mu$ M) and  $V_{max}$  (nmol/min/nmol CYP) values for rCYP2J2, 4F2 and 4F3B were 0.9 and 7.7, 0.7 and 7.9 and 3.2 and 10, respectively (data

Figure 6A shows that antibodies against CYP4F2 from two independent sources (Source A and Source B as described under *Materials*) inhibited the conversion o DB289 (3 µM) to M1 by human liver microsomes by as much as 91%. No inhibiti was observed with antibody against CYP2J2.

Figure 6B shows that the antibody against CYP4F2 from source A inhibited M1 formation by both recombinant CYP4F2 and CYP4F3B, but it did not inhibit M1 formation by recombinant CYP2J2. Given the extensive amino acid sequence homology (~ 93%) between human CYP4F2 and CYP4F3B (Christmas, et al., 2001), it is reasonable to expect that the polyclonal antibody raised against CYP4F2 cross-reacts

In order to further investigate the potential involvement of CYP2J2 and 4F2/3B, ebastine (a CYP2J2 and CYP3A4 substrate), arachidonic acid (a CYP4F2, CYP4F3B and CYP2J2 substrate), HET0016 (an arachidonic acid  $\alpha$ -hydroxylase inhibitor), and 17-ODYA (a nonselective mechanism-based arachidonic acid  $\alpha$ -hydroxylase and epoxygenase inhibitor) were evaluated for their ability to inhibit the conversion of DB289 (3 µM) to M1. These inhibitors were evaluated at concentrations ranging from 0.1 to 100 times their reported  $K_m$  or  $IC_{50}$  values.

Figure 7 shows that HET0016 inhibited M1 formation in human liver microsomes by 78% at 0.1 μM and 95% at 0.5 μM whereas 17-ODYA inhibited M1 formation by 39% at 1 μM and 84% at 10 μM. Ebastine and arachidonic did not notably inhibit M1 formation except at the highest concentrations tested. Figures 7B and 7C show that rCYP4F2 and rCYP4F3B exhibited similar inhibition profiles by all inhibitors except 17-ODYA. Figure 7D shows, somewhat unexpectedly, that rCYP2J2 was inhibited by HET0016 (53% inhibition at 0.1  $\mu$ M).

Ebastine at 1 µM appeared to be a selective inhibitor of rCYP2J2 (Figures 7B-D), but did not inhibit M1 formation by human liver microsomes (Figure 7A), indicating that CYP2J2 was not a major contributor to M1 formation by HLM. These results, though not conclusive, strongly suggested that CYP4F2 and CYP4F3B, but not CYP2J2, contribute significantly to M1 formation by human liver microsomes.

The initial reaction phenotyping experiments gave contradictory results: Based on experiments with the usual panel of recombinant CYP enzymes, it appeared that CYP1A2 would be a major contributor to the conversion of DB289 to M1 by human liver microsomes. However, experiments with chemical and antibody inhibitors and correlation analysis did not support this interpretation. The results of experiments with additional recombinant CYP enzymes and inhibitory antibodies suggest that CYP4F2 and CYP4F3B are the major enzymes in human liver microsomes responsible for converting DB289 to M1. These enzymes have previously been implicated in the metabolism of endogenous compounds, e.g. arachidonic acid and leukotriene B<sub>4</sub> (LTB<sub>4</sub>). Zimmerlin and Patten (2000) reported that the promising immunomodulatory drug FTY720 is eliminated predominantly by CYP4F-mediated  $\alpha$ -hydroxylation of the aliphatic chain. These findings not only advance our understanding of the metabolism of this new drug candidate, but, by implicating CYP4F enzymes in the metabolism of a xenobiotic, they have important implications for the conduct of reaction phenotyping experiments. Our results suggest that CYP4F2, CYP4F3B and even CYP2J2 should be included in the panel of recombinant CYP enzymes that is normally examined as part of reaction phenotyping experiments.

- 1. Anbazhagan M, et al. (2003) Synthesis of metabolites of the prodrug 2,5-bis(4-O-methoxyamidinophenyl)furan. Heterocycles 60:1133 – 1145.
- 2. Boykin DW, et al. (1996) Anti-pneumocystis activity of bis-amidoximes and bis-O-alkylamidoximes prodrugs. Bioorg Med Chem Lett 6:3017 – 3020.
- 3. Christmas P, et al. (2001) Alternative splicing determines the function of CYP4F3 by switching substrate specificity. *J Biol Chem* **276**:38166 – 38172.
- 4. Das BP and Boykin DW. (1977) Synthesis and antiprotozoal activity of 2,5-bis(4guanylphenyl)furans. *J Med Chem* **20**:531 – 536.
- 5. Hashizume T, et al. (2002) Involvement of CYP2J2 and CYP4F12 in the metabolism of ebastine in human intestinal microsomes. J Pharmacol Exp Ther 300:298 – 304.
- 6. Hashizume T, et al. (2001) A novel cytochrome P450 enzyme responsible for the metabolism of ebastine in monkey small intestine. Drug Metab Dispos 29: 798 – 805.
- 7. Saulter JY, et al. (2005) Unusual dehydroxylation of antimicrobial amidoxime pro drugs by cytochrome b5 and NADH cytochrome b5 reductase. *Drug Metab Dispos* **33**:1886 – 1893.
- 8. Stephens CE, et al. (2001) Synthesis of deuterium-labelled 2,5-bis(4-amidino phenyl)furan, 2,5-bis[4-(methoxyamidino) phenyl]furan, and 2,7-diamidinocarbazole. *J Labelled Comp Radiopharm* **44**:197 – 208.
- 9. Wang MZ, et al. CYP4F enzymes are the major enzymes in human liver microsomes that catalyze the *O*-demethylation of the antiparasitic prodrug DB289. *Drug* Metab Dispos. Epub 2006 Sept 22; doi: 10.1124/dmd.106.010587.
- 10. Wrighton SA and Stevens JC. (1992) The human hepatic cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol* **22**:1 – 21.
- 11. Zimmerlin AG and Patten CJ. (2000) Role of CYP4F in the metabolic clearance of FTY720 – Prediction of low drug to drug interaction potential. *Transplantation* **69**: S191 – S191.



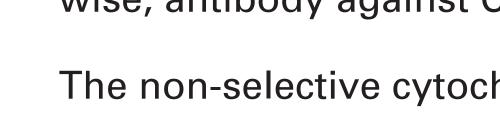
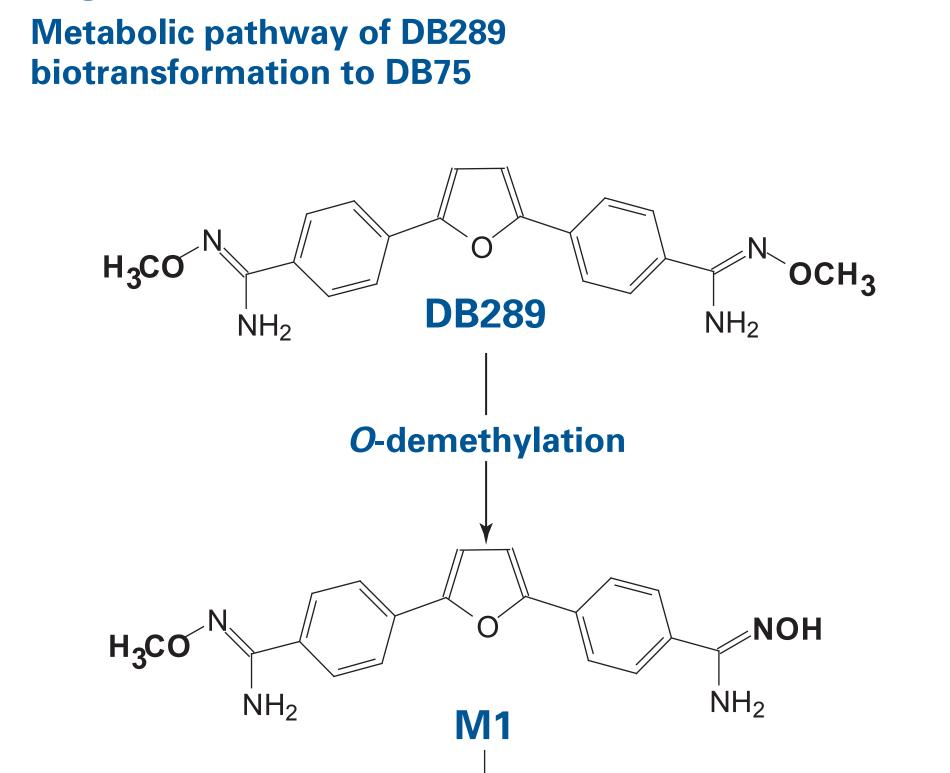
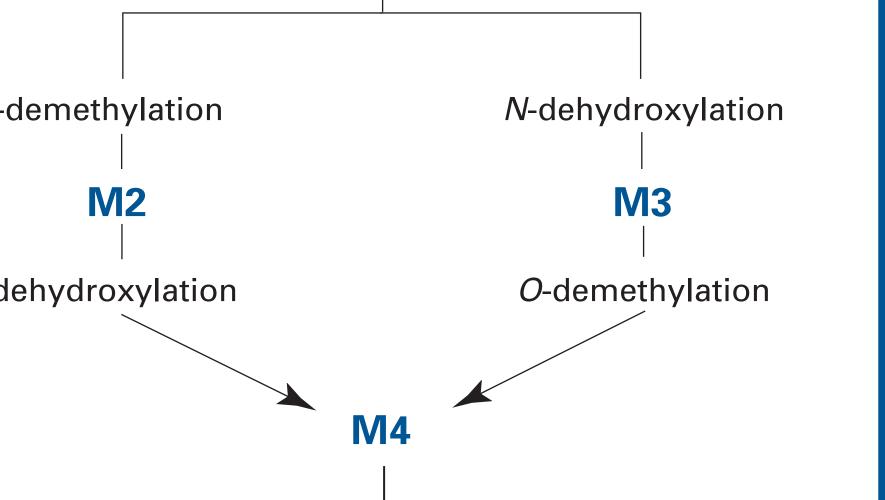
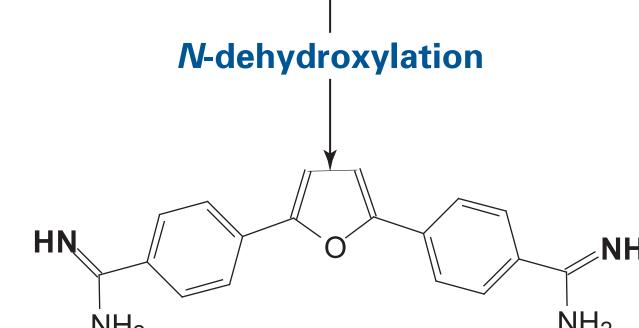
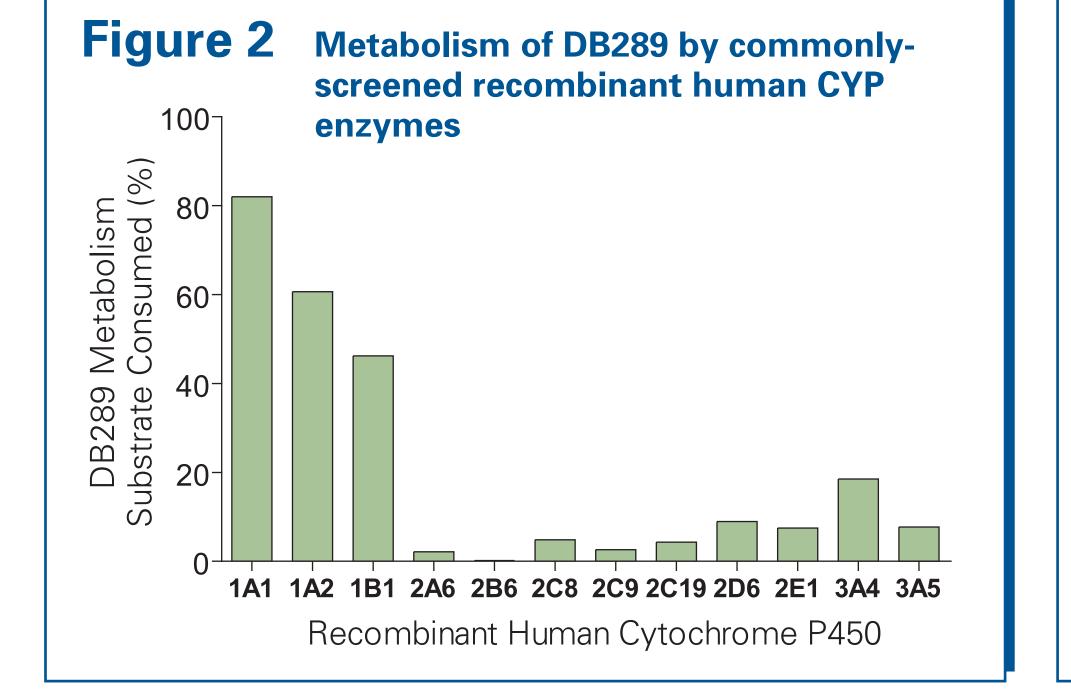


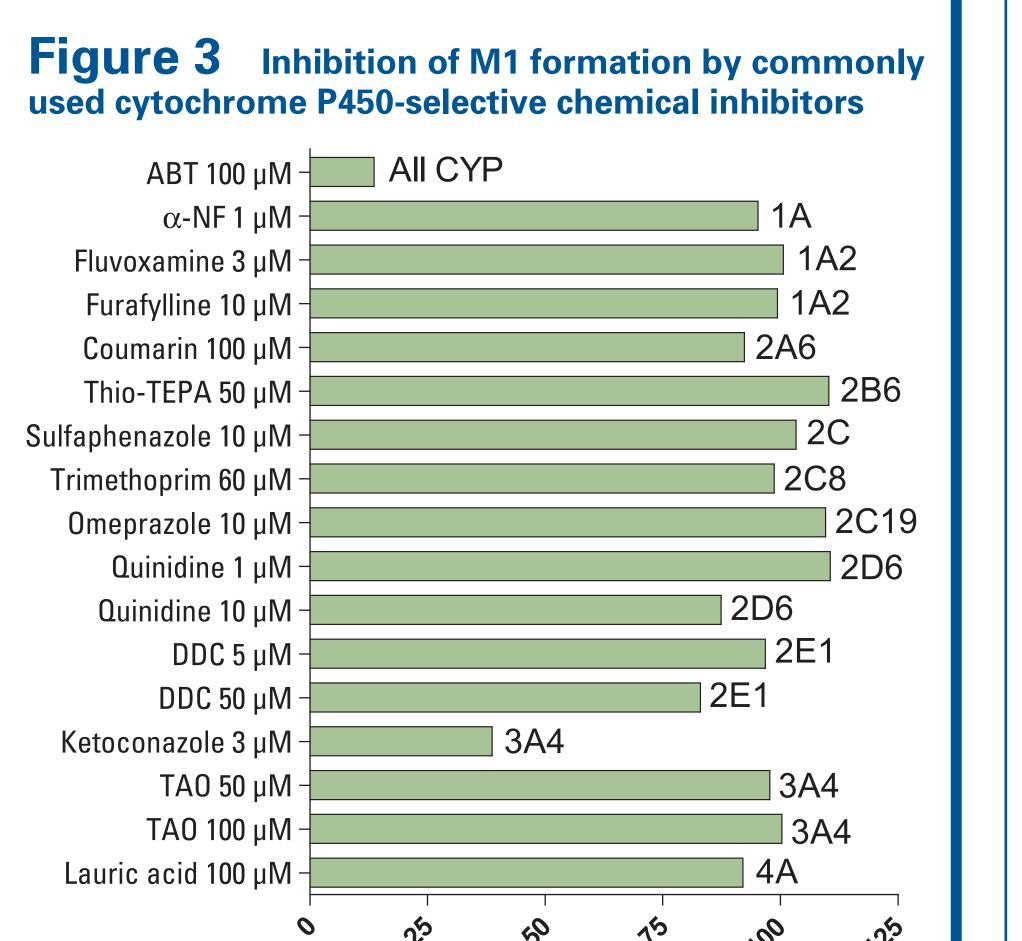
Figure 1



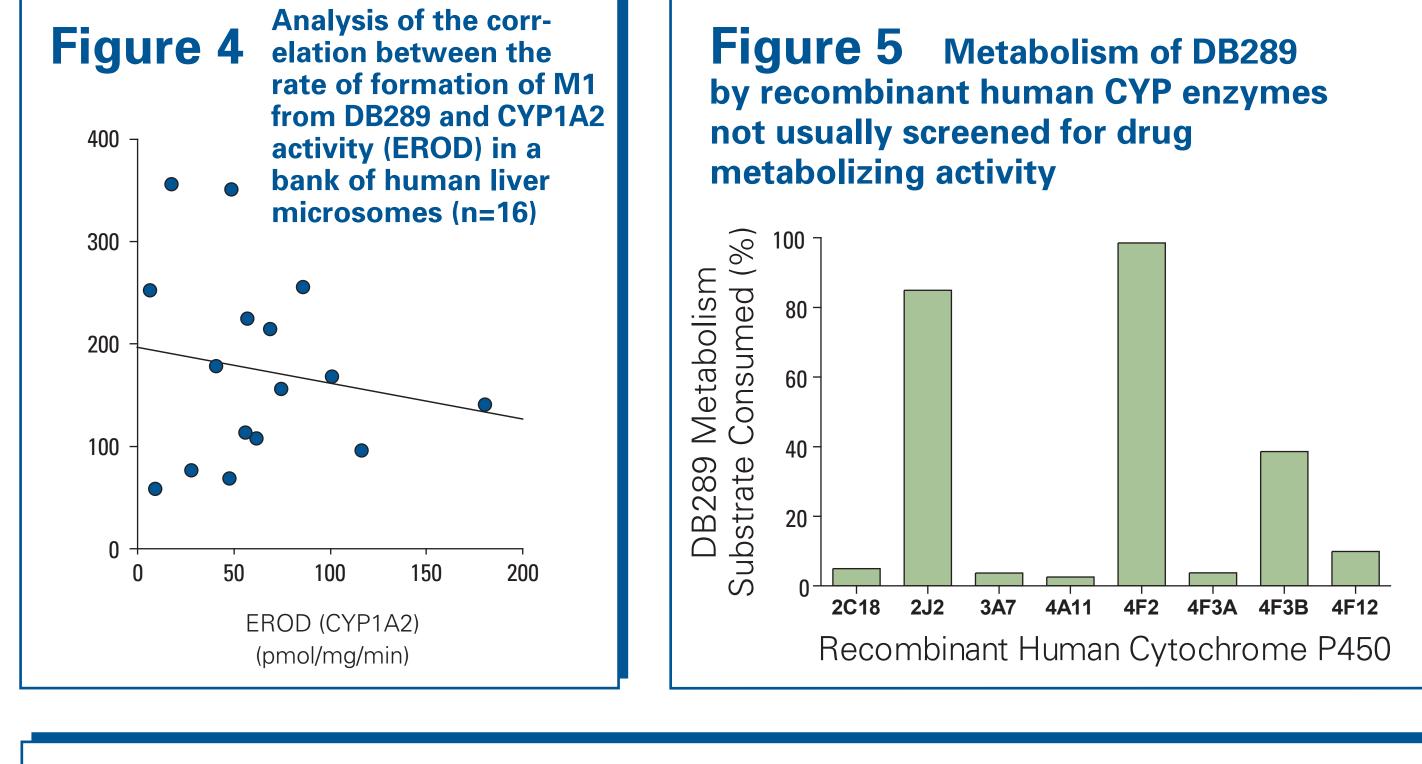


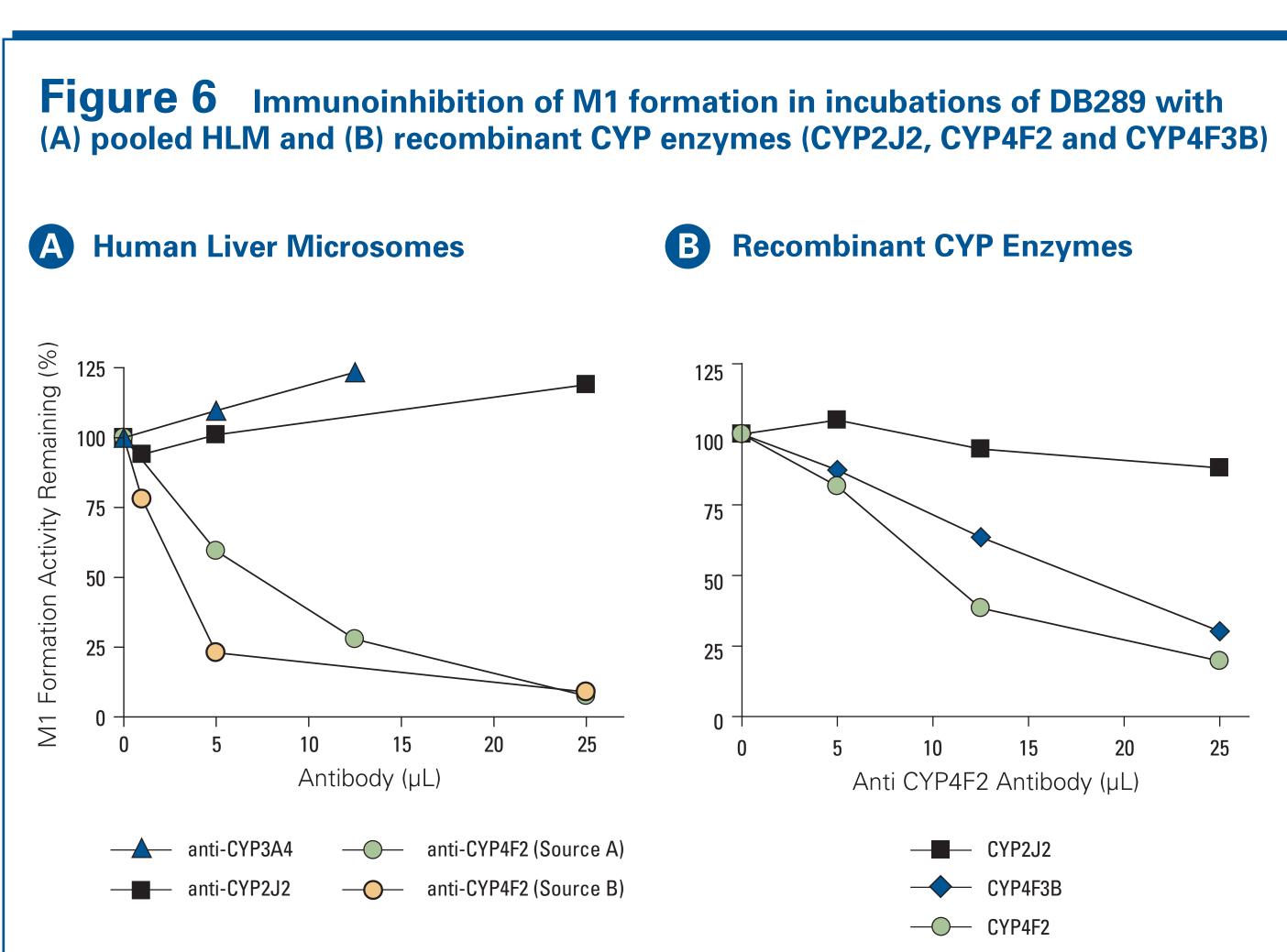


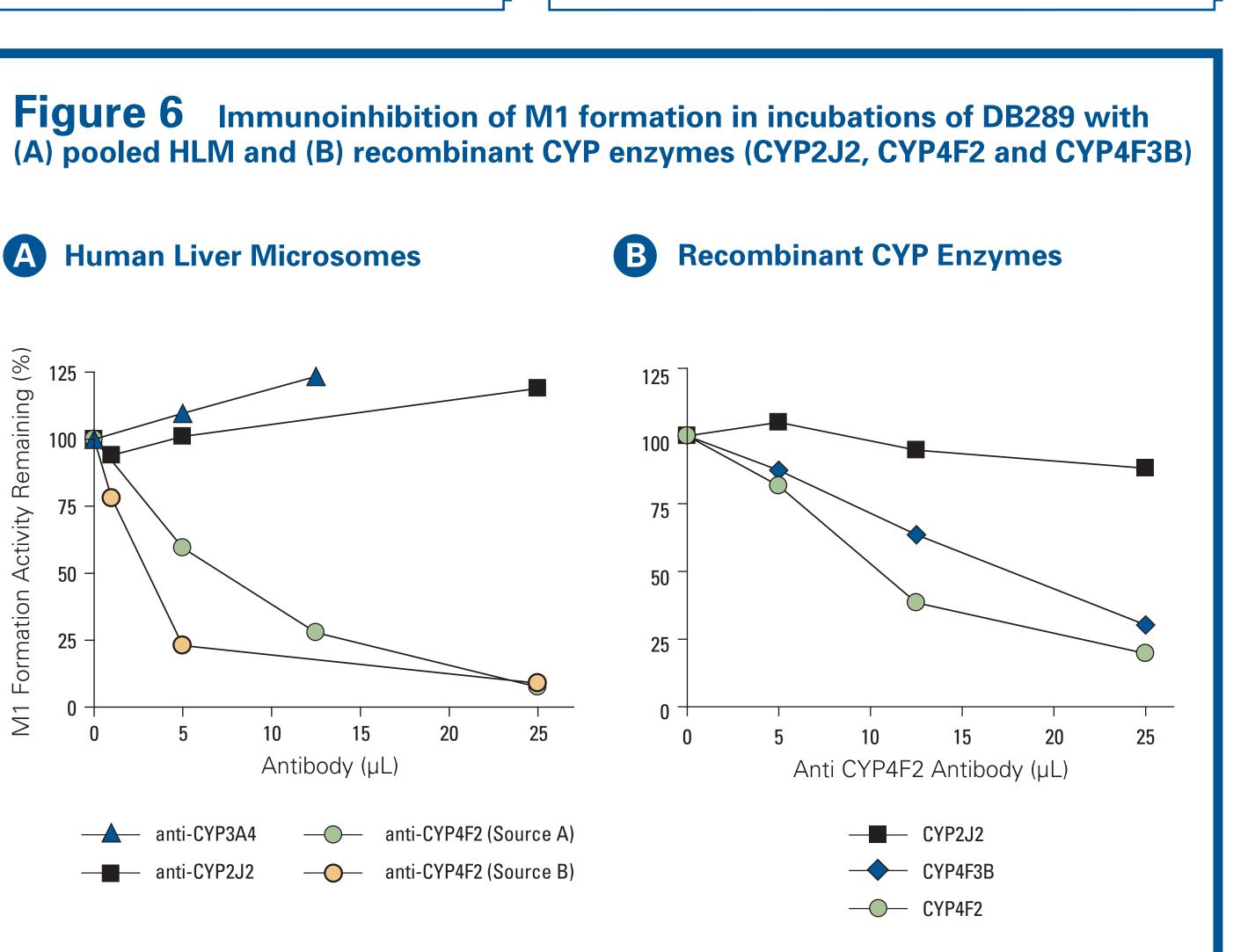




DB289 Metabolism Activity Remaining (%)







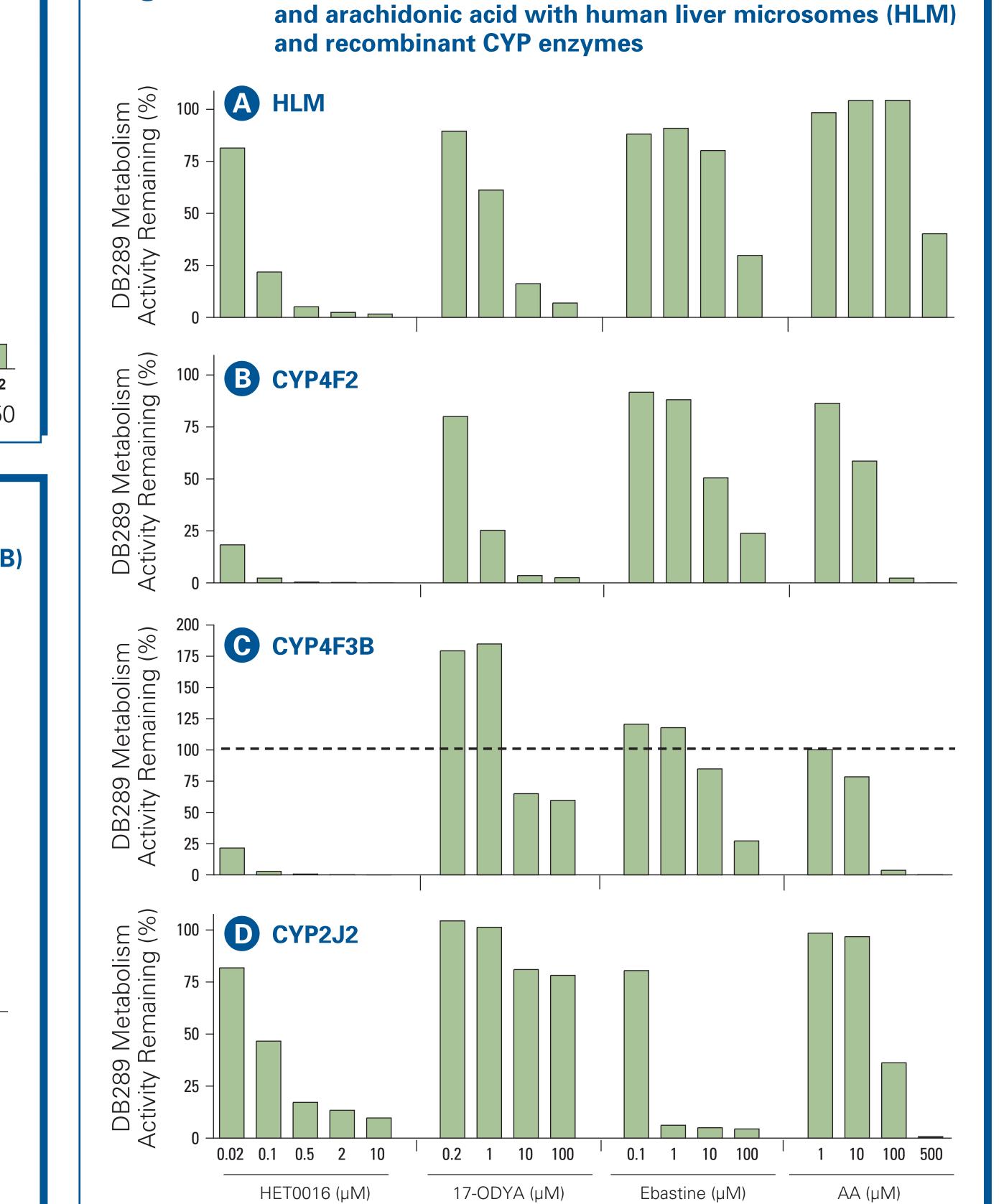


Figure 7 Inhibition of M1 formation by HET006, 17-ODYA, ebastine

