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Contribution of individual P450 isozymes to the *O*-demethylation of the psychotropic β-carboline alkaloids harmaline and harmine

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Abbreviations used:

CNS, central nervous system; CYP, cytochrome P450; HLM, human liver microsomes; MLM, mouse liver microsomes; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; 5-HT, 5-hydroxytryptamine; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

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Abstract

The psychotropic β-carboline alkaloids, showing high affinity for 5-hydroxytryptamine (5-HT), dopamine, benzodiazepine and imidazoline receptors and the stimulation of locus coeruleus neurons, are formed endogenously from tryptophan-derived indolealkylamines through the Pictet-Spengler condensation with aldehydes in both plants and mammals. Cytochromes P450 (CYP) 1A1 (18.5), 1A2 (20) and 2D6 (100) catalyzed the *O*-demethylation of harmaline, and CYP1A1 (98.5), CYP1A2 (35), CYP2C9 (16), CYP2C19 (30) and CYP2D6 (115) that of harmine (relative activities). The dehydrogenation/aromatization of harmaline to harmine was not carried out by aromatase (CYP19), CYP1A2, CYP2C9, CYP2D6, CYP3A4, pooled recombinant P450s, or by human liver microsomes (HLM). Kinetic parameters were calculated for the *O*-demethylations mediated by each isozyme and by pooled HLM. K_{cat} (min^{-1}) and K_m (μM) values for harmaline were CYP1A1 (10.8, 11.8), CYP1A2 (12.3, 13.3), CYP2C9 (5.3, 175), CYP2C19 (10.3, 160), and CYP2D6 (39.9, 1.4), and for harmine were CYP1A1 (45.2, 52.2), CYP1A2 (9.2, 14.7), CYP2C9 (11.9, 117), CYP2C19 (21.4, 121), and CYP2D6 (29.7, 7.4). Inhibition studies using monoclonal antibodies confirmed that CYP1A2 and CYP2D6 were the major isozymes contributing to both harmaline (20% and 50% respectively) and harmine (20% and 30%) *O*-demethylations in pooled HLM. The turnover numbers for CYP2D6 are amongst the highest ever reported for a CYP2D6 substrate. Finally, *CYP2D6*-transgenic mice were found to have increased harmaline and harmine *O*-demethylase activities as compared to wild-type mice. These findings suggest a role for polymorphic CYP2D6 in the pharmacology and toxicology of harmine and harmaline.

The β-carboline alkaloids are present in plants and have been of interest due to their psychotropic properties (Picada et al., 1997). They may be formed endogenously from tryptophan-derived indolealkylamines through the Pictet-Spengler condensation with simple aldehydes or with pyruvic acid in mammals, including humans (Airaksinen and Kari, 1981; Melchior and Collins, 1982). Moreover, certain β-carbolines, such as pinoline, tryptoline, 6-hydroxy-tetrahydro-β-carboline, harman and norharman have been reported as normal constituents of human tissues and body fluids. Their levels in humans are usually elevated after drinking alcohol. The association of β-carbolines with alcohol dependence and brain damage has been suggested (Melchior and Collins, 1982; Collins, 2002).

Endogenous and exogenous β-carboline alkaloids were reported to exert a wide spectrum of psychopharmacological and behavioral effects in the brain (Airaksinen and Kari, 1981). Most β-carbolines are strong reversible inhibitors of monoamine oxidase (MAO). Among them, harmaline and harmine exhibit the most potent inhibition towards purified MAO-A activity (Kim et al., 1997), and these are the principal active agents in *Peganum harmala*, a plant that has been used in traditional medicine for two millennia (Lamchouri et al., 2002). In addition, the psychotropic Amazonian plant mixture *ayahuasca* comprises the β-carboline-rich vine *Banisteriopsis caapi* mixed with the *N*,*N*-dimethyltryptamine (DMT)-containing hallucinogenic plant *Psychotria viridis*. Originally, it was proposed that the psychotropic properties of *ayahuasca* resided in the DMT, but it is now becoming clear that the β-carbolines themselves may contribute to

the hallucinogenic properties of the "tea", rather than simply acting as an inhibitor of MAO to elevate the plasma levels of DMT (Freedland and Mansbach, 1999).

β-Carbolines modulate the levels of amine neurotransmitters and their metabolites in the central nervous system (Iurlo et al., 2001), inducing behavioral changes. MAO inhibition also leads these β-carboline alkaloids to induce hypothermic effects probably through a serotonergic mechanism (Abdel-Fattah et al., 1995). Hallucinogenic effects of harmaline and harmine are suggested as a result of their binding to 5-HT_{2A} and 5-HT_{2C} receptors, and tremorgenic properties are due to their interactions with benzodiazepine receptors (Lutes et al., 1988; Glennon et al., 2000; Husbands et al., 2001). Harmaline has also been proposed as an endogenous ligand for imidazoline receptors (Husbands et al., 2001), and shown to stimulate locus coeruleus neuronal activity, which may underlie some of the behavioral effects of these β-carbolines (Ruiz-Durantez et al., 2001).

The neurotoxic properties of β-carboline alkaloids may account for their associations with Parkinson's disease (PD). Tryptophan-derived β-carbolines are similar as *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in structure, which is known to induce immediate and irreversible Parkinsonism through its neurotoxic metabolite, a quaternary ion (MPP^+). Various studies indicate that the 2,9-di-*N*-methylated β-carboline cations, that are neurotoxic comparable to MPP^+, also induce mitochondrial energy depletion and oxidative stress in nigrostriatum (Collins and Neafsey, 1985; Collins et al., 1987; Collins et al., 1992; Collins, 2002). Moreover, the bioactivated potentially neurotoxic *N*-

methylated β-carbolinium ions are reported to be present in human brain (Matsubara et al., 1993).

The metabolic bioactivation of carcinogenic α-carbolines and γ–carbolines, such as 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), has been extensively studied, because these compounds are present in cooked food (Matsubara et al., 1993; Raza et al., 1996; Matsubara, 1998; Pfau and Marquardt, 2001). In contradistinction to the so-called heterocyclic amine food mutagens, the metabolism of these potential neurotoxic β-carboline alkaloids is expected to be a detoxication process, and may play an important role in protection against brain damage (Slotkin and DiStefano, 1970a; Slotkin and DiStefano, 1970b; Slotkin et al., 1970; Zetler et al., 1974; Burke and Upshall, 1976; Tweedie and Burke, 1987).

We have therefore examined the metabolism of harmaline and harmine, and estimated the relative contribution by individual P450 isozymes to their *O*-demethylation. The results might not only provide insights into individual diathesis to chemical neurotoxins, but also the physiological potential of P450 isozymes that are expressed in human brain, beyond the detoxication of drugs and other xenobiotic chemicals.

Materials and methods

Chemicals and Enzymes. Harmaline, harmine, harmalol, harmol, pinoline, phenacetin, reduced nicotinamide adenine dinucleotide phosphate (NADPH), 4-amidinophenylmethanesulfonyl fluoride (APMSF), disodium ethylenediaminetetraacetate (EDTA), and 60% perchloric acid were purchased from Sigma (St. Louis, MO). HPLC solvents and other chemicals were of the highest grade commercially available and were used as received. Recombinant human P450 Supersomes™ and P450 insect control microsomes, pooled human liver microsomes (coded H161) were bought from BD Discovery Labware, Inc. (Woburn, MA). The monoclonal antibodies raised against human CYP2D6 (MAb 50-1-3), CYP1A1 (MAb 1-7-1), CYP1A2 (MAb 26-7-5), CYP2C9 (MAb 763-15b) and CYP2C19 (MAb 1-7-4-8) were characterized previously (Gelboin et al., 1999; Krausz et al., 2001).

Preparation of Mouse and Human Liver Microsomes. All mice used for liver microsomes preparation were maintained under controlled temperature (23±1°C) and lighting (lights on 6:00 A.M. - 6:00 P.M.) with food and water provided *ad libitum*. Adult males at 2-3 months old were used in the experiments, which were conducted under the National Institute of Health guidelines for the use and care of laboratory animals. *CYP2D6*-transgenic mice were characterized previously (Corchero et al., 2001). For preparation of mouse liver microsomes (MLM), mice were killed by CO_2 asphyxiation, and livers were excised and washed with ice-cold washing solution (250 mM sucrose, 10 mM potassium phosphate, 1 mM EDTA and 1 mM APMSF, pH 7.4). Livers from 3 male mice were pooled, minced with scissors, and homogenized using a motor-driven Teflon-

tipped pestle in the washing solution. The homogenates were centrifuged at 9,000 g for 20 min at 4^0C and the resultant supernatants were centrifuged at 100,000 g for 60 min at 4°C. The microsomal pellets were resuspended in ice-cold freezing solution (100 mM potassium phosphate, 20% (v/v) glycerol, 1 mM EDTA, 1 mM APMSF, pH 7.4), aliquoted and stored at –80°C for future use. Four individual HLM prepared in the laboratory (Krausz et al., 2001) were equally mixed as pooled HLM (pHLM). Protein concentrations were determined using a BCA Protein Assay kit (Pierce Chemical Co. Rockford, IL, USA), following the manufacturer's instructions. Cytochromes P450 contents were determined according to the method described by Omura and Sato (Omura and Sato, 1964).

Incubation Reactions with Recombinant P450 Isoforms. Each incubation reaction was carried out in 100 mM potassium phosphate, pH 7.4, containing 5 pmol of cDNA-expressed P450 enzyme, 10 μM of substrate in a final volume of 200 μL. Reactions were initiated by the addition of 20 μL of 10 mM NADPH after 5 min pre-incubation at 37°C. Incubations were terminated by the addition of 10 μL of 60% perchloric acid after 5 min incubation. The mixtures were vortexed for 20 s, then centrifuged at 14,000 g for 10 min and the supernatants transferred to new vial and directly injected for HPLC analysis. Reactions for LC-MS/MS analysis were quenched with 50 μL of ice-cold 100 mM sodium hydroxide solution, added 20 μL of pinoline (internal standard, 10 μM in methanol), and extracted with 2 mL of methyl t-butyl ether and 1 mL of ethyl acetate mixture. The extracts were evaporated and reconstituted with 50% methanol solution containing 0.2% formic acid. The final reconstitutes were transferred to new vials and

injected for LC-MS/MS and/or LC-MS analysis. The concentration for both harmaline and harmine was fixed at 10 μM for screening the activities with individual P450 isozymes. For kinetic analysis, harmaline and harmine concentrations ranged from 0 to 2,000 μM when incubated with CYP1A1, CYP1A2, CYP2C9, and CYP2C19, and from 0 to 200 μM with CYP2D6. To test the dehydrogenation of harmaline and harmalol, their final concentrations were fixed at 50 μM and incubations conducted with 5 pmol of P450 isozyme for 20 min. Reactions were terminated with 50 μL of 100 μM sodium hydroxide and subjected to extractions as described above. Phenacetin (20 μL of 10 μM in methanol) was used as internal standard. All the reactions were performed in duplicate.

Incubation Reactions with Human and Mouse Liver Microsomes. Incubation reactions were carried out in 100 mM potassium phosphate, pH 7.4, containing pooled liver microsomes with 20-80 μg of hepatic protein, NADPH at a final concentration of 1 mM, and the monoclonal antibody when necessary in a final volume of 200 μL. Reaction mixtures were pre-incubation at 37°C for 5 min, then initiated by the addition of harmaline or harmine. Reactions were terminated by the addition of 10 μL of 60% perchloric acid or 50 μL of 100 mM sodium hydroxide after 10 min incubation, and processed as above for HPLC or LC-MS/MS analysis. Harmaline concentration was fixed at 50 μM to examine its possible 3,4-dehydrogenation in pooled HLM, and phenacetin (20 μL of 10 μM) was used as internal standard. The concentrations of harmaline and harmine ranged from 0 to 500 μM for the kinetic analysis with pooled human liver microsomes. Harmaline and harmine concentrations were fixed at 10 μM for comparison their *O*-demethylation activities between wild-type and *CYP2D6*-transgenic pooled

MLM, and the immunoinhibition studies in pooled HLM and MLM. Monoclonal antibody against CYP2D6 (20 μL) was used to examine its effect on harmaline and harmine *O*-demethylation in wild-type and *CYP2D6*-transgenic MLM. Antibodies against CYP1A1, CYP1A2, CYP2C9, CYP2C19 and CYP2D6 (20 μL of each) were used combinatorially for immunoinhibition studies (Gelboin et al., 1999). Anti-lysozyme (HyHel) was used as a control for nonspecific binding. All reactions were performed in duplicate.

Identification and Quantitation of Metabolites by HPLC and LC-MS/MS. HPLC analysis was carried out on an Agilent 1100 series HPLC system consisting of the online vacuum degasser, quaternary pump, autosampler, thermostatted column compartment, fluorescence detector, and diode-array detector. The Agilent 1100 series HPLC System was controlled with Agilent ChemStation and a handheld control module. A Regis 250 mm × 4.6 mm I.D. Rexchrom phenyl 5 μ column (Morton Grove, IL) was used to separate the metabolites. The flow rate through the column at ambient temperature was 1.0 mL/min. Separation of harmaline and its metabolites was achieved with a mobile phase containing 40% Buffer A (0.1% trifluoroacetic acid in water) and 60% Buffer B (40% v/v aqueous acetonitrile). Isocratic elution with 35% Buffer A and 65% Buffer B was applied for the separation of harmine and its metabolites. The excitation and emission wavelengths of the fluorescence detector were set at 250 and 480 nm respectively for the detection of harmaline and its metabolites, and at 350 and 490 nm respectively for the analysis of harmine and its metabolites. The detection limit for harmalol and harmol was 1 pmol under the experimental conditions were used.

Calibration curves were linear from 1 to 2000 pmol for harmalol and from 5 to 2000 pmol for harmol. Intra-day and inter-day coefficients of variation were less than 5%.

LC-MS/MS and LC-MS analysis of harmaline, harmine, and their metabolites was performed on a PE SCIEX API 2000 ESI triple-quadrupole mass spectrometer (Perkin-Elmer/ABI, Foster City, CA) controlled by Analyst software. A Luna 3 μ C18 50 mm × 4.6 mm I.D. Phenomenex® column (Torrance, CA) was used to separate harmaline, harmine, their metabolites, and pinoline, which was used as internal standard for quantitation. For the separation of harmaline, harmaline, their metabolites and pinoline internal standard, the flow rate through the column at ambient temperature was 0.2 mL/min with 50% methanol and 50% water containing 0.1% formic acid (Buffer C). Isocratic elution at 0.2 mL/min with 70% methanol and 30% Buffer C was used to separate harmaline, harmalol, harmine, harmol and phenacetin internal standard. The mass spectrometer was operated in the turbo ion spray mode with positive ion detection. The turbo ion spray temperature was maintained at 350°C, and a voltage of 4.8 kV was applied to the sprayer needle. Nitrogen was used as the turbo ion spray and nebulizing gas. The detection and quantitation of substrates, metabolites of interest, and the internal standards were accomplished by multiple reactions monitoring (MRM) with the transitions m/z 215.2/174.2 for harmaline, 201.2/160.2 for harmalol, 213.2/198.2 for harmine, 199.1/171.1 for harmol, 203.2/174.2 for pinoline, 180.1/110.0 for phenacetin. The MS/MS conditions were optimized automatically using the Analyst software for each chemical. The calibration curves were linear for harmalol, harmol, harmaline, harmine concentrations ranging from 0.2 to 20 μM. Calibration and regression of the curves were

completed using $1/x^2$ weighting. The recoveries of these compounds ranged from 85 to 110%. Intra-day and inter-day coefficients of variation were less than 10%. The identification of the unknown hydroxylated metabolites was accomplished by selected ion monitoring (SIM) with the protonated molecular ions of m/z 231.2 and 229.2, which were absent in the reactions with insect control microsomes, or P450 isoforms and pooled HLM incubations without NADPH.

Data Analysis. Results are expressed as the mean ± SEM. Values were compared with unpaired t test, and the difference was considered significant if the probability (P value) was less than 5%. Enzyme Michaelis-Menten parameters, K_m and V_{max}, were estimated by nonlinear regression (GraphPad Prizm™ 3.02, San Diego, CA). Initial estimates for nonlinear regression were generated graphically using Eadie-Hofstee plots (V_0 versus $V_0/[S]$). Linear regression analyses were conducted using Microsoft Excel 2000.

Results

HPLC and LC-MS/MS Analysis. Harmaline was metabolized to two unidentified metabolites as well as its *O*-demethylated metabolite harmalol by pooled HLM (Figure 1A). The unidentified metabolites were confirmed as hydroxylated metabolites by LC-MS single ion monitoring analysis. Harmine, the other β-carboline alkaloid, was metabolized to harmol and two other unknown metabolites by pooled HLM (Figure 1B). These unknown metabolites were identified as hydroxylated harmine by LC-MS analysis, which is consistent with a previous work (Tweedie and Burke, 1987). In this study, the metabolites were assigned as 6-, 3- or 4-hydroxylated harmine (Tweedie and Burke, 1987). However, the exact hydroxylation positions on harmaline and harmine were not determined.

Harmaline and Harmine Metabolism by cDNA-Expressed Human P450 Isozymes. To investigate the involvement and relative contribution of major human P450 enzymes in harmaline and harmine *O*-demethylations, screening experiments were carried out using recombinant human P450 enzymes. Harmalol and harmol were detected in the incubations of 10 μM harmaline and harmine, respectively, with recombinant human CYP1A1, CYP1A2, CYP2C9, CYP2C19 and CYP2D6 (Figure 2). The uncharacterized hydroxylated metabolites were detected only in the reactions of harmaline and harmine with recombinant CYP1A1 and CYP1A2 (Figure 1). In addition, incubations of harmaline with recombinant aromatase (CYP19), CYP1A2, CYP2C9, CYP2D6, CYP3A4, pooled recombinant P450 isozymes, or pooled HLM did not yield any harmine, demonstrating that the theoretical aromatization of harmaline to harmine (Figure 3)

through 3,4-dehydrogenation was not mediated by these P450 isozymes. CYP19 also did not produce harmol from harmalol (Figure 3).

Kinetic Study of Harmaline and Harmine *O*-Demethylation. As expected, harmaline and harmine *O*-demethylation in pooled HLM showed biphasic kinetics indicating that more than one P450 isozymes were involved in their biotransformations (Figure 4). One-enzyme and two-enzyme kinetic parameters were estimated, which revealed an apparent one-enzyme K_m value of around 84 μM for harmaline *O*-demethylation, and about 92 μM for harmine *O*-demethylation (Table 2). Steady-state kinetic parameters were also individually estimated for CYP1A1, CYP1A2, CYP2C9, CYP2C19, and CYP2D6 (Table 3). Interestingly, CYP1A1- and CYP1A2-catalyzed harmaline and harmine *O*-demethylations exhibited atypical kinetics (Figure 5). These data were fitted to a substrate inhibition kinetics algorithm [$V=V_{max}/(1+K_m/S+S/K_i)$], and the calculated atypical kinetic parameters are listed in Table 4.

Estimation of The Relative Contribution of P450 Isozymes to Harmaline and Harmine Metabolism in Pooled HLM by Immunoinhibition Analysis. To evaluate the relative contribution of CYP1A1, CYP1A2, CYP2C9, CYP2C19 and CYP2D6 to the metabolism of harmaline and harmine, immunoinhibition analysis was performed with pooled HLM using well-characterized monoclonal antibodies. As shown in Figure 6A, CYP2D6 contributed about 20%, CYP1A2 around 50%, CYP2C9 about 15% to harmaline *O*-demethylation. For harmine *O*-demethylation (Figure 6B), around 20% was contributed by CYP2D6, 30% by CYP1A2, and 20% by CYP2C9. In addition, CYP1A2

was identified as the major isozyme contributing to the hydroxylations of harmaline and harmine (data not shown), which was consistent with the results obtained from screening experiments (Figure 1).

Evaluation the Difference of Harmaline and Harmine *O*-Demethylation in Wild-Type and *CYP2D6*-Transgenic Mice *in vitro*. To examine the role of CYP2D6 in harmaline and harmine *O*-demethylations activities, liver microsomes were prepared from wild-type and *CYP2D6*-transgenic mice and activities determined. *CYP2D6*-transgenic microsomes showed about a 6-fold higher harmaline *O*-demethylation activity than the wild-type microsomes (Figure 7). In addition, the increased activity was completely inhibited by the CYP2D6 monoclonal antibody. Harmine *O*-demethylation activity was also increased about 20% (P=0.032) in *CYP2D6*-transgenic as compared to wild-type microsomes (Figure 7). Similarly, the increased harmine *O*-demethylation activity was also totally blocked by the CYP2D6 monoclonal antibody. These results are consistent with an important role for CYP2D6 in harmaline and harmine *O*-demethylations.

Discussion

Endogenous and exogenous β-carboline alkaloids exhibit a variety of biochemical, psychopharmacological and behavioral effects in animals and man (Airaksinen and Kari, 1981). They are also thought to have genotoxic, mutagenic and cytotoxic activities (Picada et al., 1997; Boeira et al., 2002; Collins, 2002; Matsubara et al., 2002). *N*-methylation activation of β-carbolines leads to a similar pattern of neurotoxicity as MPTP, which induces irreversible Parkinsonism. The oxidation of these β-carboline alkaloids to readily excretable products (Mulder and Hagedoorn, 1974; Tan et al., 1990), appear to play a key role in the protection of cells such as neurons in brain.

In humans, P450 isozymes are expressed and active not only in liver, but also in extrahepatic tissues including the brain (Riedl et al., 1998; Hedlund et al., 2001; Strobel et al., 2001; Miksys et al., 2002). Cytochrome P450s belonging to the CYP1A, CYP2C, CYP2D, and CYP3A subfamilies have all been detected in human brain. These CNS P450 isozymes could substantially influence the biotransformations of endogenous and exogenous neurotoxins. Some β-carbolines, such as 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, that have been assumed to be formed endogenously, in this case from tryptophan and acetaldehyde, and can also be absorbed from the diet and enter the mammalian brain (Fukushima et al., 1992). Other β-carbolines, for example 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline, the condensation product of 5-HT and acetaldehyde, are not detectable in mammalian brain (Beck and Lundman, 1983), suggesting that they are neither formed endogenously, nor enter the brain from the diet. However, this simplistic model of dietary sources, blood-brain barrier passage, and

endogenous formation needs to be reevaluated in the light of the findings reported here. Clearly, various β-carbolines may be formed in mammalian brain from abundant indole precursors such as 5-HT and tryptophan, but experiments that are designed simply to detect these compounds may yield negative results if the possibility of further metabolism by brain P450s is not considered. Some of the turnover values reported here are so high as to render difficult the detection of certain β-carbolines in brain tissues. Additionally, β-carbolines are substrates for *N*-methyl transferases (Matsubara et al., 1993; Gearhart et al., 2002) and the ability of the hydroxylated β-carbolines to be *O*-methylated by mammalian hydroxyindole *O*-methyltransferases (HIOMT) has never, to our knowledge, been tested. The solution to this problem would be to screen brain tissues for the full range of non-hydroxylated, hydroxylated and *O*-methylated β-carbolines using the remarkable resolving power of techniques such as LC-MS/MS.

The data from this study clearly demonstrated the involvement of CYP1A1, CYP1A2, CYP2C9, CYP2C19 and CYP2D6 in the metabolism of harmaline and harmine. Kinetic studies and immunoinhibition analysis with monoclonal antibodies indicated that CYP1A2 and CYP2D6 are the major P450 isozymes contributing to their metabolism. The role of CYP2D6 was further confirmed by the analysis of *CYP2D6*-transgenic mice. Of note is that *O*-demethylated metabolites, harmalol and harmol, unlike harmaline and harmine themselves, are not tremor inducers. Therefore, *O*-demethylation of these β-carbolines may be an important detoxication process protecting neurons against chemical damage. Moreover, such *O*-demethylation detoxication reactions may compete with the toxifying reactions of *N*-methylation that are thought to lead to intermediates that may

trigger Parkinson's disease (Collins and Neafsey, 1985; Collins et al., 1992; de Meester, 1995; Aoyama et al., 2000; Gearhart et al., 2000; Gearhart et al., 2002).

Most importantly, these studies have demonstrated the important contribution of CYP2D6 to the *O*-demethylation of harmaline and harmine, although its role in their total clearance can not be determined by this study. Since humans are exposed to β-carbolines in the diet, for example in fresh ground coffee (Herraiz, 2002), and this group of compounds is potentially neurotoxic, there may exist profound differences in β-carboline sensitivity between extensive (EM) and poor (PM) metabolizers for the CYP2D6 polymorphism (Mahgoub et al., 1977). A recent report (Miksys et al., 2002) demonstrated clear expression of CYP2D6 in neurons such as pyramidal Purkinje cells and that the expression of CYP2D6 is considerably elevated in alcoholics, except in a subject who was a PM who had to detectable neuronal CYP2D6. These later individuals have elevated biosynthesis of β-carbolines due to their alcohol intake and conversion to acetaldehyde, but do not have the potential for neuroprotective detoxication afforded by CYP2D6, normally elevated in alcoholism (Miksys et al., 2002). Whether or not this is a paradigm for chemically-induced neurotoxicity in general, remains to be investigated. Studies of the association between CYP2D6 genotype and Parkinson's disease have produced varied results, probably because many of the studies employed small numbers of patients. However, a meta-analysis of 11 studies showed a small (odds ratio 1.47) but highly significant (P=0.01) association between the poor metabolizer genotypes and Parkinson's disease (McCann et al., 1997). The etiology of Parkinson's disease is not known and many candidate chemical causes have been proposed since the discovery by

Bill Langston and his colleagues that a Parkinson's syndrome could be induced by an identifiable environmental chemical, MPTP and that it's metabolite MPP^+ was the likely ultimate neurotoxin (Langston and Ballard, 1983; Langston and Ballard, 1984; Langston et al., 1984). While MPTP is activated to MPP^+ by MAO-B, it is detoxicated by *N*-demethylation, largely by CYP2D6. Thus there exist many commentaries predicting a role for polymorphic CYP2D6 in MPTP-induced Parkinson's disease. However, very few individuals are exposed to MPTP and this is not a plausible cause of the common manifestations of Parkinson's disease. Nevertheless, building on the MPTP/CYP2D6 paradigm, one may readily envisage how more common exposures may interact with this polymorphic enzyme to precipitate neurotoxicity syndromes such as Parkinson's disease. Obvious candidates are both the exogenous and endogenously-formed β-carbolines.

In this study, harmaline and harmine *O*-demethylation mediated by CYP1A1 and CYP1A2 enzymes showed substrate inhibition kinetics. Atypical enzyme kinetics were also reported for P450-catalyzed reactions, such as substrate inhibition and activation of CYP3A4 (Korzekwa et al., 1998; Shou et al., 2001). These findings have led to a better understanding of the active sites of P450 enzymes, including multiple sites for substrate binding (Shou et al., 2001).

It is noteworthy that while harmaline *O*-demethylation is carried out preferentially by CYP2D6, other P450 isozymes approach the activity of CYP2D6 with respect to harmine *O*-demethylation. This difference between the isozyme preference of these two β-carbolines is further demonstrated in the experiments using MLM, whereby the *CYP2D6*-

transgenic MLM had a high CYP2D6-specific harmaline *O*-demethylase activity, but a considerably poorer CYP2D6-specific harmine *O*-demethylase activity. These observations are consistent with the relative affinities of the two substrates (harmaline $Km_{rCYP2D6}$[2] 1.41 μM, harmine $Km_{rCYP2D6}$ 7.42 μM, Table 3; hamaline $Km_{HLMCYP2D6}$[3] 18.9 μM, harmine $Km_{HLMCYP2D6}$ 62.1 μM, Table 2). Thus, the difference between the two substrates is almost certainly due to the less basic nature of harmine (pKa 7.70[4]) compared to hamaline (pKa 9.80[4]). Indeed, it was recently proposed that CYP2D6 substrates must contain an ionized nitrogen center at cellular pH to bind to the Glu-216 residue for catalytic activity (Kirton et al., 2002). Based upon the foregoing difference in pKa values, at pH 7.4, for example, harmaline will be over 100-times more ionized than harmine.

In summary, the cytochromes P450 that *O*-demethylate the exogenous β-carbolines harmaline and harmine have been identified. The predominant isozymes are CYP1A2 and polymorphic CYP2D6. Plant extracts containing the β-carboline alkaloids have been used in traditional medicine for millennia and are of considerable ethnopharmacological interest and importance. Both South American *ayahuasca* and Asian *Peganum harmala* seeds employ the pharmacological properties of the β-carboline alkaloids to exert both their healing and psychotropic properties. There remains the intriguing possibility that the pharmacologic and toxicological properties of these traditional medicines are related to the CYP2D6 polymorphism. Finally, we propose that CYP2D6 could potentially ameliorate the neurotoxicity of both endogenous and exogenous β-carbolines by restricting the metabolic flux through the *N*-methylation pathways.

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Footnotes

[1] Present address: U Háje 1651, 252 63 Roztoky u Prahy, Czech Republic

[2] K_m for recombinant CYP2D6 (insect cells)

[3] K_m for CYP2D6 (high affinity component) with two-enzyme kinetics in H161 HLM

[4] The Merck Index, Twelfth Edition, 1998.

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Figure Legends

Figure 1. HPLC traces of harmaline (A) and harmine (B), and their corresponding metabolites formed by pooled human liver microsomes (pHLM), and individual P450 isozymes. The excitation and emission of the fluorescence detector were set at 250 and 480 nm respectively for analysis of harmaline reactions, and at 350 and 490 nm respectively for harmine reactions.

Figure 2. Relative harmaline (A) and harmine (B) *O*-demethylation activity catalyzed by cDNA-expressed human P450 isozymes. Incubations were performed with 5 pmol of each P450 isozyme in 100 mM potassium phosphate buffer, pH 7.4, at 37^0C for 5 min. Final substrate concentrations were fixed at 10 μM. Values represent the mean, and the vertical lines represent the errors of mean (± SEM) from duplicate incubations.

Figure 3. Summary of the metabolism of harmaline and harmine by human P450 isozymes.

Figure 4. Eadie-Hofstee plots of harmaline (A) and harmine (B) *O*-demethylations in pooled human liver microsomes (■ pHLM; ▲ H161) showing biphasic kinetics. Incubation reactions were carried out at 37^0C for 10 min. Values represent the mean of duplicate incubations.

Figure 5. CYP1A1- and CYP1A2-catalyzed harmaline (A) and harmine (B) *O*-demethylations showing substrate inhibition (■CYP1A1; ▼CYP1A2). Reactions containing 5 pmol of each enzyme lasted for 10 min at 37^0C.

Figure 6. Harmaline (A) and harmine (B) *O*-demethylation activities affected by monoclonal antibodies directed against CYP1A1, CYP1A2, CYP2C9, CYP2C19, and CYP2D6 (MAb) in pooled human liver microsomes (pHLM and H161).

Figure 7. Comparison of harmaline (A) and harmine (B) *O*-demethylation activities between wild-type (WT) and *CYP2D6*-transgenic (2D6) pooled mouse liver microsomes (MLM) from 3 mice for each group, and the effects of the CYP2D6 monoclonal antibody on these reactions. Open bars represent the control reactions with anti-lysozyme for nonspecific binding and filled bars represent the reactions with antibody.

Table 1. Chemical structures and common names of some biogenic β-carboline alkaloids.

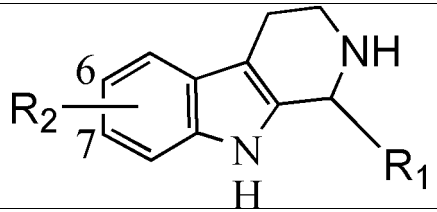
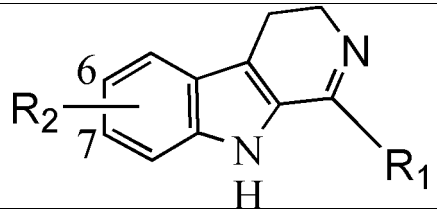
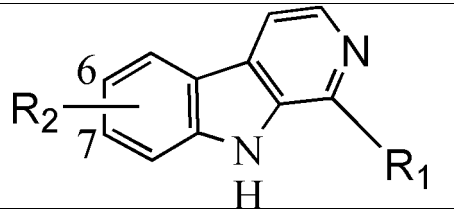
R_1	R_2			
H	H	Tryptoline	Norharmalan	Norharman
H	6-MeO	Pinoline	6-MeO-dihydro-β-carboline	6-MeO-β-carboline
Me	H	Tetrahydroharman	Harmalan	Harman
Me	7-MeO	Tetrahydroharmine	Harmaline	Harmine

Table 2. Michaelis-Menten kinetic parameters estimated for harmaline and harmine *O*-demethylations in pooled human liver microsomes. H161was purchased from BD Discovery Labware, Inc. (Woburn, MA), and pHLM was prepared in our laboratory. Reactions in duplicate were incubated in phosphate buffer, pH=7.4 for 10 min at 37^0C. Final harmaline and harmine concentrations were from 0 to 500 μM in the reactions.

			One-enzyme Kinetics			Two-enzyme Kinetics		
Substrate	HLM	Metabolite	Vmax pmol/mg protein/min	Km μM	R^2	Km (high affinity) μM	Km (low affinity) μM	R^2
Harmaline	H161	Harmalol	2110±143	84.1±17.3	0.947	18.9±9.53	>1000	0.978
	pHLM		948±46.0	83.4±12.3	0.972	11.4±2.25	606±178	0.998
Harmine	H161	Harmol	2880±78.1	91.3±7.35	0.992	62.1±23.3	>1000	0.994
	pHLM		1140±46.3	93.5±11.1	0.983	21.9±19.2	312±274	0.989

Table 3. Michaelis-Menten kinetic parameters estimated for harmaline and harmine *O*-demethylations catalyzed by P450 isozymes.

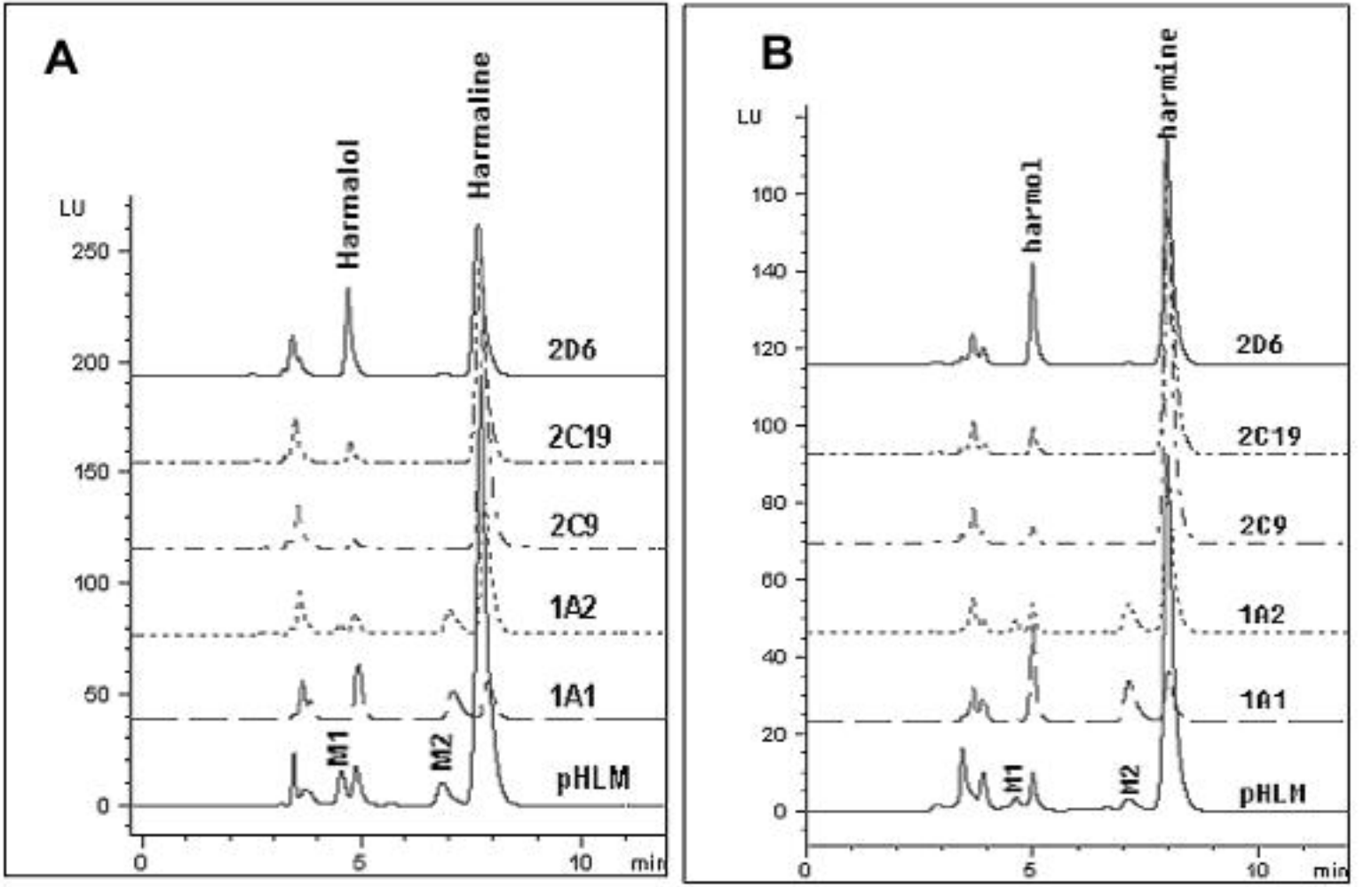
Each incubation reaction contained 5 pmol recombinant P450 isozyme, and lasted at 37^0C for 5 min. Harmaline and harmine final concentrations ranged from 0 to 2,000 μM with CYP1A1, CYP1A2, CYP2C9 and CYP2C19, and from 0 to 200 μM with CYP2D6.

Substrate	P450 isozyme	Metabolite	Vmax pmol/pmol P450/min	Km μM	Vmax/Km μL/pmol P450/min
	CYP1A1		10.8±0.539	11.8±1.90	0.915
	CYP1A2		12.3±0.594	13.3±2.41	0.925
Harmaline	CYP2C9	Harmalol	5.28±1.18	175±157	0.0302
	CYP2C19		10.3±1.88	160±109	0.0644
	CYP2D6		39.9±1.38	1.41±0.238	28.3
	CYP1A1		45.2±1.15	52.2±3.70	0.866
	CYP1A2		9.23±0.611	14.7±3.60	0.628
Harmine	CYP2C9	Harmol	11.9±1.18	117±43.7	0.102
	CYP2C19		21.4±3.19	121±67.0	0.177
	CYP2D6		29.7±0.909	7.42±1.04	4.00

Table 4. Atypical kinetic parameters estimated for harmaline and harmine *O*-demethylations catalyzed by CYP1A1 and 1A2 isozymes by fitting the data into the equation [$V=V_{max}/(1+K_m/S+S/K_i)$] describing substrate inhibition.

Substrate	P450 isozyme	Metabolite	Vmax pmol/pmol P450/min	Km μM	Ki μM	R^2
Harmaline	CYP1A1	Harmalol	22.4±6.44	59.4±31.5	442±241	0.815
	CYP1A2		13.7±1.48	16.5±5.03	1150±387	0.835
Harmine	CYP1A1	Harmol	87.5±16.4	139±38.8	308±88.6	0.971
	CYP1A2		10.1±0.923	17.8±4.83	2160±750	0.879

Fig. 1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harmol
harmine
2D6
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Fig. 2

Fig. 3

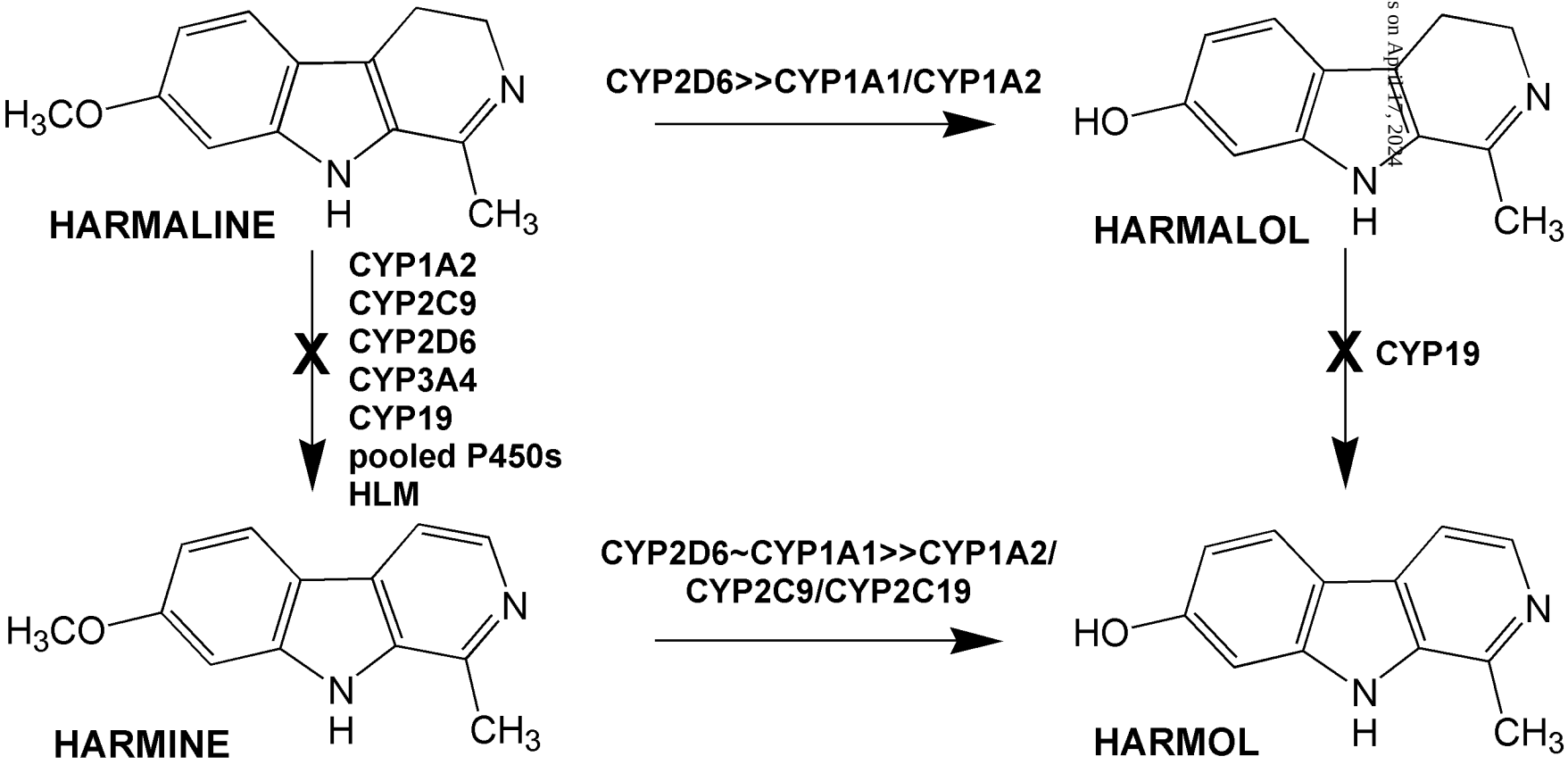

Fig. 4

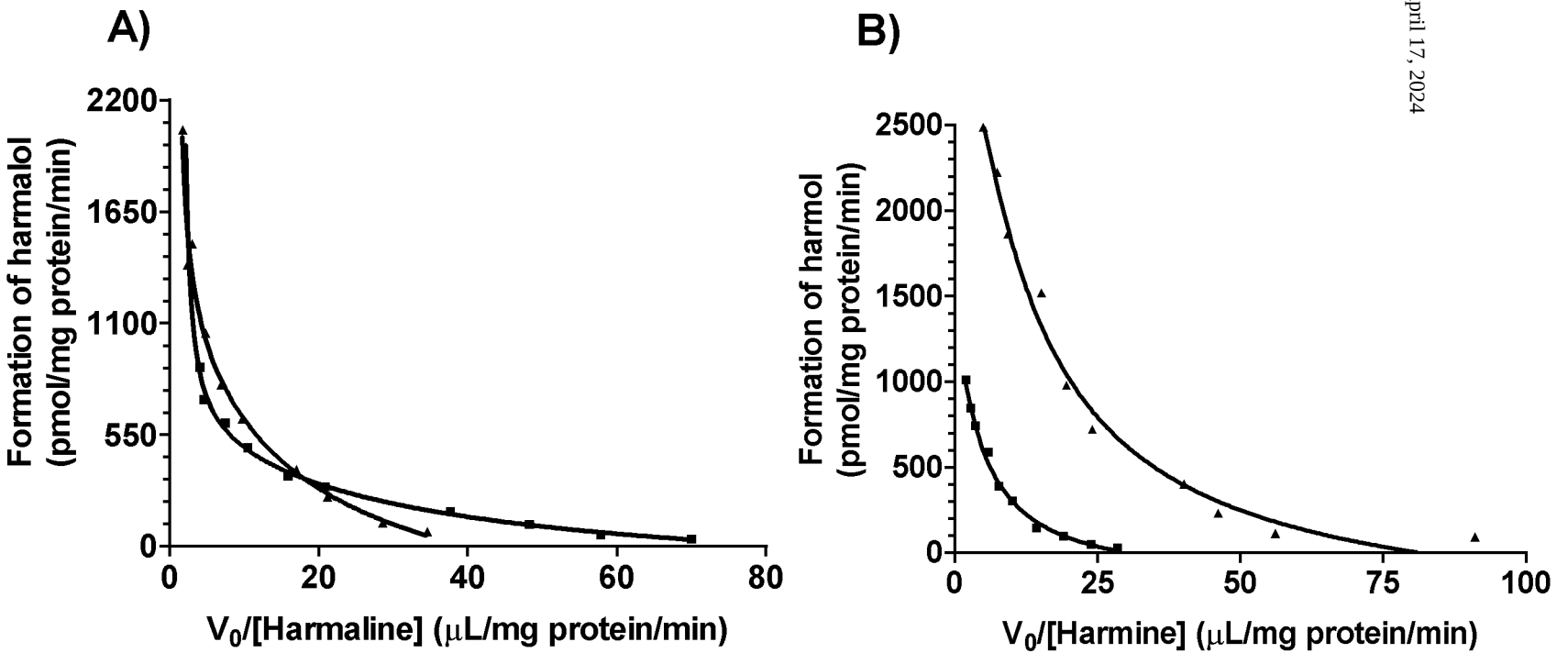

Fig. 5

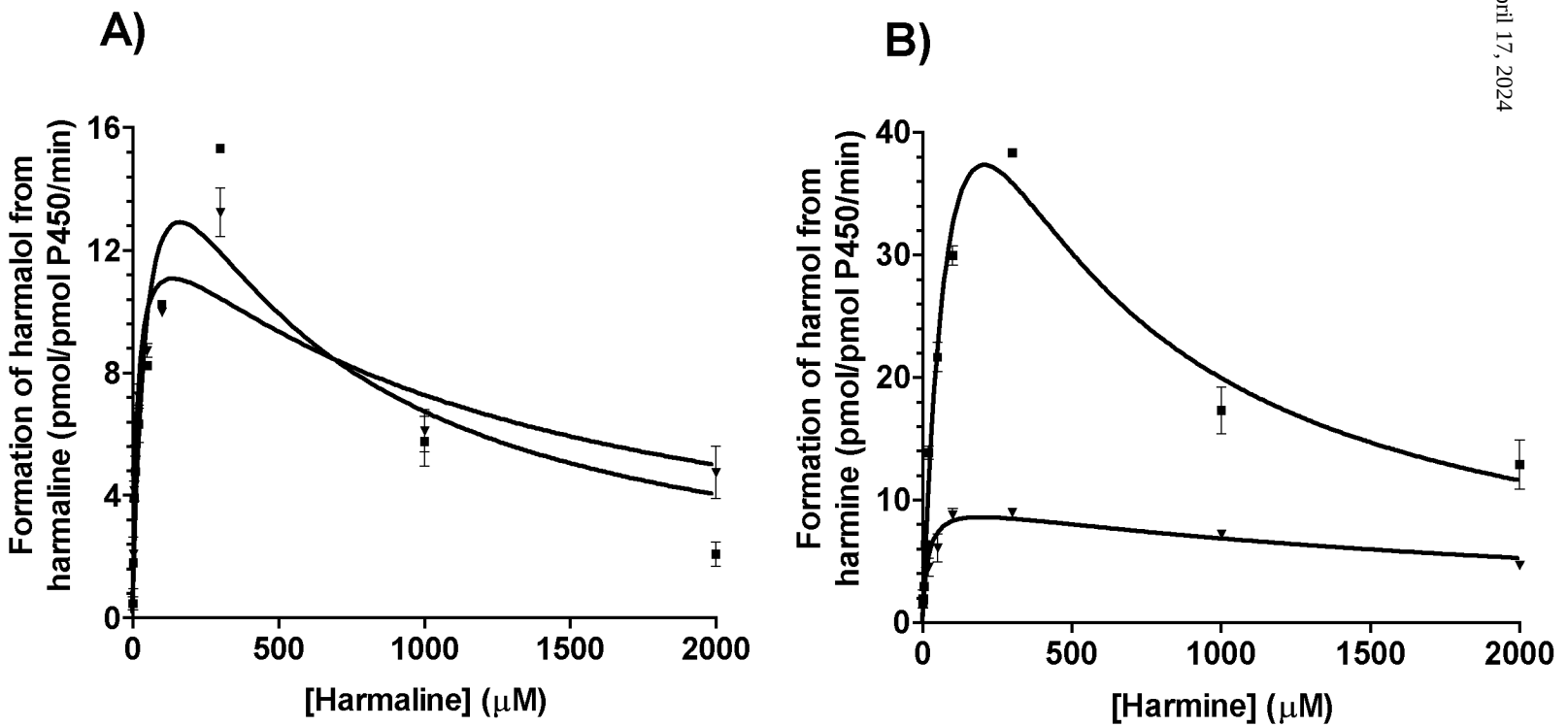

Fig. 6

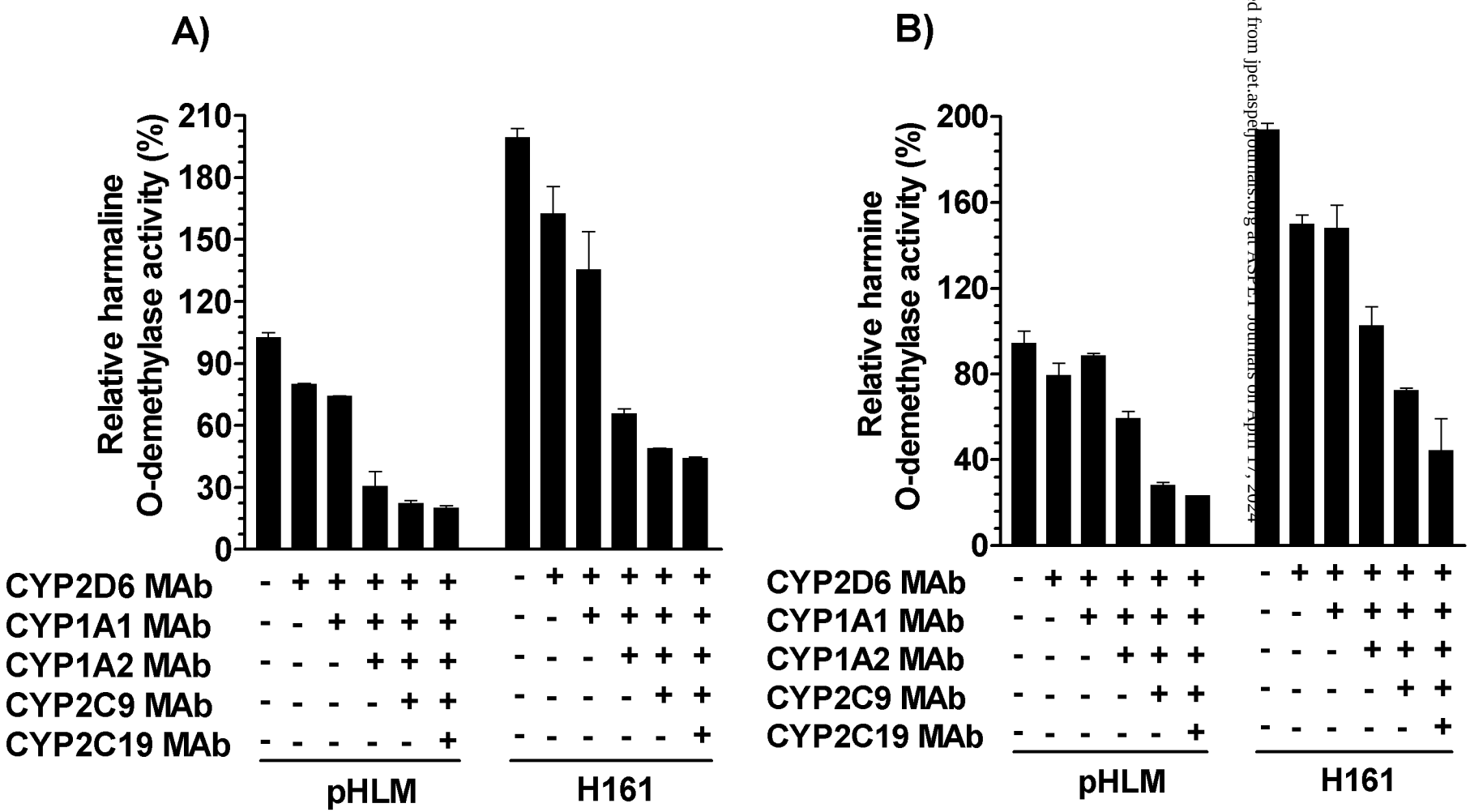

Fig. 7