

Contribution of Individual Cytochrome P450 Isozymes to the O-Demethylation of the Psychotropic β -Carboline Alkaloids Harmaline and Harmine

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Received November 19, 2002; accepted December 30, 2002

ABSTRACT

The psychotropic β -carboline alkaloids, showing high affinity for 5-hydroxytryptamine, 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 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) catalyzed 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 cytochromes P450, or human liver microsomes (HLMs). Kinetic parameters were calculated for the O-demethylations mediated by each isozyme and by pooled HLMs. K_{cat} (min^{-1})

and K_m (μM) values for harmaline were: CYP1A1, 10.8 and 11.8; CYP1A2, 12.3 and 13.3; CYP2C9, 5.3 and 175; CYP2C19, 10.3 and 160; and CYP2D6, 39.9 and 1.4. Values for harmine were: CYP1A1, 45.2 and 52.2; CYP1A2, 9.2 and 14.7; CYP2C9, 11.9 and 117; CYP2C19, 21.4 and 121; and CYP2D6, 29.7 and 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 HLMs. The turnover numbers for CYP2D6 are among 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 with 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 (Table 1), 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 depen-

dence 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 (Table 1) exhibit the most potent inhibition toward 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* or *Diplopterys cabrerana*. Originally, it was proposed that the psychotropic properties of *ayahuasca* resided in the

J.R.I. received a grant from U. S. Smokeless Tobacco Company.

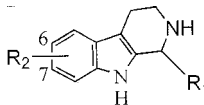
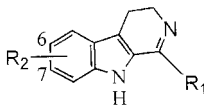
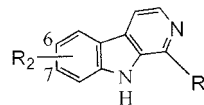
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Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

DOI: 10.1124/jpet.102.047050.

ABBREVIATIONS: MAO, monoamine oxidase; DMT, *N,N*-dimethyltryptamine; 5-HT, 5-hydroxytryptamine; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP⁺, 1-methyl-4-phenylpyridinium; P450, cytochrome P450; APMSF, 4-amidinophenylmethanesulfonyl fluoride; HPLC, high performance liquid chromatography; Mab, monoclonal antibody; MLM, mouse liver microsomes; HLM, human liver microsomes; pHLM, pooled HLM; LC-MS/MS, liquid chromatography-tandem mass spectrometry; $K_{m, \text{CYP2D6}}$, K_m for recombinant CYP2D6 (insect cells); $K_{m, \text{HLMCYP2D6}}$, K_m for CYP2D6 (high-affinity component) with two-enzyme kinetics in H161 HLMs.

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

R_1	R_2			
H	H	Tryptoline	Norharmalan	Norharman
H	6-CH ₃ O	Pinoline	6-CH ₃ O-dihydro- β -carboline	6-CH ₃ O- β -carboline
CH ₃	H	Tetrahydroharman	Harmalan	Harman
CH ₃	7-CH ₃ O	Tetrahydroharmine	Harmaline	Harmine

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 tremorogenic 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 has been 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. Tryptophan-derived β -carbolines are similar to *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, which are neurotoxic compared with MPP⁺, also induce mitochondrial energy depletion and oxidative stress in nigrostriatum (Collins and Neafsey, 1985; Collins et al., 1987, 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-5H-pyrido[4,3-*b*]indole, 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,b; 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 cytochrome P450 (P450) isozymes to their O-demethylation. The results might provide insights not only 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, NADPH, 4-aminodiphenylmethanesulfonyl fluoride (APMSF), EDTA, and 60% perchloric acid were purchased from Sigma-Aldrich (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 Gentest (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 763-15-5), 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 microsome preparations were maintained under controlled temperature (23 \pm 1°C) and lighting (lights on 6:00 AM to 6:00 PM) with food and water provided ad libitum. Adult males, 2 to 3 months old, were used in the experiments, which were conducted under the National Institutes 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 CO₂ 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 three 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 9,000g for 20 min at 4°C, and the resultant supernatants were centrifuged at 100,000g 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), following the manufacturer's instructions. Cytochrome P450 contents were determined according to the method described by 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 μ M substrate in a final volume of 200 μ L. Reactions were initiated by the addition of 20 μ L of 10 mM NADPH after 5 min preincubation at 37°C. Incubations were terminated by the addition of 10 μ L of 60% perchloric acid after 5 min incubation. The mixtures were vortexed for 20 s and then centrifuged at 14,000g for 10 min; then, the supernatants were transferred to a new vial and directly injected for HPLC analysis. Reactions for LC-MS/MS analysis were

quenched with 50 μ l of ice-cold 100 mM sodium hydroxide solution, to which was added 20 μ l of pinoline (internal standard, 10 μ M in methanol), and extracted with 2 ml of methyl *tert*-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 μ M for screening the activities with individual P450 isozymes. For kinetic analysis, harmaline and harmine concentrations ranged from 0 to 2000 μ M when incubated with CYP1A1, CYP1A2, CYP2C9, and CYP2C19, and from 0 to 200 μ M with CYP2D6. To test the dehydrogenation of harmaline and harmalol, their final concentrations were fixed at 50 μ M, and incubations were conducted with 5 pmol of P450 isozyme for 20 min. Reactions were terminated with 50 μ l of 100 μ M sodium hydroxide and subjected to extractions as described above. Phenacetin (20 μ l of 10 μ M 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 to 80 μ g of hepatic protein, NADPH at a final concentration of 1 mM, and the monoclonal antibody when necessary in a final volume of 200 μ l. Reaction mixtures were preincubated at 37°C for 5 min, then initiated by the addition of harmaline or harmine. Reactions were terminated by the addition of 10 μ l of 60% perchloric acid or 50 μ l 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 μ M to examine its possible 3,4-dehydrogenation in pooled HLM, and phenacetin (20 μ l of 10 μ M) was used as internal standard. The concentrations of harmaline and harmine ranged from 0 to 500 μ M for the kinetic analysis with pooled human liver microsomes. Harmaline and harmine concentrations were fixed at 10 μ M for comparison of 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 μ l) was used to examine its effect on harmaline and harmine *O*-demethylation in wild-type and CYP2D6-transgenic MLMs. Antibodies against CYP1A1, CYP1A2, CYP2C9, CYP2C19, and CYP2D6 (20 μ l 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 an Agilent ChemStation and a handheld control module (Agilent Technologies, Palo Alto, CA). A Regis 250 mm \times 4.6 mm i.d. REXCHROM phenyl 5 μ m column (Regis, 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 used. Calibration curves were linear from 1 to 2000 pmol for harmalol and from 5 to 2000 pmol for harmol. Intraday and interday coefficients of variation were less than 5%.

LC-MS/MS and LC-MS analysis of harmaline, harmine, and their metabolites was performed on a PerkinElmerSciex API 2000 ESI triple-quadrupole mass spectrometer controlled by Analyst software

(PerkinElmerSciex Instruments, Boston, MA). A Luna 3 μ m C18, 50 mm \times 4.6 mm i.d., Phenomenex column (Phenomenex, 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 internal standards were accomplished by multiple reactions monitoring 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, and 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, and harmine, with concentrations ranging from 0.2 to 20 μ M. Calibration and regression of the curves were completed using $1/x^2$ weighting. The recoveries of these compounds ranged from 85 to 110%. Intraday and interday coefficients of variation were less than 10%. The identification of the unknown hydroxylated metabolites was accomplished by selected ion monitoring 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 \pm S.E.M. 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 (Prism 3.02; GraphPad, 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 (Fig. 1A).

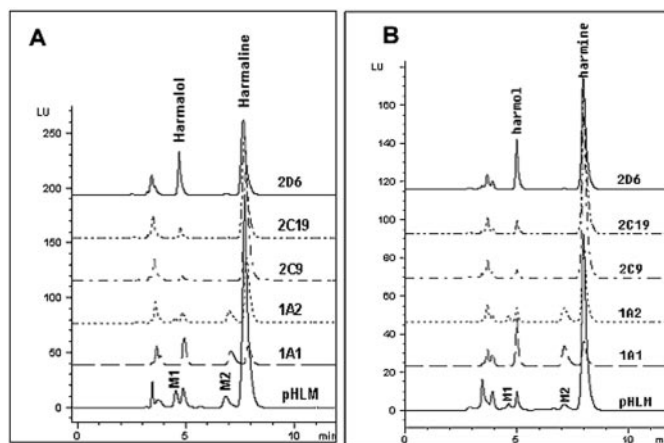


Fig. 1. HPLC traces of harmaline (A) and harmine (B), and their corresponding metabolites formed by pHLMs and individual P450 isozymes. The excitation and emission of the fluorescence detector were set at 250 and 480 nm, respectively, for analysis of harmaline reactions, and at 350 and 490 nm, respectively,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

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 (Fig. 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 μ M harmaline and harmine, respectively, with recombinant human CYP1A1, CYP1A2, CYP2C9, CYP2C19, and CYP2D6 (Fig. 2). The uncharacterized hydroxylated metabolites were detected only in the reactions of harmaline and harmine with recombinant CYP1A1 and CYP1A2 (Fig. 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 (Fig. 3) through 3,4-dehydrogenation was not mediated by these P450 isozymes. CYP19 also did not produce harmol from harmalol (Fig. 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 isozyme was involved in their biotransformations (Fig. 4). One-enzyme and two-enzyme kinetic parameters were estimated, which revealed an apparent one-enzyme K_m value of around 84 μ M for harmaline *O*-demethylation, and about 92 μ M for harmine *O*-demethylation (Table 2). Steady-state kinetic parameters were also individually estimated for CYP1A1, CYP1A2, CYP2C9,

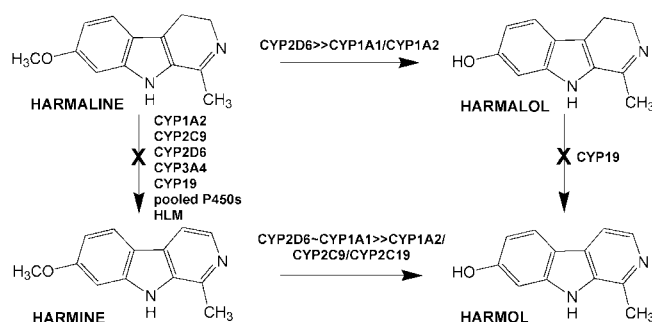


Fig. 3. Summary of the metabolism of harmaline and harmine by human P450 isozymes.

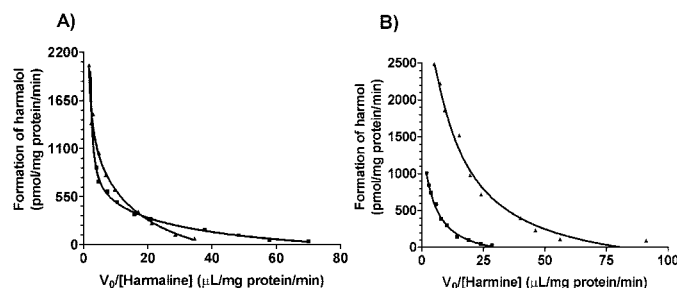


Fig. 4. Eadie-Hofstee plots of harmaline (A) and harmine (B) *O*-demethylations in pooled human liver microsomes (■, pHLM; ▲, H161) showing biphasic kinetics. Incubation reactions were carried out at 37°C for 10 min. Values represent the mean of duplicate incubations.

CYP2C19, and CYP2D6 (Table 3). Interestingly, CYP1A1- and CYP1A2-catalyzed harmaline and harmine *O*-demethylations exhibited atypical kinetics (Fig. 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 Fig. 6A, CYP2D6 contributed about 20%, CYP1A2 around 50%, and CYP2C9 about 15% to harmaline *O*-demethylation. For harmine *O*-demethylation (Fig. 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 (Fig. 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*-demethylation activities, liver microsomes were prepared from wild-type and CYP2D6-transgenic mice and activities were determined. CYP2D6-transgenic microsomes showed about a 6-fold higher harmaline *O*-demethylation activity than the wild-type microsomes (Fig. 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 with wild-type

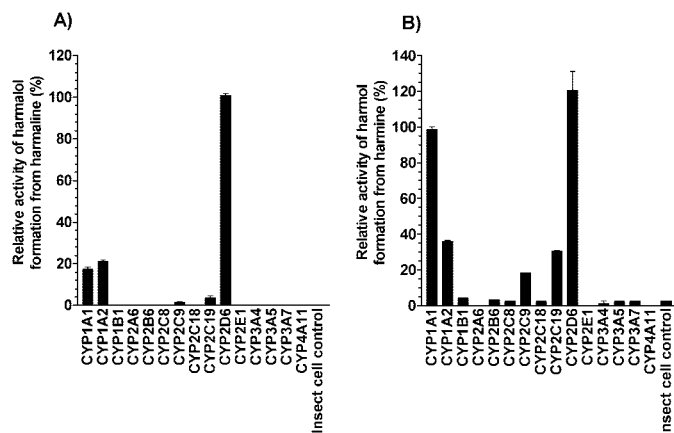


Fig. 2. Relative harmaline (A) and harmine (B) *O*-demethylation activity catalyzed by cDNA-expressed human P450 isozymes. Incubations were performed with 5 pmol of each P450 isozyme in 100 mM potassium phosphate buffer, pH 7.4, at 37°C for 5 min. Final substrate concentrations were fixed at 10 μ M.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

TABLE 2

Michaelis-Menten kinetic parameters estimated for harmaline and harmine *O*-demethylations in pooled human liver microsomes

H161 was purchased from BD Gentest (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°C. Final harmaline and harmine concentrations were from 0 to 500 μ M in the reactions.

Substrate	HLM	Metabolite	One-Enzyme Kinetics			Two-Enzyme Kinetics		
			V_{\max}	K_m	R^2	K_m (high affinity)	K_m (low affinity)	R^2
			pmol/mg protein/min	μ M		μ M	μ M	
Harmaline	H161	Harmalol	2110 \pm 143	84.1 \pm 17.3	0.947	18.9 \pm 9.53	>1000	0.978
	pHLM		948 \pm 46.0	83.4 \pm 12.3	0.972	11.4 \pm 2.25	606 \pm 178	0.998
Harmine	H161	Harmol	2880 \pm 78.1	91.3 \pm 7.35	0.992	62.1 \pm 23.3	>1000	0.994
	pHLM		1140 \pm 46.3	93.5 \pm 11.1	0.983	21.9 \pm 19.2	312 \pm 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 of recombinant P450 isozyme and lasted at 37°C for 5 min. Harmaline and harmine final concentrations ranged from 0 to 2000 μ M with CYP1A1, CYP1A2, CYP2C9, and CYP2C19, and from 0 to 200 μ M with CYP2D6.

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

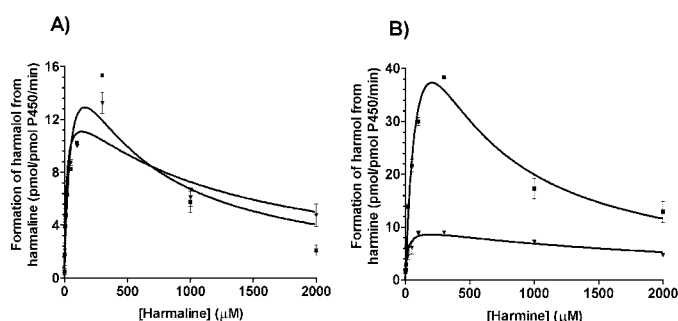


Fig. 5. CYP1A1- and CYP1A2-catalyzed harmaline (A) and harmine (B) *O*-demethylations showing substrate inhibition (■, CYP1A1; ▼, CYP1A2). Reactions containing 5 pmol of each enzyme lasted for 10 min at 37°C.

microsomes (Fig. 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 humans (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 pattern of neurotoxicity similar to that of MPTP, which induces irreversible parkinsonism. The oxidation of these β -carboline alkaloids to readily excretable products (Mulder and Hagedoorn, 1974; Tan et al., 1990), appears 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 central nervous system 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, 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 neither are 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*-methyltransferases (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 has never, to our knowledge, been tested. The solution to this problem would be to screen brain tissues for the full range of nonhydroxylated, hydroxylated, and *O*-methylated β -carbo-

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	V_{\max} <i>pmol/pmol P450/min</i>	K_m μM	K_i 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

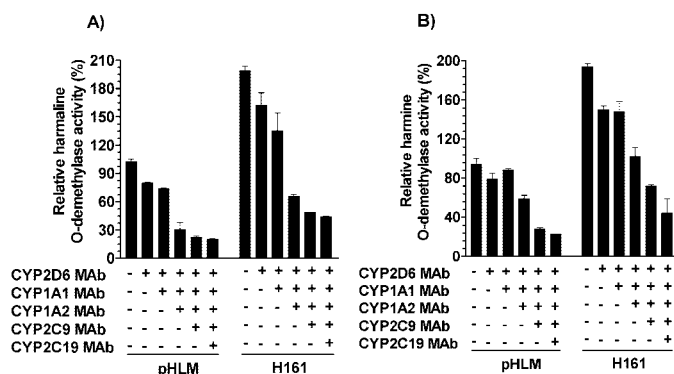


Fig. 6. Harmaline (A) and harmine (B) *O*-demethylation activities affected by monoclonal antibodies directed against CYP1A1, CYP1A2, CYP2C9, CYP2C19, and CYP2D6 (Mab) in pooled human liver microsomes (pHLM and H161).

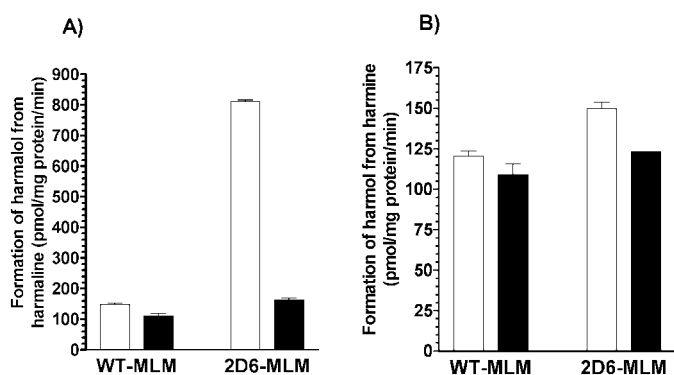


Fig. 7. Comparison of harmaline (A) and harmine (B) *O*-demethylation activities between wild-type (WT) and CYP2D6-transgenic (2D6) pooled mouse liver microsomes (MLM) from three mice for each group, and the effects of the CYP2D6 monoclonal antibody on these reactions. Open bars represent the control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

lines 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, 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 cannot be determined by this study. Since humans are exposed to β -carbolines in the diet, for example, in fresh ground coffee (Herraz, 2002), and this group of compounds is potentially neurotoxic, there may exist profound differences in β -carboline sensitivity between extensive and poor 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 poor metabolizer who had to detectable neuronal CYP2D6. These latter 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 its metabolite, MPP⁺, was the likely ultimate neurotoxin (Langston and Ballard, 1983, 1984; Langston et al., 1984). Whereas 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 exogenously and endogenously formed β -carbolines.

In this study, harmaline and harmine *O*-demethylation mediated by CYP1A1 and CYP1A2 enzymes showed sub-

strate 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 whereas 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 K_m rCYP2D6, 1.41 μ M, harmine K_m rCYP2D6, 7.42 μ M, Table 3; harmaline K_m HLMCYP2D6, 18.9 μ M, harmine K_m HLMCYP2D6, 62.1 μ M, Table 2). Thus, the difference between the two substrates is almost certainly due to the less basic nature of harmine (pK_a 7.70) compared with harmaline (pK_a 9.80) (Budavri et al., 1996). 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 (Kirtton et al., 2002). Based upon the foregoing difference in pK_a 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.

Acknowledgments

We thank Dr. Timothy S. Tracy, West Virginia University, for helpful discussions.

References

- Abdel-Fattah AF, Matsumoto K, Gammaz HA, and Watanabe H (1995) Hypothermic effect of harmala alkaloid in rats: involvement of serotonergic mechanism. *Pharmacol Biochem Behav* **52**:421–426.
- Airaksinen MM and Kari I (1981) β -Carbolines, psychoactive compounds in the mammalian body. Part II: Effects. *Med Biol* **59**:190–211.
- Aoyama K, Matsubara K, Okada K, Fukushima S, Shimizu K, Yamaguchi S, Uezono T, Satomi M, Hayase N, Ohta S, et al. (2000) *N*-methylation ability for azaheterocyclic amines is higher in Parkinson's disease: nicotinamide loading test. *J Neural Transm* **107**:985–995.
- Beck O and Lundman A (1983) Occurrence of 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-beta-carboline in tissues and body fluids of rat. *Biochem Pharmacol* **32**:1507–1510.
- Boeira JM, Viana AF, Picada JN, and Henriques JA (2002) Genotoxic and recombinogenic activities of the two beta-carboline alkaloids harman and harmine in *Saccharomyces cerevisiae*. *Mutat Res* **500**:39–48.
- Budavri S, O'Neil MJ, Smith A, Heckelman PE, Kinneary JF, and O'Neill M (1996) *The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals*, 12th ed, CRC Press LLC, Boca Raton, FL.
- Burke MD and Upshall DG (1976) Species and phenobarbitone-induced differences

- in the kinetic constants of liver microsomal harmine *O*-demethylation. *Xenobiotica* **6**:321–328.
- Collins M (2002) Alkaloids, alcohol and Parkinson's disease. *Parkinsonism Relat Disord* **8**:417–422.
- Collins MA and Neafsey EJ (1985) Beta-carboline analogues of N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP): endogenous factors underlying idiopathic parkinsonism? *Neurosci Lett* **55**:179–184.
- Collins MA, Neafsey EJ, Cheng BY, Hurley-Gius K, Ung-Chhun NA, Pronger DA, Christensen MA, and Hurley-Gius D (1987) Endogenous analogs of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: indoleamine derived tetrahydro-beta-carbolines as potential causative factors in Parkinson's disease. *Adv Neurol* **45**:179–182.
- Collins MA, Neafsey EJ, Matsubara K, Cobuzzi R Jr, Albores R Jr, Fields J, and Rollema H (1992) Indole-N-methylation of beta-carbolines: the brain's bioactivation route to toxins in Parkinson's disease? *Ann NY Acad Sci* **648**:263–265.
- Corchero J, Granvil CP, Akiyama TE, Hayhurst GP, Pimprale S, Feigenbaum L, Idle JR, and Gonzalez FJ (2001) The CYP2D6 humanized mouse: effect of the human CYP2D6 transgene and HNF4alpha on the disposition of debrisoquine in the mouse. *Mol Pharmacol* **60**:1260–1267.
- de Meester C (1995) Genotoxic potential of beta-carbolines: a review. *Mutat Res* **339**:139–153.
- Freedland CS and Mansbach RS (1999) Behavioral profile of constituents in ayahuasca, an Amazonian psychoactive plant mixture. *Drug Alcohol Depend* **54**:183–194.
- Fukushima S, Matsubara K, Akane A, and Shiono H (1992) 1-Methyl-tetrahydro-beta-carboline-3-carboxylic acid is present in the rat brain and is not increased after acute ethanol injection with cyanamide treatment. *Alcohol* **9**:31–35.
- Gearhart DA, Collins MA, Lee JM, and Neafsey EJ (2000) Increased beta-carboline 9N-methyltransferase activity in the frontal cortex in Parkinson's disease. *Neurobiol Dis* **7**:201–211.
- Gearhart DA, Neafsey EJ, and Collins MA (2002) Phenylethanolamine N-methyltransferase has beta-carboline 2N-methyltransferase activity: hypothetical relevance to Parkinson's disease. *Neurochem Int* **40**:611–620.
- Gelboin HV, Krausz KW, Gonzalez FJ, and Yang TJ (1999) Inhibitory monoclonal antibodies to human cytochrome P450 enzymes: a new avenue for drug discovery. *Trends Pharmacol Sci* **20**:432–438.
- Glennon RA, Dukat M, Grella B, Hong S, Costantino L, Teitler M, Smith C, Egan C, Davis K, and Mattson MV (2000) Binding of beta-carbolines and related agents at serotonin (5-HT(2)) and 5-HT(1A), dopamine (D(2)) and benzodiazepine receptors. *Drug Alcohol Depend* **60**:121–132.
- Hedlund E, Gustafsson JA, and Warner M (2001) Cytochrome P450 in the brain; a review. *Curr Drug Metab* **2**:245–263.
- Herraz T (2002) Identification and occurrence of the bioactive beta-carbolines norharman and harman in coffee brews. *Food Addit Contam* **19**:748–754.
- Husbands SM, Glennon RA, Gorgerat S, Gough R, Tyacke R, Crosby J, Nutt DJ, Lewis JW, and Hudson AL (2001) Beta-carboline binding to imidazoline receptors. *Drug Alcohol Depend* **64**:203–208.
- Iurlo M, Leone G, Schilström B, Linner L, Nomikos G, Hertel P, Silvestrini B, and Svensson H (2001) Effects of harmine on dopamine output and metabolism in rat striatum: role of monoamine oxidase-A inhibition. *Psychopharmacology (Berl)* **159**:98–104.
- Kim H, Sablin SO, and Ramsay RR (1997) Inhibition of monoamine oxidase A by beta-carboline derivatives. *Arch Biochem Biophys* **337**:137–142.
- Kirtton SB, Kemp CA, Tomkinson NP, St-Gallay S, and Sutcliffe MJ (2002) Impact of incorporating the 2C5 crystal structure into comparative models of cytochrome P450 2D6. *Proteins* **49**:216–231.
- Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ, and Tracy TS (1998) Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry* **37**:4137–4147.
- Krausz KW, Goldfarb I, Butters JT, Yang TJ, Gonzalez FJ, and Gelboin HV (2001) Monoclonal antibodies specific and inhibitory to human cytochromes P450 2C8, 2C9 and 2C19. *Drug Metab Dispos* **29**:1410–1423.
- Lamchouri F, Settaf A, Cherrah Y, El Hamidi M, Thigui N, Lyoussi B, and Hassar M (2002) Experimental toxicity of Peganum harmala seeds. *Ann Pharm Fr* **60**:123–129.
- Langston JW and Ballard P (1984) Parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): implications for treatment and the pathogenesis of Parkinson's disease. *Can J Neurol Sci* **11**:160–165.
- Langston JW and Ballard PA Jr (1983) Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *N Engl J Med* **309**:310.
- Langston JW, Irwin I, Langston EB, and Forno LS (1984) 1-Methyl-4-phenylpyridinium ion (MPP+): identification of a metabolite of MPTP, a toxin selective to the substantia nigra. *Neurosci Lett* **48**:87–92.
- Lutes J, Lorden JF, Beales M, and Oltmans GA (1988) Tolerance to the tremorogenic effects of harmaline: evidence for altered olivo-cerebellar function. *Neuropharmacology* **27**:849–855.
- Mahgoub A, Idle JR, Dring LG, Lancaster R, and Smith RL (1977) Polymorphic hydroxylation of debrisoquine in man. *Lancet* **2**:584–586.
- Matsubara K (1998) [Metabolic activation of azaheterocyclics induced dopaminergic toxicity: possible candidate neurotoxins underlying idiopathic Parkinson's disease]. *Nippon Hoigaku Zasshi* **52**:301–305.
- Matsubara K, Aoyama K, Suno M, and Awaya T (2002) N-methylation underlying Parkinson's disease. *Neurotoxicol Teratol* **24**:593.
- Matsubara K, Collins MA, Akane A, Ikebuchi J, Neafsey EJ, Kagawa M, and Shiono H (1993) Potential bioactivated neurotoxins, N-methylated beta-carbolinium ions, are present in human brain. *Brain Res* **610**:90–96.
- McCann SJ, Pond SM, James KM, and Le Couteur DG (1997) The association between polymorphisms in the cytochrome P-450 2D6 gene and Parkinson's disease: a case-control study and meta-analysis. *J Neurol Sci* **153**:50–53.

- Melchior C and Collins MA (1982) The route and significance of endogenous synthesis of alkaloids in animals. *Crit Rev Toxicol* **9**:313–356.
- Miksys S, Rao Y, Hoffmann E, Mash DC, and Tyndale RF (2002) Regional and cellular expression of CYP2D6 in human brain: higher levels in alcoholics. *J Neurochem* **82**:1376–1387.
- Mulder GJ and Hagedoorn AH (1974) UDP glucuronyltransferase and phenolsulfo-transferase in vivo and in vitro. Conjugation of harmol and harmalol. *Biochem Pharmacol* **23**:2101–2109.
- Omura T and Sato R (1964) The carbon-monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**:2370–2378.
- Pfau W and Marquardt H (2001) Cell transformation in vitro by food-derived heterocyclic amines Trp-P-1, Trp-P-2 and N(2)-OH-PhIP. *Toxicology* **166**:25–30.
- Picada JN, da Silva KV, Erdtmann B, Henriques AT, and Henriques JA (1997) Genotoxic effects of structurally related beta-carboline alkaloids. *Mutat Res* **379**:135–149.
- Raza H, King RS, Squires RB, Guengerich FP, Miller DW, Freeman JP, Lang NP, and Kadlubar FF (1996) Metabolism of 2-amino-alpha-carboline. A food-borne heterocyclic amine mutagen and carcinogen by human and rodent liver microsomes and by human cytochrome P4501A2. *Drug Metab Dispos* **24**:395–400.
- Riedl AG, Watts PM, Jenner P, and Marsden CD (1998) P450 enzymes and Parkinson's disease: the story so far. *Mov Disord* **13**:212–220.
- Ruiz-Durantez E, Ruiz-Ortega JA, Pineda J, and Ugedo L (2001) Stimulatory effect of harmaline and other beta-carbolines on locus coeruleus neurons in anaesthetized rats. *Neurosci Lett* **308**:197–200.
- Shou M, Lin Y, Lu P, Tang C, Mei Q, Cui D, Tang W, Ngui JS, Lin CC, Singh R, et al. (2001) Enzyme kinetics of cytochrome P450-mediated reactions. *Curr Drug Metab* **2**:17–36.
- Slotkin T and DiStefano V (1970a) Urinary metabolites of harmine in the rat and their inhibition of monoamine oxidase. *Biochem Pharmacol* **19**:125–131.
- Slotkin TA and DiStefano V (1970b) A model of harmine metabolism in the rat. *J Pharmacol Exp Ther* **174**:456–462.
- Slotkin TA, DiStefano V, and Au WY (1970) Blood levels and urinary excretion of harmine and its metabolites in man and rats. *J Pharmacol Exp Ther* **173**:26–30.
- Strobel HW, Thompson CM, and Antonovic L (2001) Cytochromes P450 in brain: function and significance. *Curr Drug Metab* **2**:199–214.
- Tan TM, Sit KH, and Wong KP (1990) UDP-glucuronyltransferase activity toward harmol in human liver and human fetal liver cells in culture. *Anal Biochem* **185**:44–50.
- Tweedie DJ and Burke MD (1987) Metabolism of the beta-carbolines, harmine and harmol, by liver microsomes from phenobarbitone- or 3-methylcholanthrene-treated mice. Identification and quantitation of two novel harmine metabolites. *Drug Metab Dispos* **15**:74–81.
- Zetler G, Back G, and Iven H (1974) Pharmacokinetics in the rat of the hallucinogenic alkaloids harmine and harmaline. *Naunyn-Schmiedeberg's Arch Pharmacol* **285**:273–292.

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