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0022-3565/96/2772-0595$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 277, No. 2
Printed in U.S.A.

JPET 277:595–603, 1996

Selective Mechanism-Based Inactivation of Rat CYP2D by 4-Allyloxymethamphetamine

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Accepted for publication December 14, 1995

ABSTRACT

The high selectivity of amphetamine and its derivatives for CYP2D-mediated oxidations suggested the use of the phenylisopropylamine skeleton as a template for a selective inhibitor of this important enzyme. Accordingly, 4-allyloxymethamphetamine (ALLMA) was synthesized and its ability to selectively inactivate CYP2D was investigated both in *in vitro* and *in vivo* experiments. Incubation studies with rat liver microsomes demonstrated that this compound suppressed the CYP2D-mediated methylenedioxymethamphetamine (MDMA) demethylation in a time- and dose-dependent manner and that the inhibition required the presence of NADPH. The development of irreversible inhibition was associated with oxidation at position 4 of the aromatic ring, the common site of CYP2D-mediated oxidation of this group of compounds. In *in vivo* studies, single doses of ALLMA (1–10 mg/kg) were administered to adult male Sprague-Dawley rats and liver microsomes were obtained 3 hr later. Methamphetamine *p*-hydroxylation and low K_m MDMA demethylation activities, both mediated by CYP2D, were reduced by more than 80% after a dose of 10 mg/kg. Cytochrome P-450 reactions attributed to P-450s other than CYP2D, such as aniline *p*-hydroxylation, the high K_m system of MDMA demethylation and the N-demethylation of methamphetamine, benzphetamine, aminopyrine and erythromycin, all appeared to be minimally affected. The importance of aromatic ring oxidation in the metabolism is such that inhibition of CYP2D would be expected to cause a significant change in the pharmacokinetics of these compounds. The kinetics of MDMA metabolic activity in microsomes from ALLMA-pretreated rats were comparable to those from female Dark-Agouti rats, an animal model for CYP2D1 deficiency.

The CYP2D subfamily of cytochrome P-450s is important for the metabolism of drugs containing a basic functionality, such as the amphetamines (Kumagai *et al.*, 1994; Law and Moody, 1994; Lin *et al.*, 1995), β-blockers (Lennard *et al.*, 1986; Otton *et al.*, 1988; Suzuki *et al.*, 1992; Masubuchi *et al.*, 1993) and antidepressants (Coutts, 1994; Brosen and Gram, 1989), and thus may play a critical role in the toxicity or therapeutic action of these compounds. One of the unique features of the human form of CYP2D is its polymorphism which is expressed as a deficiency in certain individuals (Eichelbaum and Gross, 1990; Meyer *et al.*, 1990; Gonzalez and Meyer, 1991). These individuals, called poor metabolizers, are more sensitive to CYP2D substrates, an effect attributable to the increased bioavailability of a given dose. The importance of this subfamily suggested that a selective and irreversible inhibitor might be a useful probe to investigate the turnover and the participation of CYP2D in selective metabolic pathways.

Prior studies have indicated that the amphetamine structure (phenylisopropylamine) has a relatively high and selective affinity for this enzyme subfamily, as amphetamine (Law and Moody, 1994), 4-methoxyamphetamine (Kitchen *et al.*, 1979), MDMA (Kumagai *et al.*, 1994) and MeAmp (Lin *et al.*, 1995) all are substrates for CYP2D and undergo oxidation on the aromatic ring. Moreover, the metabolism of these compounds by CYP2D is the dominant pathway at low substrate concentration. These observations suggested that an inactivating function attached to a phenylisopropylamine skeleton might be a potential starting point in the development of an irreversible inhibitor of CYP2D. The present report describes initial studies of ALLMA, containing a MeAmp skeleton with an allyloxy function on the aromatic ring (fig. 1), as a CYP2D mechanism-based inhibitor. The selection was based on the finding that oxidation of substituents on position 4 of the aromatic ring was the common CYP2D reaction for these phenylisopropylamines, and that the N-methyl function appeared to confer a low K_m value (Kumagai *et al.*, 1994; Lin *et al.*, 1995). The allyl function has been shown to inhibit cytochrome P-450 in other structures such as diallylsulfide (Brady *et al.*, 1991) and allylisopropylacet-

Received for publication July 25, 1995.
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ABBREVIATIONS: MDMA, methylenedioxymethamphetamine; MeAmp, methamphetamine; ALLMA, 4-allyloxymethamphetamine; DHMA, dihydroxymethamphetamine; *p*-OH-MeAmp, *p*-hydroxymethamphetamine; SD, Sprague-Dawley; DA, Dark Agouti; PCA, perchloric acid; SOD, superoxide dismutase; GC, gas chromatography; MS, mass spectrometry; FID, flame ionization detector; TFAA, trifluoroacetic anhydride; TFA, trifluoroacetic acid.

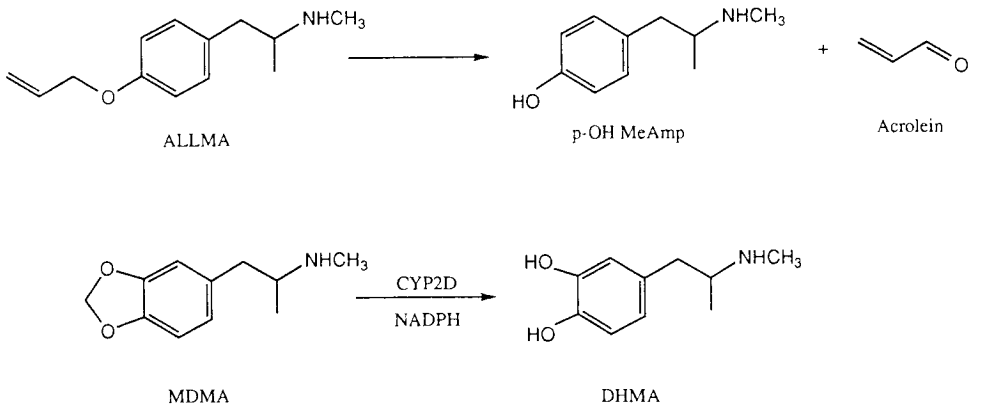

Fig. 1. Structures of ALLMA and MDMA and their CYP2D metabolites.

amide (Ortiz de Montellano *et al.*, 1984; Levin *et al.*, 1972) through metabolic activation.

Materials and Methods

Chemicals. MDMA and (+)-MeAmp were obtained from the Research Technology Branch of The National Institute on Drug Abuse (Rockville, MD). (−)-MeAmp was prepared from (−)-ephedrine by the procedure described by Kishi *et al.* (1983). DHMA was synthesized according to the method of Smissman and Borchardt (1971). Quinine was obtained from the Aldrich Chemical Co. Inc. (Milwaukee, WI). Benzphetamine, bufuralol and 4-hydroxyphentermine were gifts from the Upjohn Co. (Kalamazoo, MI), Hoffmann-La Roche (Nutley, NJ) and Pennwalt Co. (Rochester, NY), respectively. All other chemicals used were obtained from common commercial sources.

Synthesis of (±)-ALLMA. 4-Allyloxybenzaldehyde was prepared by refluxing equimolar (0.05 mol) amounts of 4-hydroxybenzaldehyde and allylbromide with 6.9 g of potassium carbonate in 25 ml of acetone for 5 hr. The reaction mixture was poured into 100 ml of water and the product was extracted from the aqueous mixture with ether. The ether extracts were combined, dried (K_2CO_3), then evaporated to yield 3 g of a white solid which was characterized by its NMR spectrum. Two g of the 4-allyloxybenzaldehyde was then converted to the corresponding nitrostyrene by condensation with nitroethane by refluxing 1 hr in 5 ml of nitroethane containing 1 g of ammonium acetate. The reaction mixture was then poured onto ice and the mixture was extracted with ether. The ether extract was evaporated and the residue was passed through a silica gel column and eluted with dichloromethane. The eluate, upon evaporation, was a dark yellow solid, characterized as the nitrostyrene by its IR and NMR spectrum. Next, 3 g (0.0137 mol) of the nitrostyrene in ether (10 ml) was added dropwise to an ether solution of lithium aluminum hydride (2.2 g, 0.058 mol), after which the mixture was stirred at room temperature for 2 hr. The excess hydride was decomposed by the slow addition of $MgSO_4$-$10H_2O$. The product was extracted with ether and the product was precipitated by addition of ethereal HCl to the extract. The crude hydrochloride obtained was recrystallized from a methanol-ether mixture.

The 4-allyloxy amphetamine obtained above (0.5 g) was heated in ethyl formate (65°C) overnight. The reaction mixture was evaporated and the residue was purified by passage through a silica gel column and elution with 5% methanol in dichloromethane. The formamide obtained, a clear oil, was then reduced with lithium aluminum hydride as described above and was converted to its HCl salt by solution in ether followed by the addition of HCl. Calculated for $C_{13}H_{20}NOCl$: C, 64.58; H, 8.33; N, 5.79. Found: C, 64.84; H, 8.46; N, 5.73. MS: (EI of TFA derivative) *m/z* 301.

Synthesis of (±)-*p*-OH-MeAmp. 4-Hydroxymethamphetamine and its deuterium-substituted analog were synthesized from 4-benzylnitrostyrene, prepared by condensing 4-benzyloxybenzaldehyde with nitroethane as described above. The nitrostyrene was reduced with lithium aluminum hydride to (±)-4-benzyloxyamphetamine. This product was N-formylated with ethyl formate as described above and the formyl group was reduced to the N-methyl group with lithium aluminum hydride to generate 4-benzyloxymethamphetamine. The 4-benzyloxy group was removed by catalytic hydrogenation (Pd/Charcoal). 4-Hydroxy-N-$^2H_2$-methyl amphetamine (d_2-*p*-OH-MeAmp) was prepared by the same sequence except that $LiAl^2H_4$ was used to reduce the formyl function. The products were identified by mass spectral analysis of their O-, N-ditriflouroacetyl derivatives.

Hepatic microsomal preparation. Male SD and female DA rats (2 months of age), obtained from Bantin-Kingman (Fremont, CA), were used for the preparation of hepatic microsomes. Hepatic microsomes were isolated from the 9,000 × *g* supernatant fraction of liver homogenate by centrifugation at 105,000 × *g* for 60 min. The microsomal pellet thus obtained was resuspended in 0.1 M pyrophosphate buffer (pH 7.4) and again was centrifuged at 105,000 × *g* for 30 min as described previously (Kumagai *et al.*, 1994). The resulting pellets were stored at −80°C and resuspended in 1.15% KCl (w/v) before assay.

Inactivation of cytochromes P-450 by ALLMA *in vivo*. Rats were injected i.p. with ALLMA at a dose of 1 to 10 mg/kg and sacrificed by decapitation 3 hr later. Liver microsomes were prepared as described above and examined for changes in protein and cytochrome P-450 content as well as cytochrome P-450 catalytic activities.

Inactivation of P-450s by ALLMA *in vitro*. Two-stage incubation methods were conducted to examine the effect of ALLMA on cytochrome P-450 catalyzed reactions *in vitro*. Unless stated otherwise, the first incubation contained the following components: ALLMA, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.6), an NADPH generating system (0.5 mM $NADP^+$, 8 mM glucose-6-phosphate, 5 mM magnesium chloride and 1 U of glucose-6-phosphate dehydrogenase), and microsomes (1–2 mg of protein per ml of incubation mixture). The reaction was conducted at 37°C for 0 to 30 min in a total volume of 1 ml for the dilution method (Brady *et al.*, 1987) or 10 ml for the centrifugation method. In the former method, aliquots of the incubation mixture were diluted by a factor of 10 in the second stage incubation (catalytic assay), whereas in the latter method, microsomal proteins from the first incubation were washed by centrifugation at 105,000 × *g* for 60 min, followed by resuspension and recentrifugation. The microsomal pellet was then resuspended in 1.15% KCl (w/v) and used in the second stage reaction to determine the changes in the cytochrome P-450 catalytic activities. Aliquots from the first incubation mixture were quenched with PCA and analyzed for the amount of ALLMA remaining and *p*-OH-MeAmp formed. The competitive inhibition capability of ALLMA over the range of 0.1 to 3 μM also was determined to assess inhibition by ALLMA carried over in the dilution process.

Enzymatic assays. The incubation mixture consisted of the same concentration of buffer and cofactors as described above with the substrate under consideration in a final volume of 1 ml. SOD (100 U) was added to the MDMA incubation mixtures to prevent further oxidation of its catechol metabolite to *o*-quinone (Hiramatsu *et al.*, 1990). At the end of the incubation period, the reactions were terminated by the addition of PCA to a final concentration of 2.5%. A substrate concentration of 10 μM was used in the MDMA demethylation reaction, unless otherwise noted. The reaction was carried out for 5 min and the amount of DHMA formed was monitored by high-pressure liquid chromatography-electrochemical detection under the conditions described previously (Kumagai *et al.*, 1994). Aminopyrine (5 mM), benzphetamine (1 mM) and erythromycin (0.4 mM) were incubated for 10 min and N-demethylation was assessed by the formaldehyde produced using the method of Nash (1953). *p*-Hydroxylation of aniline (8 mM) was carried out for 20 min and was determined by the method of Imai *et al.* (1966). MeAmp was incubated for 5 min and its N-demethylation and *p*-hydroxylation products were measured by GC-MS as described elsewhere (Hiratsuka *et al.*, 1995). All incubations were conducted at 37°C.

Analysis of ALLMA and *p*-OH-MeAmp

ALLMA and one of its metabolites, *p*-OH-MeAmp, were extracted and converted to their trifluoroacetyl amide and amide-ester derivatives, respectively, for GC/FID and GC/MS based analysis. In the analysis, the ALLMA incubation mixture was quenched with 0.5 ml of 7.5% PCA after a fixed incubation time and 10 nmol of 4-hydroxyphentermine and 5 nmol of d_2-*p*-OH MeAmp were added as the internal standards for ALLMA and *p*-OH-MeAmp, respectively.

Extraction for ALLMA. The reaction mixture was alkalinized with 1.2 ml of 10% sodium carbonate and extracted with 5 ml of dichloromethane. The upper aqueous layer was extracted further for *p*-OH-MeAmp as described below. The dichloromethane layer was evaporated under a stream of nitrogen to 100 μl and then treated with 100 μl of TFAA at 60°C for 15 min. The excess TFAA was removed by allowing evaporation in a fume hood. The residue was reconstituted in 60 μl of anhydrous acetonitrile and a 2-μl aliquot was injected into the GC equipped with a FID for analysis of ALLMA.

Extraction for *p*-OH-MeAmp. The aqueous layer was re-extracted with 5 ml of isopropanol-dichloromethane (1:4) after addition of 1.2 g of sodium chloride. This more polar solvent mixture provides better recoveries of *p*-OH-MeAmp. The organic layer obtained was evaporated to dryness under a stream of nitrogen, the residue was reconstituted in 50 μl of acetonitrile, then treated with 100 μl of TFAA for 15 min at 60°C. The residual TFAA was removed by evaporation under nitrogen and the residue was reconstituted in 80 to 120 μl of acetonitrile. A 1-μl aliquot of the acetonitrile solution was injected into the GC/MS system.

GC conditions for ALLMA analysis. Analysis of ALLMA (as its TFA derivative) was performed on a Hewlett Packard 5890GC equipped with a Hewlett-Packard fused silica capillary column with cross-linked methyl silicone, 0.32 mm (inside), 0.52 μm and 12 m. The GC temperature program began at 60°C and was increased to 190°C at a rate of 10°C/min. The flow rate was 4.3 ml/min. Under these conditions, the retention times for the TFA derivatives of 4-hydroxyphentermine (internal standard) and ALLMA were 6.72 and 12.49 min, respectively.

GC/MS conditions for *p*-OH-MeAmp analysis. A HP 5971A GC/MS system, equipped as described above, was used to measure *p*-OH-MeAmp (as the TFA derivative). The mass spectrometer was operated in its specific ion detection mode and analyte was quantitated by comparing the response at *m/z* 153.95 (for the TFA derivative of *p*-OH-MeAmp) and *m/z* 156.00 (for the TFA derivative of the internal standard, d_2-*p*-OH-MeAmp). The temperature profile was similar to that for the GC-FID system, starting at 65°C and increasing to 195°C at a rate of 25°C/min. Under these conditions, the retention time of the *p*-OH-MeAmp N-, O-bis TFA derivative was 5.6 min.

Other assays. Protein concentration was determined by the method of Bradford (1976), using bovin serum albumin as the reference standard. Cytochrome P-450 content was measured spectrally by the method of Omura and Sato (1964).

Results

***In vitro* characterization of CYP2D inactivation by ALLMA.** Initial studies examining the inhibitory properties of ALLMA utilized the MDMA demethylation reaction (MDMA to DHMA, fig. 1) in rat liver microsomes. Prior studies (Kumagai *et al.*, 1994) had shown this reaction to be catalyzed by members of the cytochrome P-450 CYP2D subfamily with a K_m value of 1 to 3 μM. The reaction also can be catalyzed by cytochrome P-450 isoforms of the CYP2B subfamily, but with a K_m value in the millimolar range. Accordingly, a concentration of 10 μM MDMA was used for studies of CYP2D-mediated reaction.

Two protocols were used to expose the microsomes to ALLMA and to assess microsomal activity in the absence, or at minimal concentrations, of residual inhibitor. In the first protocol, microsomes, fortified with an NADPH generating system, were incubated with ALLMA at an initial concentration of 10 μM for 30 min. After exposure, the microsomes were washed by centrifugation, resuspension and recentrifugation to remove residual ALLMA. The resulting pellet was then resuspended and examined for changes in MDMA demethylation. As shown in figure 2, preincubation with ALLMA reduced low concentration (2–50 μM) MDMA demethylation without affecting the reaction occurring at higher concentrations. A nonselective autoinactivation by the NADPH generating system was observed when the microsomes were incubated with the NADPH generating system alone. When the NADPH generating system was excluded during the first incubation with ALLMA, the MDMA demethylation activity

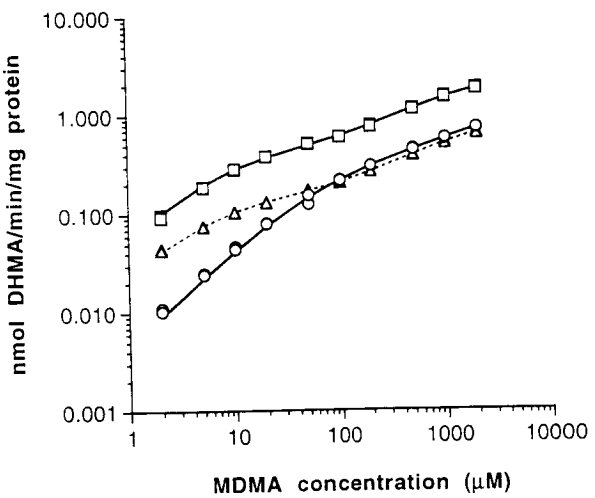

Fig. 2. Effect of ALLMA (10 μM) on the kinetics of MDMA demethylation in untreated rat liver microsomes. Untreated rat liver microsomes were preincubated at 37°C for 30 min under conditions listed below. The microsomes were then washed by centrifugation and the pellets obtained were then assayed in duplicate for MDMA demethylenase activity over a substrate concentration ranging from 2 μM to 2 mM. □, preincubated with ALLMA. △, preincubated with NADPH. ○, preincubated with both ALLMA and NADPH. Individual data points are shown with lines representing the predicted values, obtained by nonlinear regression procedures described under "Methods."

was identical to a control incubated in the absence of ALLMA and the NADPH generating system.

The second protocol for evaluation of ALLMA activity utilized a preincubation, as described above, followed by removal of an aliquot of the incubation mixture, dilution by 10-fold and evaluation of demethylation in a second incubation. This procedure has been used in studies with phencyclidine (Brady *et al.*, 1987), and was used here to determine the temporal effects of ALLMA. Figure 3 shows the time course of ALLMA metabolism during the preincubation period and the associated loss of MDMA (10 μM) demethylation activity, determined in the second stage incubation mixture. There was no significant consumption of ALLMA during a 30-min incubation in the absence of an NADPH generating system. Quinine, a selective rat CYP2D inhibitor, was coincubated with ALLMA to determine the role of CYP2D in ALLMA metabolism. Although the formation of *p*-OH-MeAmp was blocked completely, some ALLMA consumption occurred (fig. 3A).

The loss of demethylation caused by ALLMA was dependent on the time of preincubation and, as shown in figure 3B, more than 85% of control CYP2D activity was lost in 12 min. However, about 48% of the CYP2D activity also is lost by an NADPH-dependent autoinactivation process over a 30-min period. This inactivation was not affected by agents that reduced active oxygen species as 100 U of SOD reduced the loss of activity by only about 10% from the loss and catalase (300 U) had no significant effect (data not shown).

Next, a concentration-effect study was performed in which ALLMA consumption, *p*-OH-MeAmp formation and CYP2D inactivation were determined after incubating various concentrations of ALLMA with microsomes for 10 min. At this time point, ALLMA-dependent reduction of CYP2D activity could be observed with minimal autoinactivation. The formation of *p*-OH-MeAmp and the inactivation of MDMA demethylation both reached a plateau at ALLMA concentrations between 15 to 20 μM, whereas overall ALLMA consumption continued to rise, presumably because of other metabolic pathways not dependent on CYP2D (fig. 4). Thus, under these conditions, the enzyme catalyzing *p*-OH-MeAmp formation appeared to be inhibited, whereas other pathways of ALLMA metabolism remain functional.

Experiments were then performed to assess overall ALLMA metabolism and the selectivity of the loss in cytochrome P-450 activity. Over 88% of the added ALLMA (15 nmol/ml) were consumed after a 10-min incubation with 1 mg/ml of microsomal protein. As shown in table 1, the drop in both ALLMA consumption and *p*-OH-MeAmp formation upon addition of a CYP2D inhibitor (quinine) or substrate (bufuralol) appeared to be stoichiometrically equivalent within experimental error, suggesting that *p*-OH-MeAmp was probably the only CYP2D product formed. Inasmuch as only 38 and 41% of the total ALLMA consumption was accounted for by the formation of *p*-OH-MeAmp in male and female microsomes, respectively, the consumption of ALLMA was not due solely to CYP2D catalysis. The data also suggested that a male-predominant enzyme is involved in the metabolism of ALLMA by other pathway(s), because the formation of *p*-OH-MeAmp showed no sex-related differences. Metabolism of ALLMA *via* N-demethylation was examined because sex-related differences, favoring the male, have been observed in this reaction with several other MeAmp derivatives (unpublished observations). The primary N-demethylation product, 4-allyloxyamphetamine, was detected by GC; however, quantitation of this metabolite has not been successful because of the interference of another signal. Thus, it is not clear at this point whether pathways other than N-demethylation and *p*-OH-MeAmp formation are contributing to the consumption of ALLMA.

Incubations examining the protective effect of bufuralol

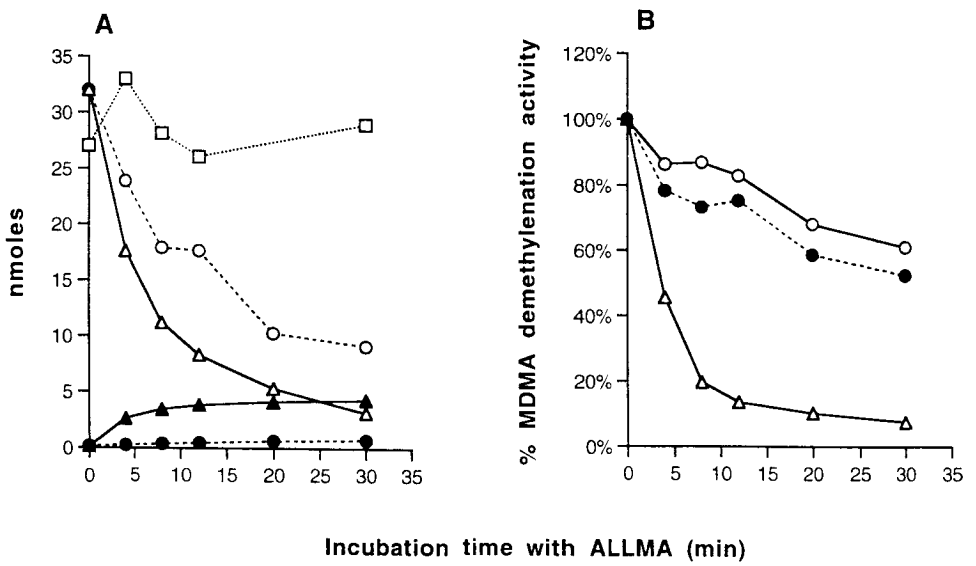

Fig. 3. Time course of ALLMA metabolism and its effect on MDMA demethylation in rat liver microsomes. A, ALLMA consumption and *p*-OH-MeAmp formation ALLMA (30 μM) were incubated with rat liver microsomes at 37°C for time intervals of 0 to 30 min under conditions described under "Materials and Methods." Aliquots of the incubation mixture were then analyzed for ALLMA consumption (○, □ and △) and *p*-OH-MeAmp formation (● and ▲) in the absence (▲/solid lines) and presence (○ and ●/dashed lines) of 30 μM quinine. □, the levels of ALLMA remaining when incubated in the absence of NADPH. Each point is the average of two determinations. B, the time-dependent inactivation of MDMA (10 μM) demethylation activity. Demethylation activity was determined in aliquots of the incubation mixture after dilution of an aliquot 10-fold using procedures described under "Methods." ●, reincubation mixture contained in NADPH. ○, preincubation mixture contained in NADPH and SOD. △, preincubation mixture contained in NADPH, SOD and ALLMA. Each point is the average of two determinations.

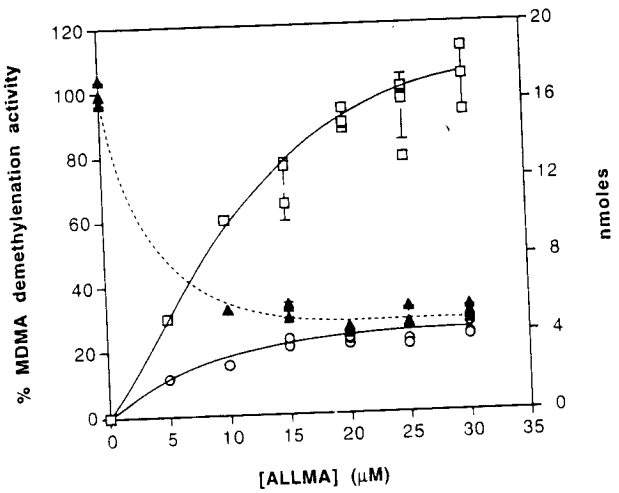

Fig. 4. Dose-dependent ALLMA metabolism and its effect on MDMA demethylation. Rat liver microsomes were preincubated with 5 to 30 μM of ALLMA at 37°C for 10 min, the mixture was diluted 10-fold, then assayed for MDMA (10 μM) demethylation activity. □, nanomoles of ALLMA consumed; ○, nanomoles of *p*-OH-MeAmp formed; ▲, percentage of MDMA demethylenase activity.

were carried out also. Bufuralol is a CYP2D substrate and will inhibit the oxidation of ALLMA by CYP2D competitively. It also is removed more readily than quinine by washing. A 1-ml aliquot of each 10 ml of reaction mixture was used to determine the level of ALLMA remaining and the *p*-OH-MeAmp formed (table 1). The remaining microsomes were centrifuged, washed twice and examined further for the protection effect of bufuralol against suppression of CYP2D activity by ALLMA and the specificity of the inhibition. As shown in figure 5, the suppression of MDMA demethylation was only 21 ± 11% in the presence of 30 μM bufuralol, compared to 73 ± 5% in its absence. Under conditions in which ALLMA reduced CYP2D activity by 73%, benzphetamine N-demethylation, catalyzed mainly by CYP2C11 and CYP2B1 (Ryan and Levin, 1990; Soucek and Gut, 1992), and aniline *p*-hydroxylation, catalyzed by CYP1A and CYP2E1 (Ryan and Levin, 1990; Soucek and Gut, 1992), were reduced by 13 ± 4 and 17 ± 7%, respectively. Both inhibitions were apparently reversed by 30 μM bufuralol.

Selectivity of the inactivation of CYP2D *in vivo*. To assess the *in vivo* effects of ALLMA, male SD rats were given i.p. doses of 1, 5 and 10 mg/kg of ALLMA and were sacrificed 3 hr later. The effect of ALLMA on the catalytic activity of CYP2D and other cytochrome P-450 enzymes in liver microsomes was then determined (table 2). ALLMA produced a dose-dependent inhibition of low K_m MDMA demethylation and, at a dose of 10 mg/kg, suppressed MeAmp *p*-hydroxylation to levels comparable to the reduction of MDMA demethylation. The other reactions examined appeared to be refractory to inhibition or, in the cases of aminopyrine and erythromycin N-demethylation, considerably less sensitive.

A dose-dependent increase in the protein level per gram of microsome pellet was observed. The total cytochrome P-450 content per gram of microsomal pellet also was elevated after an ALLMA dose larger than 1 mg/kg (table 3). There were, however, no significant changes in either the liver weight per animal or in the weight of microsomal pellet obtained per gram of liver tissue after the treatment.

The kinetics of MDMA demethylation in microsomes obtained from untreated and ALLMA-pretreated male SD rats were compared to those of untreated female DA rats, a rat strain generally used as a CYP2D-deficient model (Al-Dabbagh *et al.*, 1981; Suzuki *et al.*, 1991; Masubuchi *et al.*, 1991b; Law and Moody, 1994). Prior studies from this laboratory indicated that MDMA demethylation exhibited two-site kinetics consistent with the presence of high-affinity and low-affinity sites of oxidation (Kumagai *et al.*, 1994). A comparison of the kinetic parameters of the three sets of microsomes is shown in table 4; in the analysis, the model reflecting changes in V_{max} only was tested, because irreversible inhibition by a catalytic inhibitor should cause only a reduction of V_{max}. Thus, the data from the three groups of rat livers were pooled and analyzed by nonlinear regression procedures for fit to a model describing three groups of enzymes with common K_m values but differing in V_{max}. The results showed that the V_{max} values for the high-affinity (low K_m) site were 8- and 35-fold lower in the DA rat and ALLMA-pretreated rat, respectively, when compared to that of untreated SD rats. DA rats exhibit a 1.8-fold lower V_{max} value for the high K_m system, but there was no significant difference between the ALLMA-pretreated and untreated SD rat microsomes. The data obtained are presented as Eadie-Hofstee plots (fig. 6) with the lines representing the values predicted by the model.

Discussion

The results presented in this paper showed that ALLMA is converted to an irreversible inhibitor by a CYP2D- and

TABLE 1

Effects of quinine and bufuralol on the metabolism of ALLMA

Quinine (1 and 5 μM) was coincubated with 15 μM ALLMA in a 1-ml reaction mixture for 10 min at 37°C to examine its effect on ALLMA metabolism by male and female SD rat liver microsomes. Bufuralol (30 μM) and ALLMA (15 μM) were incubated under the same conditions except that a 10-ml reaction mixture and only male SD liver microsomes were used. The microsomal proteins were separated and washed twice by centrifugation and then assayed for cytochrome P450 activities (results shown in fig. 5). Each value is the mean ± S.D. of triplicate incubations expressed as nanomoles per milligram of protein per 10 min. The differences between male *vs.* female activity are noted as * for $P < .05$ and ** for $P < .01$.

	ALLMA Consumption	Decrease in Consumption	*p*-OH-MeAmp Formation	Decrease in Formation
Male			5.66 ± 0.80	
Control	14.83 ± 0.51		3.43 ± 0.80	2.23 ± 0.99
Quinine, 1 μM	13.35 ± 1.32	1.48 ± 1.21	1.42 ± 0.21	4.25 ± 0.76
Quinine, 5 μM	10.23 ± 1.19	4.60 ± 1.11	2.11 ± 0.78	3.55 ± 0.95
Bufuralol, 30 μM	10.27 ± 0.66	4.56 ± 0.69		
Female			5.15 ± 0.17	
Control	12.48 ± 0.03**		3.02 ± 0.04	2.13 ± 0.15
Quinine, 1 μM	10.12 ± 0.41*	2.36 ± 0.37	1.12 ± 0.18	4.03 ± 0.21
Quinine, 5 μM	5.08 ± 2.80*	7.40 ± 2.17		

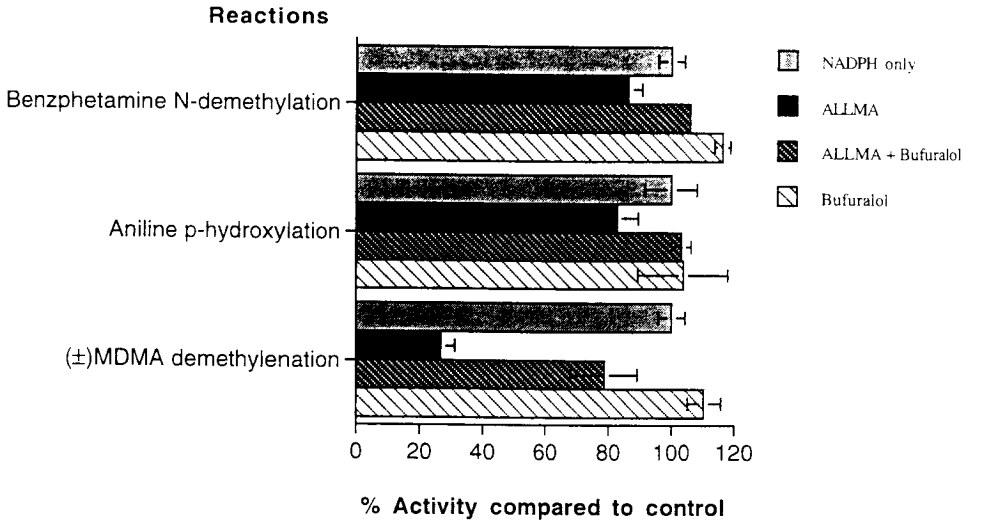

Fig. 5. Effect of bufuralol on the inhibition effect of ALLMA. Male SD rat liver microsomes were preincubated with 15 μM ALLMA in the presence or absence of 30 μM bufuralol, according to the conditions described in table 1. The reactions examined were carried out under conditions described under "Materials and Methods." Each value is the mean ± S.D. of three to four samples expressed as the percentage of activity compared to that of control microsomes, in which the first incubation was conducted in the absence of both ALLMA and bufuralol. The control activities were 2.33 ± 0.19, 22.15 ± 0.94 and 0.21 ± 0.01 nmol of product per min/mg of protein for aniline, benzphetamine and MDMA, respectively. An additional set of controls with bufuralol replacing ALLMA in the first incubation was carried out to assess the effect of bufuralol alone.

TABLE 2

Selective inhibition of rat liver microsome activities by ALLMA pretreatment

ALLMA was administered to male SD rats *via* i.p. injection at doses ranging from 1 to 10 mg/kg. Liver microsomes were prepared after 3 hr and examined for metabolic activities. Each value is the mean ± S.D. of the number of determinations indicated in parentheses or as results from duplicates. ND, not determined.

Reaction	[S]	P-450 Enzymes Involved	Dose of Allyloxymethamphetamine (i.p.)		
			1 mg/kg	5 mg/kg	10 mg/kg
			Activity compared to control microsomes[a]		
	mM				
Aniline *p*-hydroxylation	8	CYPA1/2, CYP2E1	102.1 ± 6.4% (*n* = 4)	88.3 ± 7.7% (*n* = 4)	151.5 ± 7.8% (*n* = 4)
Aminopyrine N-demethylation	5	CYP2B1	81.0 ± 6.3% (*n* = 4)	90.3 ± 8.2% (*n* = 4)	74.0 ± 2.1% (*n* = 4)
Benzphetamine N-demethylation	1	CYP2B1, CYP2C11	97.2 ± 31.7% (*n* = 10)	110.1 ± 13.8% (*n* = 11)	108.4 ± 3.4% (*n* = 12)
Erythromycin N-demethylation	0.4	CYP3A	80.3 ± 3.4% (*n* = 4)	66.5 ± 11.9% (*n* = 4)	83.9 ± 4.0% (*n* = 4)
MDMA demethylenation	0.01	CYP2D	77.1 ± 8.6% (*n* = 13)	23.9 ± 4.2% (*n* = 13)	14.4 ± 0.6% (*n* = 4)
	10	CYP2B1/2	92.4 ± 3.2% (*n* = 9)	78.4 ± 4.3% (*n* = 9)	101.0 ± 2.2% (*n* = 4)
MeAmp *p*-hydroxylation	0.01 (+)	CYP2D	ND	ND	19.1%, 17.7%
	0.01 (−)	CYP2D	ND	ND	20.0%, 20.9%
MeAmp N-demethylation	0.01 (+)	CYP2B, CYP2C11, FMO	ND	ND	155.1%, 151.0%
	0.01 (−)	CYP2B, CYP2C11	ND	ND	157.4%, 161.7%

[a] From rats injected with 0.9% saline.

NADPH-dependent process, which also is dependent upon incubation time and ALLMA concentration. This inactivation process is highly selective for CYP2D and can be blocked by coincubating with another CYP2D substrate, bufuralol. This process appears to be mediated through oxidation at position 4 of the aromatic ring, as the time course of inhibition correlates with the generation of the *p*-OH-MeAmp and the reversible protection by bufuralol, the CYP2D inhibitor, reduces *p*-OH-MeAmp and inhibitory action similarly. However, *p*-OH-MeAmp, at concentrations comparable to those generated here (10 μM), had no effect on MeAmp oxidation (unpublished data), suggesting that the actual inhibitory species is an oxidative cleavage product or a reactive intermediate in the generation of *p*-OH-MeAmp. There still remains, however, the possibility that another metabolite, generated by a completely different pathway, could be responsible for the inhibition.

The slight inhibition of benzphetamine N-demethylation and aniline *p*-hydroxylation observed under these conditions (fig. 5) could be due to the minor role of CYP2D1 in the two reactions, as reported by Soucek and Gut (1992), who described results from reconstituted system studies with purified cytochrome P-450 isoforms.

When administered to intact animals, ALLMA (>1 mg/kg) appeared to increase both protein and total cytochrome P-450 levels. The basis for this increase in such a short period o

TABLE 3

Effect of ALLMA on protein and cytochrome P-450 content

ALLMA was administered to male SD rats *via* i.p. injection at doses of 1, 5 and 10 mg/kg and the level of protein and cytochrome P-450 were examined in liver microsomes prepared 3 hr postinjection. The microsomal pellets were weighed before storage. Control rats were pretreated with saline under the same conditions.

ALLMA	mg Protein/g Microsome	nmol P-450/g Microsome	nmol P-450/mg Protein
Control	34.1	61.5	1.80
1 mg/kg	50.8	52.7	1.04
5 mg/kg	53.9	79.1	1.47
10 mg/kg	74.0	158.2	2.14

time is unclear. However, as the catalytic activities of the microsomes were normalized to protein content, changes in the protein level without changes in the content of the specific cytochrome P-450 enzyme catalyzing the reaction could result in an apparent decrease in activity mediated by that enzyme. This may account for the 10 to 35% drop in aminopyrine and erythromycin N-demethylation activities, because the effects do not appear to be dose-dependent in the manner of the CYP2D-mediated reactions. On the other hand, the activity for MeAmp N-demethylation and aniline *p*-hydroxylation increased with 10 mg/kg of ALLMA pretreatment, which suggested that some of the enzymes (*i.e.*, cytochrome P-450 enzymes or FMO) involved in these reactions may be increased after the treatment. Taken together, the data suggest that, although ALLMA appears to inactivate CYP2D specifically, it also elicits other effects *in vivo* that result in an increase of some P-450s and other proteins as well.

Compounds such as propranolol and phencyclidine have been shown to be activated by CYP2D to form reactive metabolic intermediates that irreversibly bind to microsomal proteins (Owens *et al.*, 1993; Masubuchi *et al.*, 1994) and cause a decrease in CYP2D-mediated reactions (Masubuchi *et al.*, 1991a; Hiratsuka *et al.*, 1995). However, the inactivation of cytochrome P-450 by phencyclidine was rather nonspecific; reactions mediated by CYP2C11 (Hiratsuka *et al.*, 1995) and members of the CYP2B subfamily (Osawa and Coon, 1989; Crowley and Hollenberg, 1992) also were inhibited in the process. Propranolol, on the other hand, appears to resemble ALLMA more in its selective impairment of CYP2D activity after repetitive p.o. administration (Masubuchi *et al.*, 1991a). Furthermore, an increase in the N-desisopropylase activity was observed after propranolol pretreatment.

Several groups have conducted studies to evaluate the relevance of female DA rats as a model for human CYP2D6-poor metabolizers with debrisoquine (Kahn *et al.*, 1985), bufuralol (Boobis *et al.*, 1986) and metoprolol (Barham *et al.*, 1994) as probes. Interstrain differences also were investigated in a number of reactions mediated by other P-450 isoforms (Barham *et al.*, 1994). These findings suggested that although the DA rat may be useful in providing a first indi-

TABLE 4

Kinetic values for (±)-MDMA demethylenation

MDMA demethylenation was examined in liver microsomes obtained from untreated male SD rats (24 data points), ALLMA-pretreated male SD rats (38 data points) and female DA rats (33 data points) over a substrate concentration range from 1 to 10 μM. Reactions were carried out at 37°C for 5 min, as described under "Materials and Methods." All three sets of data were then pooled and analyzed for the best fit of the low- and high-K_m enzyme systems involved and the differences of their V_{max}. The kinetic parameters ± asymptotic S.D. were obtained using a nonlinear regression program (BMDP) after logrithmic transformation.

Microsome Preparation	Low-K_m System		High-K_m System	
	K_m	V_{max}	K_m	V_{max}
	μM	*nmol/min/mg of protein*	*μM*	*nmol/min/mg of protein*
Untreated male SD	1.453 ± 0.302	0.457 ± 0.030	665.804 ± 53.388	2.253 ± 0.142
ALLMA-treated male SD[a]		0.013 ± 0.002		2.484 ± 0.107
Untreated female DA		0.056 ± 0.005		1.277 ± 0.075

[a] Rat liver microsomes prepared from rats pretreated with 10 mg/kg of ALLMA for 3-hr postinjection time point.

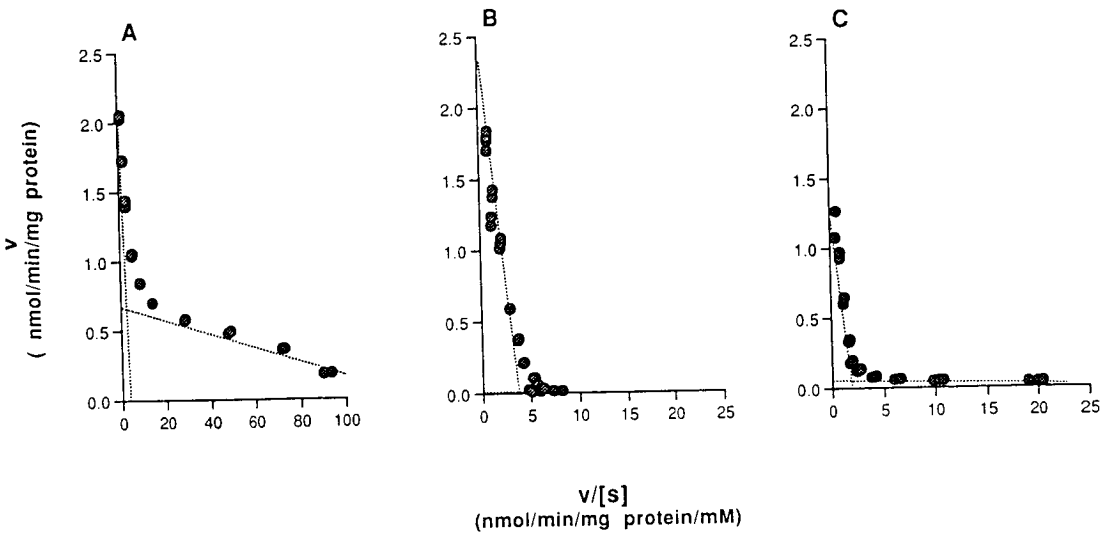

Fig. 6. Eadie-Hofstee Plot for MDMA demethylation by (A) untreated male SD, (B) ALLMA-pretreated male SD and (C) female DA rat liver microsomes. Each point represents the individual value obtained and the dotted lines represent the predicted values as calculated by nonlinear regression.

cation of CYP2D6 substrate, there may be limitations in the interpretation because of interstrain differences observed in cytochrome P-450s from the CYP2C and CYP3A subfamilies. ALLMA-pretreated rats, on the other hand, may be better in evaluating the role of these CYP2D-mediated metabolic pathways, because a direct comparison can be made within the same strain. As shown in figure 6, although the low-K_m component appears to be almost blocked completely in preparations from ALLMA-pretreated SD rats, female DA rats appear to have a low K_m with low capacity component and a high-K_m component which also appears to be different from that of the SD rats.

The inactivation process appears to be mediated through oxidation at position 4 of the aromatic ring, because the formation of *p*-OH-MeAmp from ALLMA correlates with the inhibition observed in the CYP2D-mediated MDMA demethylation, both in terms of the time course and concentration dependency of inactivation. Furthermore, the reversible protection by bufuralol, a CYP2D substrate, reduces *p*-OH-MeAmp and inhibitory action similarly. The inactivation does not appear to be caused by *p*-OH-MeAmp, because, as stated earlier, this compound had no effect on MeAmp oxidation at comparable concentrations. This suggests that the actual inhibiting species is an oxidative cleavage product or a reactive intermediate in the generation of *p*-OH-MeAmp. Acrolein, generated after oxidation of ALLMA by CYP2D to *p*-OH-MeAmp (fig. 1), is one candidate for the inhibiting species. This α,β-unsaturated aldehyde has been shown to react readily with glutathione and thiol-containing enzymes (Esterbauer *et al.*, 1975; Cooper *et al.*, 1992) and causes a decrease the content/activity of cytochrome P-450 both *in vitro* (Gurtoo *et al.*, 1981; Marinello *et al.*, 1984) and *in vivo* (LeBlanc and Waxman, 1990; Cooper *et al.*, 1992). Maximal levels of acrolein, estimated from peak levels of *p*-OH-MeAmp, could be 3 to 4 nmol (see fig. 4) and CYP2D levels in the same mixture were in the range of 0.18 to 0.23 nmol. Thus, a high ratio of acrolein to CYP2D, about 20 to 1, would be generated, allowing even an inefficient reaction to occur with CYP2D. Alternatively, however, a one electron oxidation of the readily oxidized allyloxy group could generate a radical species that directly binds to the enzyme. The chemical details of the inhibitory process thus remain to be elucidated.

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