THE INVOLVEMENT OF CYTOCHROME P450 3A4 IN THE N-DEMETHYLATION OF L-\(\alpha\)-ACETYL METHADOL (LAAM), NORLAAM, AND METHADONE

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ABSTRACT:

The \(N\)-demethylation of LAAM, norLAAM, and methadone has been investigated in human liver microsomes and microsomes containing cDNA-expressed human P450s. Gas chromatography/mass spectrometry methods allowed detection of norLAAM and dinorLAAM formation from LAAM, dinorLAAM formation from norLAAM, and EDDP and EMDP formation from methadone. The rates of \(N\)-demethylation varied 4- to 7-fold in microsomes from four different donors with activities for LAAM and norLAAM consistently greater (5- to 14-fold) than for methadone. The \(N\)-demethylation of LAAM, norLAAM, and methadone were significantly inhibited by ketoconazole. IC\(_{50}\) could be determined for ketoconazole inhibition of LAAM and norLAAM \(N\)-demethylation of 1.6 and 1.1 \(\mu \text{M}\), respectively. The ability of ketoconazole to reduce methadone \(N\)-demethylation below 40% varied in regard to liver donor. No other P450-selective inhibitors reduced the average activities more than 43%. cDNA-expressed P450 3A4 \(N\)-demethylated LAAM, norLAAM, and methadone at greater rates than the other cDNA-expressed P450s studied (1A2, 2C9, 2D6, or 2E1). P450 3A \(N\)-demethylation of LAAM, norLAAM, and methadone exceeded the next most active P450, respectively, by at least 2.5, 9.6, and 13.4 times when expressed per milligram protein and by 18.2, 6.0, and 6.1 times when expressed per nanomole P450. These results suggest that P450 3A4 is the primary site of \(N\)-demethylation of LAAM, norLAAM, and methadone in human liver. Although other enzymes may also be capable of \(N\)-demethylating these compounds, identification of specific enzymes, except P450 3A4, has yet to be established. Knowledge of these enzymatic pathways is essential for assessment of the impact of metabolic drug-drug interactions on therapeutic success and/or adverse events.

Methadone, an opiate receptor agonist, at appropriate doses prevents opiate withdrawal without producing euphoria in the tolerant individual (1, 2). This characteristic of methadone has made it a suitable medication to maintain opiate users while they attempt to terminate opiate abuse. The ability of methadone to prevent withdrawal, however, has a relatively short half-life, requiring daily administration of the drug. This, in turn, requires either daily visits to a treatment facility or disbursement of the medication. The latter is unsuitable for maintenance of drug dependency medications such as methadone. This work was supported in part by US PHS contract N01-DA1–9205.

1 Abbreviations used are: LAAM, \(L\)-\(\alpha\)-acetylmethadol; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EMDP, 2-ethyl-5-methyl-3,3-diphenylpyrrole; DEDTC, diethylidithiocarbamate; P450, cytochrome P450; the P450 nomenclature used is that recommended by the P450 nomenclature Committee (54).

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al. (21) have presented substantive data on P450s involved in methadone N-demethylation, these studies used substrate concentrations much greater than those expected in the clinical setting. As LAAM is a newly approved drug, we felt it important to investigate the prototype drug, methadone, under the same experimental conditions.

Materials and Methods

Reagents. LAAM HCl, LAAM HCl-(2,3,3-heptane-d₃), norLAAM HCl, norLAAM HCl-(acetoxy-d₃), dinorLAAM HCl, dinorLAAM HCl-(acetoxy-d₃), methadone HCl, EDDP perchlorate, EDDP perchlorate-(2,2,2-ethyl-d₄), EMDP HCl, and EMDP HCl-(2,2,2-ethyl-d₄) were prepared at Research Triangle Institute (Research Triangle Park, NC) and provided by the National Institute on Drug Abuse (Rockville, MD). Methadone HCl-(1,1,1-heptan-d₃) was purchased from Radian (Austin, TX). LAAM, norLAAM, and dinorLAAM are diastereomers. Methadone, EDDP, and EMDP are racemic mixtures. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (Type XII), NADP, ketoconazole, and quinidine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfaphenazole was obtained from Ultrafine Chemicals (Manchester, UK). Sources for sodium DEDTC and ammonium hydroxide, 78:20:2 (v/v/v), the eluates were evaporated and reconstituted in 1000 ng/ml standards listed above were added. For LAAM and metabolites, samples were then buffered to pH 9.0 and extracted with N-butyl chloride; the extracts were dried and derivatized with trifluoroacetic anhydride, dried, and reconstituted with N-butyl chloride. The reconstituted residues were separated using a Finnigan model 9610 gas chromatograph with a 5% phenyl methylsilicone fused capillary column (15–20 m × 0.25-mm i.d., 0.25 µm film thickness) and detected using a Finnigan model 4500 quadrupole mass spectrometer operated in the positive ion chemical ionization mode with methane:ammonia as the reagent gas. The molecular ions of LAAM (d₄ and d₃) and the trifluoroacetates derivatives of the ammonium adducts of norLAAM (d₃ and d₄) and dinorLAAM (d₃ and d₄) were monitored, with the peak height ratios (d₃/d₄) used to calculate concentrations based on least square regression of calibrators (10–1000 ng/ml) included in each run.

Incubation Conditions. Microsomes were prepared from the human liver by differential centrifugation of homogenates as previously described (31), and stored at −80°C until used. Protein concentration of the microsomal fractions was measured using the method of Bradford (32) (Bio-Rad, Hercules, CA) with bovine serum albumin as standard. P450 content was determined from the difference spectrum of carbon monoxide-reduced vs. reduced microsomes as described by Omura and Sato (33).

Incubations were conducted at final volumes of 1.0 ml in 0.1 M phosphate buffer (pH 7.4) containing 1.0 mM EDTA and 5.0 mM MgCl₂. Buffer and inhibitor (if used/100 µl in aqueous solution) were added first, followed by microsomal protein (0.1 ml at 10 mg protein/ml) and the mixture preincubated 1–2 min at 37°C. The reaction was initiated by addition of the NADPH-generating system (final concentrations: 10 mM glucose-6-phosphate, 1.2 mM NADP, and 1.2 units of glucose-6-phosphate dehydrogenase) and substrate. Reactions were terminated by transferring the incubation vials to an ice-water bath, and after they were capped, to a −80°C freezer, where they were stored until analysis. Controls for all experiments included either incubates that did not contain the NADPH-generating system for human liver microsomes or incubates that did not contain microsomal protein for the cDNA-expressed microsomes.

Analytical Determinations. Metabolite concentrations were determined by GC/MS. These two methods have been described in detail elsewhere (20, 22); a brief description follows. To initiate both analyses, frozen incubates were thawed, immediately placed into an ice bath, and the deuterated internal standards listed above were added. For LAAM and metabolites, samples were then buffered to pH 9.0 and extracted with N-butyl chloride; the extracts were dried and derivatized with trifluoroacetic anhydride, dried, and reconstituted with N-butyl chloride. The reconstituted residues were separated using a Finnigan model 9610 gas chromatograph with a 5% phenyl methylsilicone fused capillary column (15–20 m × 0.25-mm i.d., 0.25 µm film thickness) and detected using a Finnigan model 4500 quadrupole mass spectrometer operated in the positive ion chemical ionization mode with methane:ammonia as the reagent gas. The molecular ions of LAAM (d₄ and d₃) and the trifluoroacetates derivatives of the ammonium adducts of norLAAM (d₃ and d₄) and dinorLAAM (d₃ and d₄) were monitored, with the peak height ratios (d₃/d₄) used to calculate concentrations based on least square regression of calibrators (10–1000 ng/ml) included in each run.

Statistics. Statistical differences among the effects of P450-specific inhibitors and among the activities of the cDNA-expressed P450s were tested using one-way ANOVA (p < 0.05). If the ANOVA demonstrated that a significant difference existed, differences between specific inhibitors or between cDNA-expressed P450s were determined using the Tukey test (p < 0.05) (34).

Results

N-Demethylation in Human Liver Microsomes. Previous experiments demonstrated that the addition of either 10 µM LAAM or norLAAM to human liver microsomes for 15 min resulted in detectable production of norLAAM plus dinorLAAM and dinorLAAM, respectively (20). Experiments with these two substrates were therefore carried out under these conditions, and when LAAM was used as
substrate, both norLAAM and norLAAM plus dinorLAAM production were evaluated. EDDP production from 10 μM methadone occurs at a slower rate (22), so 45 min incubations were used for methadone incubations. EMDP production was not detected and, therefore, not evaluated in human liver microsomes.

Control samples (NADPH generating system or microsomes omitted) occasionally had detectable product. The amount of product corresponded with substrate concentration. Product formation in controls was: not time dependent; similar in incubations of substrate in buffer only and microsomes lacking the NADPH generating system; and did not exceed 1% of substrate concentration (not shown). These results suggested this product formation arose from minor impurities in the substrates. These controls were therefore used as blanks.

N-Demethylation of LAAM, norLAAM, and methadone was determined in microsomes from four different donors (three used for all three drugs). The N-demethylation of norLAAM (16 to 169 pmol/min/mg protein) and LAAM (32 to 212 pmol/min/mg protein) occurred at similar rates that were consistently greater than the rates of methadone N-demethylation (4 to 25 pmol/min/mg protein) (fig. 2). Individual variation in rates of N-demethylation were observed for all three drugs. The variation was diminished but still readily apparent when activities were expressed relative to P450 rather than protein content (fig. 2).

Effect of P450 Isozyme-Selective Inhibitors. Inhibitors selective for individual P450s (35–37) were investigated for their ability to inhibit conversion of LAAM to norLAAM plus dinorLAAM, norLAAM to dinorLAAM, and methadone to EDDP by human liver microsomes prepared from four individual donors (table 1). Among the tested inhibitors, ketoconazole (3 μM) was the most potent inhibitor of LAAM, norLAAM, and methadone. Inhibition of methadone N-demethylation by ketoconazole, however, could not be statistically distinguished (Tukey post-hoc test, p < 0.05) from the inhibitory effects of ciprofloxacin or sulfaphenazole. Considerably greater variations between individual liver donors were observed for the effects of all P450 inhibitors on N-demethylation of methadone compared with LAAM or norLAAM.

Concentration dependent ketoconazole inhibition of N-demethylation was evaluated in liver preparations from two individual donors. A clear concentration dependent inhibition was observed with LAAM (10 μM) or norLAAM (10 μM) as substrates. IC₅₀ was determined for LAAM to norLAAM (1.65 μM), LAAM to norLAAM plus dinorLAAM (1.9 μM), and norLAAM to dinorLAAM (1.08 μM). A wider variation in the ability of ketoconazole to inhibit N-demethylation of 10 μM methadone was observed. Inhibition of methadone N-demethylation by 3 μM ketoconazole was 50% of controls in these two liver preparations; however, when ketoconazole concentrations were either 5 or 10 μM, increased inhibition was minimal. By contrast, 3 μM ketoconazole produced 100% inhibition in two of the other microsomes presented in table 1.

N-Demethylation by cDNA-Expressed P450s. LAAM (10 μM for 15 min), norLAAM (10 μM for 15 min), and methadone (100 μM for 45 min) were incubated with 1 mg protein/ml of commercially available microsomes that contain cDNA-expressed human P450s 1A2, 2C9, 2D6, 2E1, or 3A4. When incubated with LAAM, norLAAM, or methadone, the P450 3A4 containing microsomes produced significantly greater amounts of N-demethylation products than any of the other microsomes (fig. 3). LAAM was also N-demethylated by microsomes containing 2E1 and 2D6 to an extent that was significantly different from the other microsomes (fig. 3A). Based on the information provided by the vendor, these microsomes do not contain equal amounts of the specific P450s. The reported specific P450 content of the 2D6 (0.248 nmol/mg protein) and 2E1 microsomes (0.124 nmol/mg protein) were greater than the 3A4 microsomes (0.035 nmol/mg protein); expression of activity per nanomole P450 resulted in only the P450 3A4 microsomes producing significantly greater amounts of N-demethylation product from LAAM. When the P450 content was used to evaluate norLAAM metabolism, the 2C9 microsomes, which had particularly low amounts of P450 2C9 (7.5 pmol/mg protein), produced significantly greater amounts of norLAAM relative to P450 content (42 ± 18 pmol/min/nmol P450) than all P450s except 3A4 (254 ± 16 pmol/min/nmol P450). P450 3A4 microsomes N-demethylated methadone significantly greater than the other microsomes regardless of the manner of expressing the activity. In contrast to human liver microsomes, incubation of methadone with the cDNA-expressed P450s microsomes also resulted in EMDP production (fig. 3C). N-Demethylation product formation in P450 3A4 microsomes was substrate concentration dependent and was significantly inhibited by ketoconazole (fig. 4C).

Discussion

Incubations of LAAM with human liver microsomes produced substantially amounts of norLAAM and dinorLAAM. These findings accurately reflect the demethylation pattern observed in clinical studies that have reported that both norLAAM and dinorLAAM are present in urine and plasma at similar concentrations (5, 9, 27). This is the first complete report of LAAM N-demethylation in human liver microsomes, although demethylation has been demonstrated previously in rat tissue (16) or inferred from formaldehyde generation in liver tissue from rats (13, 19) and mice (18). The metabolism in vitro of norLAAM has not been studied previously in tissues from any species.

By contrast, incubation of methadone with human liver microsomes and the NADPH-generating system resulted in production of the first N-demethylation product, EDDP, but no detectable secondary product, EMDP, under the conditions studied. Iribarne et al. (21) also did not detect EMDP generation in human liver microsomes. Although a number of studies have been conducted on the in vitro metabolism of methadone in experimental animals, most did not discriminate between EDDP and EMDP formation as they monitored formaldehyde production (12, 14, 19). The generation of EMDP has been demonstrated in perfused rat liver studies that employed radiolabeled methadone (15, 17). The very low rate of EMDP formation was consistent with findings of much higher concentrations of EDDP than EMDP in human urine (7, 8, 22) and with a single study that found EDDP was detectable in human plasma, but that EMDP could not be detected.
The effect of P450-selective inhibitors on the N-demethylation of LAAM, norLAAM, and methadone

<table>
<thead>
<tr>
<th>Inhibitor (concentration: P450 target)</th>
<th>N-Demethylation Pathway</th>
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<tr>
<td></td>
<td>LAAM to norLAAM</td>
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<td></td>
<td>(% Control)</td>
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<tr>
<td>Ciprofloxacin (200 μM; 1A2)</td>
<td>91.3 ± 9.8a (82–103)</td>
</tr>
<tr>
<td>Sulfaphenazole (100 μM; 2C9)</td>
<td>65.6 ± 11.6b (54–79)</td>
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<tr>
<td>Quinidine (1 μM; 2D6)</td>
<td>84.3 ± 8.3c (78–97)</td>
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<tr>
<td>DEDTC (100 μM; 2E1)</td>
<td>72.1 ± 7.1d (65–82)</td>
</tr>
<tr>
<td>Ketoconazole (3 μM; 3A4)</td>
<td>3.8 ± 3.5e (0–8)</td>
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Values are the mean ± SD of determinations in microsome preparations from 4 different livers. Substrates were added at 10 μM. Incubations with LAAM, norLAAM, and methadone were for 15, 15, and 45 min, respectively. For each liver preparation the activity (pmol/min/mg protein) in the presence of the inhibitor was expressed as percent of its respective control. Control values are presented in fig. 2A. Values in parentheses are the range of inhibition.

Table 1

Fig. 3. N-Demethylation of A) 10 μM LAAM, B) 10 μM norLAAM and C) 100 μM methadone in microsomes containing cDNA-expressed human P450s.

Incubations with LAAM and norLAAM were for 15 min; those with methadone for 45 min. Values are the mean ± SD for triplicate incubations. Statistical differences within a pathway were tested using one-way ANOVA (p < 0.05) with the Tukey post-hoc test. Bars that do not share the same letter are significantly different (p < 0.05).

when the limit of quantitation was 10 ng/ml (22). It therefore seems that EDDP, relative to LAAM, norLAAM, and methadone, is a poor substrate for N-demethylation. One may speculate that the structural change from an alkyl amine to a pyrrolidine that occurs during the cyclization sterically hinders access of EDDP to the site of N-demethylation.

Human liver microsomal P450s responsible for the N-demethylation of LAAM to norLAAM plus dinorLAAM, norLAAM to dinorLAAM, and methadone to EDDP plus EMDP in vitro were investigated using both P450 selective inhibitors and cDNA-expressed P450s. Our results establish for the first time that P450 3A4 is capable of N-demethylating LAAM and norLAAM. Furthermore, we substantiate the role of P450 3A4 in methadone metabolism reported by Iribarne et al. (21). The combined inhibition and cDNA-expressed P450 experiments provide strong evidence that P450 3A4 is a major participant in the N-demethylation of LAAM, norLAAM, and methadone.

While the most appropriate concentration of substrate for metabolism studies in vitro remains to be fully determined, the general recommendation is that it should be in the range of circulating plasma concentrations (38). Iribarne et al. (21) used concentrations of methadone for correlation (2.5 mM) and inhibition (500 μM) studies far in excess of clinically achieved concentrations. Most of our studies were conducted at 10 μM, which is approximately 10-fold above therapeutic values (27, 39 – 41).

The involvement of P450s other than 3A4 in the N-demethylation of these compounds is less clear. Inhibition data presented in table 1 suggest that P450 2C9 may N-demethylate LAAM but not norLAAM and that methadone is a possible substrate for P450 1A2 and 2C9. Increasing ketoconazole to 10 μM, a concentration that completely
inhibits most P450 3A4 reactions (36, 37), failed to elicit more than 60% inhibition in the two liver preparations used. Iribarne et al. (21) also found that complete inhibition of methadone N-demethylation could not be achieved by a number of P450 3A4 inhibitors. A possible reason for this observation could be the participation of other enzymes in the N-demethylation of methadone. Iribarne et al. (21) reported that quinidine did not inhibit methadone N-demethylation (consistent with our results); however, in contrast to these authors, we observed some inhibition of methadone N-demethylation by sulfaphenazole and ciprofloxacin. The difference may arise from the fact that a 50-fold higher substrate concentration was used in their inhibition studies.

Except for P450 3A4, results of our inhibition studies are inconsistent with those observed for cDNA-expressed P450s. For example, the data presented in fig. 3 suggest several candidate P450s may be capable of N-demethylating LAAM, norLAAM, or methadone, including P450 2D6 and 2E1. Similar inconsistencies between inhibition and human heterologously expressed P450 studies were noted by Iribarne et al. for methadone N-demethylation (21); they found N-demethylation with heterologously expressed 2D6, 2C18, and 2C8, but did not find inhibition of microsomal activity by inhibitors of these P450s. Although available data suggest that multiple enzymes are responsible for N-demethylation of LAAM, norLAAM, and methadone, identification of specific enzymes, except P450 3A4, are yet to be established.

The finding that P450 3A4 is a major enzyme responsible for the N-demethylation of LAAM, norLAAM, and methadone provides information suggesting possible drug-drug interactions that could produce varying therapeutic results in drug-abusing individuals dosed with LAAM or methadone. Drug-drug interactions producing either metabolic inhibition or induction of LAAM, norLAAM, and methadone should be considered as the number of compounds known to interact with P450 3A4 are large (42–45). The list of substantiated drug interactions with methadone is small. However, rifampin (30), phenobarbital (46), and phenytoin (47), that are known P450 3A4 inducers (48), were found to induce the metabolism of methadone, producing suboptimal therapeutic results. Recently, fluvoxamine co-administration has been found to increase methadone plasma concentrations (49), suggesting it inhibits methadone metabolism. Fluvoxamine has been found to inhibit in vitro reactions of substrates of P450 3A4, 1A2 and, to a lesser extent, 2D6 (50–53). No drug-drug interactions have been reported for LAAM. This is not surprising since LAAM has only recently been approved for clinical use and its use is relatively limited.

The results of this study suggest that multiple P450s may be responsible for the metabolism of LAAM, norLAAM, and methadone; however, they indicate a major role of P450 3A4 in the N-demethylation of LAAM, its primary metabolite norLAAM, and methadone. Based on this information, it should be possible to project the likelihood of interactions for a wide range of concomitant medications. While the immediate goal is to avoid unanticipated changes in plasma drug concentrations (increases or decreases), intentional manipulations of metabolism might also be feasible (e.g. to reduce the frequency of dosing or to compress the range of interpatient variability).

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