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N-Demethylation of levo-α-acetylmethadol by human placental aromatase

Sujal V. Deshmukh, Tatiana N. Nanovskaya, Gary D.V. Hankins, Mahmoud S. Ahmed*

Department of Obstetrics and Gynecology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555 0587, USA

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Abstract

Levo-α-acetylmethadol (LAAM) is a methadone derivative used to treat the opiate addict. We previously reported on the kinetics for transplacental transfer of LAAM and its levels in the fetal circuit using the technique of dual perfusion of the placental lobule. The aim of this investigation was to identify the enzyme responsible for the biotransformation of LAAM and norLAAM and the metabolites formed in the term human placenta. Placental microsomes exhibited higher activities than the mitochondrial and cytosolic fractions in metabolizing LAAM to norLAAM. None of these subcellular fractions catalyzed the formation of dinorLAAM from either LAAM or norLAAM as determined by HPLC/UV. Evidence obtained from the effects of cytochrome P450 (CYP) inhibitors on the demethylation of LAAM to norLAAM by placental microsomes suggested that CYP 19/aromatase is the major enzyme involved. Out of 10 monoclonal antibodies raised against various CYP isoforms, only that for aromatase caused over 80% inhibition of norLAAM formation. The biotransformation of LAAM to norLAAM exhibited monophasic kinetics with apparent K_m and V_{max} values of 105 ± 57 μM and 86.8 ± 15.6 pmol mg^{-1} protein min^{-1}, respectively. The kinetic profile determined for a cDNA-expressed CYP 19 metabolism of LAAM to norLAAM was similar to that determined for placental microsomes. Taken together, the above data indicate that CYP 19/aromatase is the enzyme responsible for the *N*-demethylation of LAAM to norLAAM in term human placentas obtained from healthy pregnant women.

Keywords: Human placenta; Aromatase; LAAM; Metabolism; Opiate addict

1. Introduction

LAAM is an analog of methadone with a longer duration of action, which allows it to be administered every 2 or 3 days [1,2]. LAAM is used as an alternative to methadone in maintenance therapy of the heroin/opiate addict [3]. The long duration of LAAM action as compared with other *mu* opiate agonists is attributed to its sequential metabolism to the pharmacologically active norLAAM and dinorLAAM [4] (Fig. 1) and their elimination half-lives of 1–1.5 and 3–4 days, respectively, as compared with half a day for the parent compound [5–7]. Taken to together, these reports led investigators to consider LAAM as a prodrug. Reports on the potency of LAAM and its metabolites are in agreement that norLAAM is more potent than LAAM *in vitro* [8] and *in vivo* [6]. However, few researchers reported that dinorLAAM was more potent [6,9] and others have shown it to be less potent than LAAM [8,10].

Recently, utilizing the technique of dual perfusion of placental lobule, we reported on the role of human placenta in the transfer of LAAM and norLAAM to the fetal circuit and determined the kinetics for their transfer. Fetal transfer rates of the parent compound and its metabolite were fast and similar, but the amounts that reached the fetal circulation were different (17% for LAAM and 27% for norLAAM); the remainder was retained by the tissue—i.e. the placenta acted as a depot for the two compounds [11]. It should be noted that the concentration of the drug and its metabolite in the tissue was higher than their concentration in the maternal circuit, but neither drug was metabolized during the experimental period. The lack of detection of the tissue's metabolic activity is one of the limitations of the technique, since the drugs would have greater access to the metabolic enzymes *in vivo*. Therefore, it was necessary to

* Corresponding author. Tel.: +1-409-772-0977; fax: +1-409-772-5297.
E-mail address: maahmed@utmb.edu (M.S. Ahmed).
Abbreviations: LAAM, levo-α-acetylmethadol; CYP, cytochrome P450.

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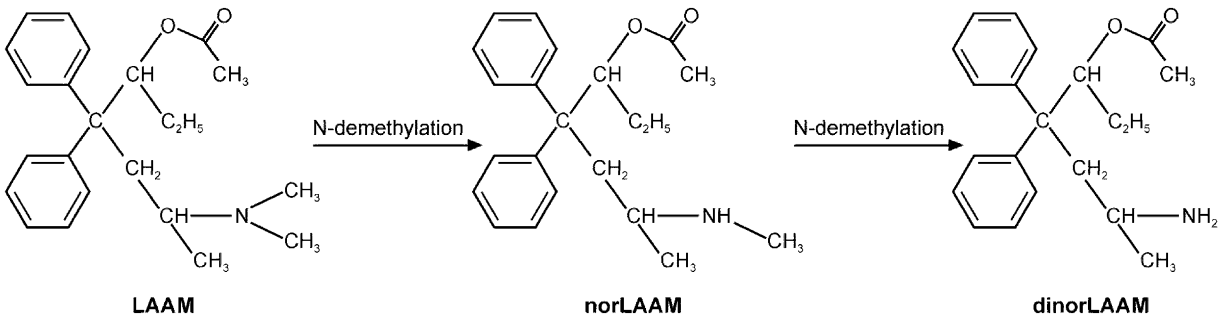

Fig. 1. Structure of LAAM and its major metabolites norLAAM and dinorLAAM.

use other techniques to determine the role of human placenta in the metabolism of LAAM.

Human placenta may act as a functional barrier protecting the fetus from drugs, environmental toxins, and other xenobiotics. The placenta expresses metabolizing enzymes similar to those in the liver and can thus affect maternal and fetal drug disposition [12–16]. Several researchers have provided a detailed account of these enzymes and their activities [17–19].

LAAM is metabolized rapidly to its active metabolites norLAAM and dinorLAAM by human liver microsomal CYP 3A4 [20,21]. However, hepatic CYP 2B6 and 2C18 are also involved in catalyzing the *N*-demethylation of LAAM [21–23]. Intestinal microsomal CYP 3A4 sequentially metabolizes LAAM to norLAAM and dinorLAAM [22].

The goal of this investigation was to identify the major enzyme responsible for the metabolism of LAAM and norLAAM, as well as the metabolites formed, in the trophoblast tissue of term human placenta.

2. Materials and methods

2.1. Chemicals and other supplies

All chemicals were purchased from Sigma Chemical Co unless otherwise mentioned. Acetonitrile was purchased from Fisher Scientific Co; LAAM, norLAAM, dinorLAAM, and norbuprenorphine were a gift from the National Institute on Drug Abuse drug supply unit. The monoclonal antibodies to CYP isoforms were a generous gift from Dr. Andrew Parkinson (XenoTech LLC). Rabbit antiserum to human placental aromatase was purchased from Hauptman–Woodward Institute. The cDNA-expressed CYP 19 system is commercially available as Supersomes^{TM} from Gentest.

2.2. Placental preparations

Placentas were obtained, from healthy pregnant women at term, immediately after delivery according to a protocol approved by the University of Texas Medical Branch Institutional Review Board. Villous tissue was dissected from each placenta, rinsed with ice-cold saline, and homogenized in 0.1 M potassium phosphate buffer, pH 7.4 (Ultra Turrax). The homogenate was used to prepare subcellular fractions by differential centrifugation; 10,000 *g* for the mitochondrial pellet, and 104,000 *g* for the microsomal pellet and the supernatant for the cytosolic. The mitochondrial and microsomal pellets were re-suspended in 0.1 M potassium phosphate buffer (pH 7.4), and their protein content was determined using a kit purchased from Bio-Rad Laboratories and BSA as a standard. Aliquots of the subcellular fractions were stored at −80° until used.

2.3. Enzyme-catalyzed reactions

The biotransformation of LAAM to norLAAM by the mitochondrial, microsomal, and cytosolic fractions of the placental tissue was determined in the following manner. The subcellular fraction (1 mg protein) was preincubated with 300 μM LAAM in 0.1 M of potassium phosphate buffer (pH 7.4, final volume 1 mL) for 5 min at 37°. The reaction was initiated by adding the NADPH-regenerating system (NADP, 0.4 mM; glucose-6-phosphate, 4 mM; glucose-6 phosphate-dehydrogenase, 1 U/mL; and $MgCl_2$, 2 mM) and incubating it for 20 min at 37°. The reaction was stopped by adding 100 μL of a 30% (w/v) trichloroacetic acid containing 18.5 μg/mL norbuprenorphine as an internal standard. The reaction solution was then centrifuged at 12,000 *g* for 10 min, and the supernatant was separated and extracted with 2 × 1.5 mL butyl chloride. The contents were shaken vigorously (vortex) for 15 min, the butyl chloride layer was separated, air-dried, and the residue was re-suspended in 250 μL of the mobile phase. An aliquot of 200 μL was used for analysis by HPLC/UV as described in Section 2.5. Similar experiments were carried out to determine the biotransformation of norLAAM to dinorLAAM using a substrate concentration as high as 1000 μM.

The kinetics were determined for the metabolism of LAAM to norLAAM by human placental microsomal fractions and the cDNA-expressed CYP 19 preparation. The concentrations of LAAM used to construct the saturation

curves ranged between 25 and 1000 μM and were incubated for 15 min with either the microsomal (1 mg/mL) or cDNA expressing human CYP 19 + reductase (concentration of about 40–100 pmol CYP/mL). The saturation curves were used to calculate the apparent K_m and V_{max} values for the reaction.

2.4. Enzyme identification

Identification of the major CYP enzyme responsible for catalyzing the metabolism of LAAM to norLAAM was achieved by: (1) compounds known as selective inhibitors for CYP isoforms, and (2) monoclonal antibodies raised against human liver or placental CYP isoforms.

2.4.1. Chemical inhibitors

The compounds reported as selective inhibitors of CYP isoforms were utilized at their respective concentrations in parentheses: α-naphthoflavone (0.1 μM), CYP 1A; sulfaphenazole (10 μM), CYP 2C; quninidine (5 μM), CYP 2D6; 4-methylpyrazole, (25 μM), CYP 2E1; nifedipine, (200 μM), CYP 3A4; ketoconazole (2.5 μM), CYP 3A; 4-hydroxyandrostenedione (1 μM), CYP 19; and aminoglutethimide (10 μM), CYP 19. Stock solutions of the inhibitors were prepared in methanol to achieve the concentration indicated above after an aliquot of 5 μL was added to the reaction mixture. LAAM was added to achieve a final concentration of 100 μM together with 1 mg/mL of the microsomal fraction. The reaction components were pre-incubated for 5 min at 37°, followed by the addition of the NADPH-regenerating system to initiate the reaction that lasted 20 min. The control reaction contained the same volume of methanol (5 μL) instead of the inhibitors.

2.4.2. Monoclonal antibodies

Monoclonal antibodies raised against human liver CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, and 3A4/5 were a generous gift from XenoTech LLC. Rabbit antiserum to human placental aromatase was purchased from Hauptman–Woodward Institute. A pool of microsomal fractions prepared from twelve placentas was used in the following experiments. In each experiment, 0.325 mg protein of the microsomal pool was incubated at room temperature for 15 min with each antibody at its concentration causing 80% inhibition of the respective CYP isoform it was raised against. LAAM was added to the reaction components to attain a final concentration of 500 μM, and the incubation continued for 20 more minutes at 37°. The control reaction contained mouse immunoglobulin G instead of the monoclonal antibodies.

2.5. Analytical procedure

The metabolites were identified by HPLC/UV. The HPLC system consisted of a Waters 600E multi-solvent delivery system, a Waters 2487 dual-wavelength absorbance detector, and a Waters 717 autosampler—all controlled by Waters Millennium[32] chromatography manager (Waters).

Samples were prepared for analysis as described in Section 2.3. A Luna 5 μm C18 column (250 mm × 4.6 mm) purchased from Phenomenex was used and was preceded by a guard column. The mobile phase was made of 3.5 g/L ammonium carbonate dissolved in a mixture of 80:20 (v/v) methanol:water. Isocratic elution was performed at a flow rate of 1 mL/min and the eluent detected at a wavelength of 218 nm. Peaks were integrated using the Waters Millennium[32] chromatography manager, and ratios of the metabolite peak area to that of the internal standard were used for all calculations. Under the described experimental conditions, the detection limits for norLAAM and dinorLAAM were 25 and 50 ng/mL, respectively. Standards were processed under identical sample conditions, and all analyses were calculated using a standard curve prepared for each experiment.

2.6. Data analysis

The data are represented as mean ± standard deviation. Kinetic analysis was done by nonlinear regression using the SPSS 11.0 for Windows software. The equation used was: $v = [S \times V_{max}]/[S + K_m]$, where v: reaction velocity; S: substrate concentration; V_{max}: maximum reaction velocity, and K_m: the Michaelis–Menten constant.

Statistical analysis of data on the effect of inhibitors on LAAM metabolism was carried out using one-way ANOVA with a Tukey's comparison and deemed significant if the P value was <0.05.

3. Results

3.1. Metabolism of LAAM and norLAAM by term human placentas

Microsomal fractions were used to establish the conditions for the enzyme-catalyzed metabolism of LAAM and identify the metabolite(s) formed. The formation of norLAAM from LAAM required the presence of an NADPH-regenerating system suggesting that the enzyme is a monooxygenase. The rate of norLAAM formation was proportional to the amount of microsomal protein to a concentration of 2 mg/mL and an incubation period of 30 min. HPLC was used to identify the product(s)/metabolite(s) formed by comparing their retention times with standards of norLAAM and dinorLAAM. Analysis of the products formed as a result of incubating LAAM with the microsomal fractions obtained from several placental preparations revealed the presence of norLAAM only—i.e. dinorLAAM was not detected under our experimental conditions. In addition, the microsomal fractions were incubated with a range of norLAAM concentrations

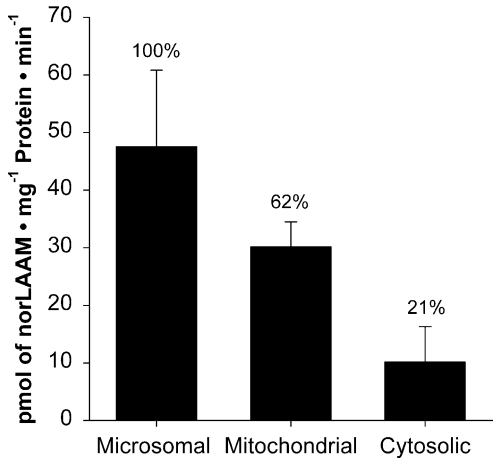

Fig. 2. The *N*-demethylation of LAAM to norLAAM by human placental subcellular fractions. Each subcellular fraction (1 mg protein) was incubated with LAAM (300 μM) at 37° for a period of 20 min. Data represent mean ± SD for the activities of the subcellular fractions from six term human placentas.

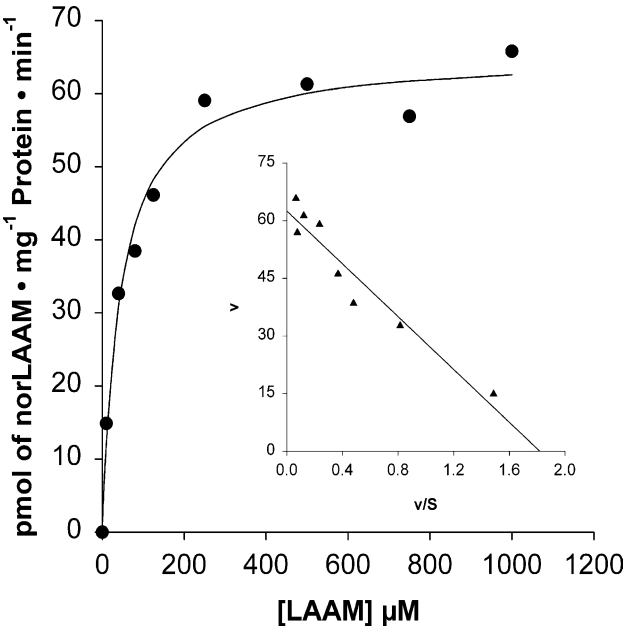

Fig. 3. A graph representing the data obtained from a typical experiment for the effect of increasing concentrations of LAAM on the rate of formation of norLAAM by placental microsomal fraction. Each concentration of LAAM was incubated with microsomal protein (1 mg/mL) for 15 min at 37°. Analysis of the data revealed a mean apparent K_m and V_{max} values of 106 ± 58 μM and 86.9 ± 15.6 pmol mg^{-1} protein min^{-1}. The inset is an Eadie–Hofstee plot of the data indicating monophasic kinetics.

between 25 and 1000 μM and under these conditions dinorLAAM was also not detected.

3.2. Subcellular distribution of enzyme activity

The microsomal, mitochondrial, and cytosolic fractions of term human placental tissue were compared for their *N*-demethylation of LAAM to norLAAM. A concentration of LAAM equal to three times its K_m value was used to account for intraplacental (between fractions of the same placenta) and interplacental (between different placentas) variability in the activity of the enzyme. The rate of norLAAM formation was highest in presence of the microsomal fractions followed by the mitochondrial and cytosolic fractions (Fig. 2). The percent of norLAAM formed by the mitochondrial and cytosolic fractions to that by the microsomal fractions was approximately 62 ± 11% and 20 ± 8%, respectively.

3.3. Kinetics of LAAM metabolism by placental microsomes

The effect of increasing substrate (LAAM) concentration on product (norLAAM) formation was investigated using microsomal preparations. Under the experimental conditions described in Section 2, less than 10% of the substrate (LAAM) was biotransformed to the product norLAAM. Plotting the rate of norLAAM formation vs. concentration of LAAM exhibited a typical Michaelis–Menten saturation curve (Fig. 3). The apparent K_m and V_{max} were calculated for the *N*-demethylation of LAAM by microsomal fractions from different placentas (Table 1) and revealed a mean of 106 ± 58 μM and 86.9 ± 15.6 pmol mg^{-1} protein min^{-1}, respectively. A 5-fold variability in the apparent K_m values was observed and ranged between 44 and 197 μM; a 2-fold variability was observed for the V_{max} values (65–112 pmol mg^{-1} protein min^{-1}). Analysis of the data using an Eadie–Hofstee plot (Fig. 3, inset) revealed monophasic kinetics and suggested the involvement of one enzyme or several enzymes with similar affinities to the substrate. The mean

Table 1
Kinetic parameters for the formation of norLAAM from LAAM in human placental microsomal fraction

Preparation #	K_m (μM)	V_{max} (pmol mg^{-1} protein min^{-1})	V_{max}/K_m[a] (μL mg^{-1} protein min^{-1})
1	197.14	84.59	0.43
2	84.73	95.8	1.13
2	44.24	65.33	1.48
4	93.9	82.6	0.88
5	62.62	81.22	1.30
6	152.31	111.69	0.73
Mean ± SD	105.82 ± 57.87	86.87 ± 15.58	0.99 ± 0.39

[a]Intrinsic clearance.

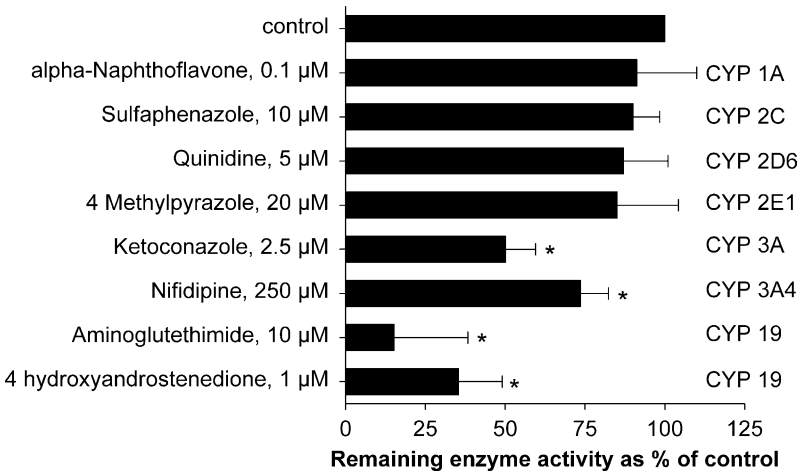

Fig. 4. The effect of inhibitors selective for CYP isoforms on the *N*-demethylation of LAAM to norLAAM by placental microsomal fractions. Each inhibitor was added to the reaction at the indicated concentration and is approximately 10 times its reported inhibition constant for that CYP isoform. LAAM at a concentration of 100 μM, equal to its apparent K_m, is coincubated with the inhibitors for 20 min at 37°. *Statistical significance of $P < 0.05$ as determined by one-way ANOVA with a Tukey's comparison.

intrinsic clearance value calculated under these *in vitro* conditions was $1.0 \pm 0.4 \times 10^{-3}$ mL mg^{-1} protein min^{-1}. The values for the kinetic parameters mentioned above represent the mean obtained using six different microsomal fractions prepared from their respective human placentas.

3.4. Inhibition of LAAM metabolism

3.4.1. Chemical inhibitors

The effect of inhibitors, selective for CYP isoforms, on the metabolism of LAAM to norLAAM by placental microsomes was utilized to identify the enzyme catalyzing the reaction. The inhibitors chosen were those selective for the previously identified CYP isoforms of term human placenta and those that are involved in the metabolism of xenobiotics. Other inhibitors included were those selective for the CYP isoforms catalyzing the metabolism of LAAM in human liver or intestine: CYP 1A, 2C, 2D6, 2E1, 3A4/5, and 19. The concentration used for each inhibitor was that reported as the most selective for a particular CYP isoform [24–26]. The concentration of LAAM used was equal to its apparent K_m as determined in this investigation (ca. 100 μM).

Four compounds (Fig. 4) caused over 25% inhibition of norLAAM formation: nifidipine (30%), ketoconazole (50%), 4-hydroxyandrostenedione (65%), and aminoglutethimide (>70%). The latter two compounds are selective for CYP 19 and caused approximately twice the inhibition caused by the CYP 3A-selective nifidipine and significantly more than ketoconazole. All other compounds tested caused less than 25% inhibition or had no effect on norLAAM formation. These data suggest that the most likely major enzyme to catalyze the metabolism of LAAM by placental microsomes is CYP 19.

3.4.2. Immunoinhibition of LAAM metabolism

Monoclonal antibodies raised against human CYP isoforms were investigated for their inhibition of the *N*-demethylation of LAAM to norLAAM by placental microsomes. The ratio of each antibody to the microsomal proteins in the reaction was that reported by the manufacturer as causing over 80% inhibition of the catalytic activity of the specific CYP isoform. The concentration of LAAM (300 μM) was in its saturation range, as determined in this investigation, to ensure maximum formation of norLAAM. The highest inhibition of norLAAM formation (ca. 75%) was observed in the presence of antibodies against CYP 19 (Fig. 5). Antibodies against CYP 2A6, 2C8, 2D6, and 2E1 caused approximately 20% inhibition. The effect of the remaining antibodies tested was statistically insignificant. Therefore, antibodies against CYP 19

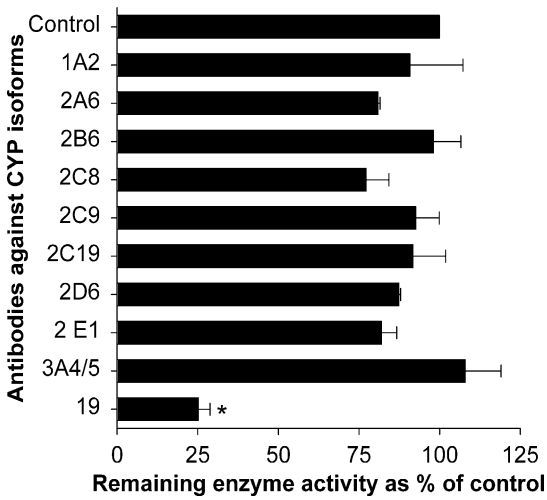

Fig. 5. Immunoinhibition of norLAAM formation by monoclonal antibodies raised against human CYP isoforms. A pool of microsomal fractions prepared from 12 placental preparations was preincubated with the various antibodies for 15 min at room temperature. LAAM and NADPH were added and the incubation continued at 37° for 20 min. The mean of three experiments is represented ±SD. *Statistical significance of $P < 0.05$ as determined by one-way ANOVA with a Tukey's comparison.

were at least three times more potent than any of the others tested—thus, confirming the effect of chemical inhibitors on norLAAM formation. Taken together, it is likely that CYP 19/aromatase is the major enzyme present in placental microsomes and is responsible for the metabolism of LAAM to norLAAM.

3.5. Kinetics of LAAM metabolism by cDNA-expressed CYP 19

3.5.1. norLAAM formation

The kinetic profile for LAAM metabolism by a preparation of cDNA-expressed CYP 19 was investigated and compared with that for placental microsomes. The concentration of LAAM and other reaction conditions were identical to that used for placental microsomes. The cDNA-expressed CYP 19 preparation catalyzed the biotransformation of LAAM to norLAAM only (dinorLAAM was not detected under the experimental conditions utilized). The increase in the concentration of LAAM was proportional to the rates of norLAAM formation and exhibited saturation kinetics with apparent K_m and V_{max} values of 116 ± 51 μM and 3.69 ± 0.014 pmol pmol^{-1} CYP 19 min^{-1}, respectively. Eadie–Hofstee plot of the data reveled monophasic kinetics (Fig. 6).

3.5.2. Effect of chemical inhibitors

The compounds that caused >25% inhibition of LAAM metabolism by placental microsomes were nifidipine, ketoconazole, 4-hydroxyandrostenedione, and aminoglutethimide. These compounds also inhibited cDNA-expressed CYP 19 conversion of LAAM to norLAAM by 35, 50, 70, and 80%, respectively (Fig. 7). The inhibition of the expressed CYP 19 by these compounds was almost quantitatively identical to their effect on the activity of placental microsomal catalysis of the reaction.

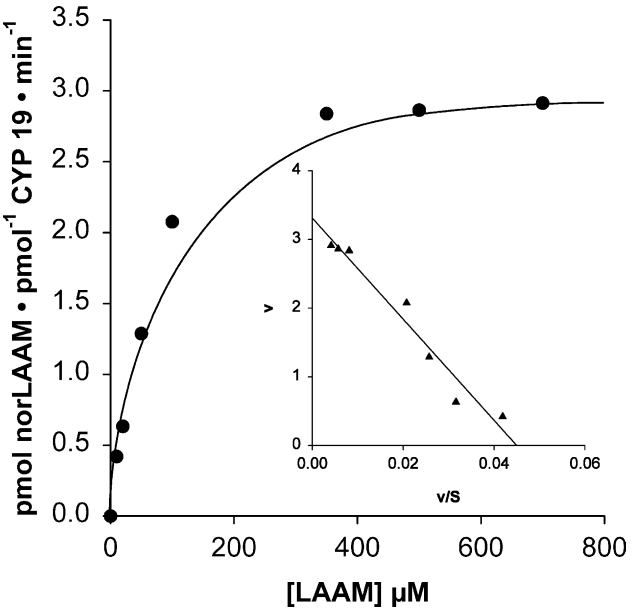

Fig. 6. A saturation curve of the *N*-demethylation of LAAM by a system of cDNA expressing CYP 19. An Eadie–Hofstee plot of the same data (inset) indicates monophasic kinetics. Analysis of the data revealed an apparent K_m and V_{max} values of 116 μM and 3.7 pmol pmol^{-1} CYP 19 min^{-1}, respectively.

4. Discussion

LAAM is an opiate agonist used for maintenance treatment of the adult opiate addict. We recently reported on the kinetics for the transplacental transfer of LAAM and norLAAM, their metabolism, and effects on the tissue utilizing the technique of dual perfusion of placental lobule [11]. The goal of this investigation was to identify the major placental enzyme responsible for the metabolism of LAAM and norLAAM, the kinetics of the reaction, and the product(s) formed.

Microsomal CYP 3A4 is the major enzyme responsible for the sequential *N*-demethylation of LAAM to norLAAM

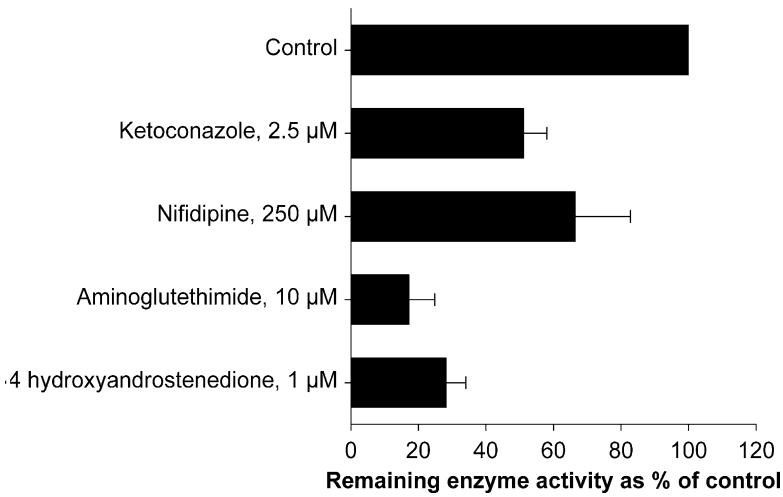

Fig. 7. The effect of inhibitors, causing >25% inhibition of norLAAM formation by placental microsomes, on the metabolism of LAAM by the cDNA expressing CYP 19 system Supersomes™. The concentration of LAAM and other experimental conditions are similar to the description for Fig. 4.

and dinorLAAM (Fig. 1) in human liver and intestine [21,22]; however, CYP 2B6 and 2C18 may also contribute to their metabolism in the liver [23]. In human placenta, CYP 3A4 expression, but not activity, was reported [27]. When LAAM was transfused in the maternal circuit of the dually perfused lobule, norLAAM was not formed/detected and when the latter was transfused under experimental conditions that favor accumulation of the metabolites formed, dinorLAAM was also not detected [11]. However, formation of minute amounts of either LAAM or norLAAM metabolites during the transfusion experiments could not be excluded because of the detection limits of the analytical method used, as well as other experimental conditions discussed previously [11].

Our data indicated that the *N*-demethylation of LAAM by trophoblast tissue homogenates required the presence of an NADPH-regenerating system, suggesting that the enzyme involved is most likely an oxygenase/CYP isoform. The three subcellular fractions of trophoblast tissue homogenates that we investigated catalyzed the demethylation of LAAM to norLAAM but at different rates; the microsomal fraction had the highest rate, followed by the mitochondrial and cytosolic fractions (Fig. 2). The mitochondrial fraction activity ranged from 50 to 70% (mean = 60%) of the activity in the microsomal fraction. A similar enzyme activity ratio of placental mitochondrial to microsomal fractions has been reported for the dealkylation and aromatization of other substrates [14,28,29].

The metabolism of norLAAM was investigated for two reasons: (1) LAAM is considered a prodrug due to its rapid first pass metabolism, and the presence of norLAAM in the circulation of a pregnant woman under treatment with the drug should be expected; (2) to determine whether increasing the concentration of norLAAM, as a substrate, may result in the formation of dinorLAAM by the microsomal fraction. We did not detect dinorLAAM when the concentration of norLAAM as a substrate was 1 mM. These findings indicate that the CYP isoform in placental microsomes is different from that in the liver and intestine, where CYP 3A4 was identified as the major enzyme catalyzing the sequential *N*-demethylation of the opiate to norLAAM and dinorLAAM [21,22].

We used the microsomal fraction to identify the major placental enzyme responsible for the metabolism of LAAM. In the reaction solution, the amounts of norLAAM formed was linear with the increase in microsomal protein up to 2 mg/mL. The relationship between LAAM as a substrate and the formation of norLAAM exhibited hyperbolic saturation kinetics (Fig. 3) with an apparent mean K_m of 105.8 ± 57 μM and a V_{max} of 86.0 ± 15.0 pmol mg^{-1} protein min^{-1}. Eadie–Hofstee plots of the data revealed monophasic kinetics, indicating that one enzyme (or more than one with similar affinity to the substrate) is catalyzing the reaction. The intrinsic clearance value from the liver [21] was 60 times greater than that calculated for the placental metabolism of LAAM (Table 1), indicating that the latter can act as a secondary line of defense for protection of the fetus.

To achieve the goal of identifying the CYP isoform catalyzing the metabolism of LAAM, a pool of microsomal fractions obtained from six placentas was used to average the interplacental differences observed in the K_m and V_{max} values (Table 1). Two types of inhibitors were utilized in the identification of the enzyme: (1) chemical inhibitors known as selective for a particular CYP isoform and (2) monoclonal antibodies against the enzymes. The concentration of each chemical inhibitor used was that reported in the literature as selective for a particular CYP isoform [24–26]. The CYP 19/aromatase-selective inhibitors, aminoglutethimide, and 4-hydroxyandrostenedione were the most potent and caused 70–75% inhibition of norLAAM formation (Fig. 4). Use of a chemical inhibitor at a concentration selective for a specific CYP isoform does not provide unequivocal identification of the enzyme because of cross inhibition of other CYP isoforms. Therefore, monoclonal antibodies raised against CYP isoforms were used to confirm the identification based on the use of chemical inhibitors. Among the monoclonal antibodies investigated, the most potent inhibitor was that raised against CYP 19 (Fig. 5), which confirmed that it was the major enzyme responsible for the *N*-demethylation of LAAM to norLAAM. These data also suggest that CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, and 3A4/5 and other metabolic enzymes may contribute less than 25% of LAAM metabolism in human placenta.

Two other types of experiments were used to substantiate and confirm the identification of CYP 19. First, we determined the kinetics of a commercially available cDNA expressing CYP 19 Supersomes™ catalysis of LAAM biotransformation to norLAAM, and second, we determined the effect of inhibitors on the same reaction. Data obtained from the first type of experiments revealed an apparent K_m value of 115.5 μM, i.e. within the range determined for the placental microsomal fractions (44.0–194.0 μM). The profile obtained on the effect of inhibitors causing >25% inhibition of norLAAM formation by the cDNA expressing CYP 19 was similar to the profile obtained for the same reaction catalyzed by the placental microsomal fractions (Fig. 7).

Therefore, all data obtained on the effects of chemical inhibitors, monoclonal antibodies, and kinetics of the *N*-demethylation of LAAM confirm that the major enzyme catalyzing the reaction in human placental microsomes is CYP 19/aromatase. Since the presence of aromatase has also been reported in the mitochondrial fraction of human trophoblast tissue [14,29], it is therefore plausible that the *N*-demethylation of LAAM in this fraction is catalyzed by the same enzyme.

In summary, aromatase/CYP 19 is the enzyme responsible for approximately 75% of LAAM *N*-demethylation to norLAAM in human placenta. The same enzyme is also responsible for the metabolism of buprenorphine to

norbuprenorphine in term human placenta [29]. CYP 19/ aromatase is a key enzyme in the conversion of androgens to estrogens in human placenta, suggesting that an interaction site exist in pregnant women if treated with LAAM for opiate dependence. At this time, it is unclear whether treatment with LAAM during pregnancy can affect maternal or neonatal outcome.

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