IN VITRO CHARACTERIZATION OF THE OXIDATIVE CLEAVAGE OF THE OCTYL SIDE CHAIN OF OLANEXIDINE, A NOVEL ANTIMICROBIAL AGENT, IN DOG LIVER MICROSONES

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ABSTRACT:
The metabolism of olanexidine [1-(3,4-dichlorobenzyl)-5-octylbiguanide], a new potent biguanide antiseptic, was investigated in dog liver microsomes to characterize the enzyme(s) catalyzing the biotransformation of olanexidine to C-C bond cleavage metabolites. Olanexidine was initially biotransformed to monohydroxylated metabolite 2-octanol (DM-215), and DM-215 was subsequently oxidized to diol derivatives threo-2,3-octandiol (DM-221) and erythro-2,3-octandiol (DM-222). Diols were further biotransformed to a ketol derivative and C-C bond cleavage metabolite (DM-210, hexanoic acid derivative), an in vivo end product, in the incubation with dog liver microsomes. The formations of DM-215, DM-221, DM-222, and DM-210 followed Michaelis-Menten kinetics, and Eadie-Hofstee analysis of the metabolite formation activity confirmed single-enzyme Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ values for the formation of DM-210 appeared to be 2.42 $\mu$M and 26.6 pmol/min/mg in the oxidation of DM-221 and 2.48 $\mu$M and 30.2 pmol/min/mg in the oxidation of DM-222. The intrinsic clearance ($V_{max}/K_m$) of the C-C bond cleavage reactions was essentially the same with either DM-221 or DM-222 as substrate. These oxidative reactions were significantly inhibited by quinidine, a selective inhibitor of CYP2D subfamilies, indicating the metabolic C-C bond cleavage of the octyl side chain of olanexidine to likely be mediated via the CYP2D subfamily in dog liver microsomes. This aliphatic C-C bond cleavage by cytochrome P450s may play an important role in the metabolism of other drugs or endogenous compounds possessing aliphatic chains.

Olanexidine monohydrochloride hemihydrate (OPB-2045) is a newly synthesized antimicrobial agent (Tsubouchi et al., 1997) that has potent microbical activity toward fungi (yeast), Gram-negative bacteria (Pseudomonas aeruginosa), and Gram-positive bacteria including methicillin-resistant Staphylococcus aureus and vancomycin-resistant Enterococci at low concentrations (Ohguro et al., 1997; Sakagami et al., 1999).

The absorption, distribution, metabolism, and excretion of olanexidine have been reported for rats and dogs after subcutaneous administration (Kudo et al., 1998a,b,c,d). Olanexidine is eliminated principally by metabolism in rats and dogs (Kudo et al., 1998c,d), and four metabolites have been structurally identified in dog urine as 6-[5-(3,4-dichlorobenzyl)-1-biguanidino] hexanoic acid (DM-210(1)), 4-[5-(3,4-dichlorobenzyl)-1-biguanidino] butanoic acid (DM-212), 5-[5-(3,4-dichlorobenzyl)-1-biguanidino] pentanoic acid (DM-213), and 3,4-dichlorobenzoic acid (Fig. 1) (Kudo et al., 1998c). Interestingly, these metabolites, except 3,4-dichlorobenzoic acid, are each a methylene chain shorter with a carboxy end, and they are predominant metabolites of olanexidine in both rats (Kudo et al., 1998d) and dogs (Kudo et al., 1998c).

Previous in vitro studies in rat and dog liver preparations demonstrated the degraded products of olanexidine to be produced by C-C bond cleavage following sequential oxidative reactions (monohydroxylations, dihydroxylation, and ketol formation at the octyl side chain, Fig. 1), but not $\beta$-oxidation (Umehara et al., 2000). These reactions required NADPH as a cofactor, and the C-C bond cleavage reaction was inhibited by cytochrome P450 inhibitors, indicating cytochrome P450 as an enzyme possibly involved in the metabolism of olanexidine (Umehara et al., 2000).

Some cytochrome P450 enzymes integrated into biosynthetic pathways possess catalytic activity not only in conventional hydroxylation reactions but also in the C-C bond cleavage reactions (Akhtar et al., 1993; Ortiz de Montellano, 1995). The C-20–C-22 bond of cholesterol is cleaved by a mitochondrial cytochrome P450 (CYP11A1) (Shikita and Hall, 1974; Byron and Gut, 1980; Ortiz de Montellano, 1995), and removal of the pregnenolone side chain to produce dehydroandrosterone is mediated by the cytochrome P450 system (CYP17) (Corina et al., 1991; Miller et al., 1991). However, no information is available regarding the enzyme(s) involved in the C-C
bond cleavage reactions in the metabolism of drugs containing aliphatic chains.

This study was conducted to characterize the enzyme(s) catalyzing the metabolic conversion of olanexidine into C-C bond cleavage metabolites using in vitro metabolic intermediates of olanexidine as substrate.

Materials and Methods

Chemicals. Olanexidine monohydrochloride hemihydrate, DM-210 dihydrochloride, 8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-2-octanol dihydrochloride (DM-215 dihydrochloride), threo-8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-2,3-octandiol dihydrochloride (DM-221 dihydrochloride), and erythro-8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-2,3-octandiol dihydrochloride (DM-222 dihydrochloride) were obtained from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). β-NADPH, α-naphthoflavone, troleandomycin, quinidine, and sulfaphenazole were purchased from Sigma Chemical Co. (St. Louis, MO). S-Mephenytoin was purchased from Ultrafine Chemical Co. (Manchester, UK). Pentobarbital was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Sodium N,N-diethyldithiocarbamate trihydrate was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents and solvents were of analytical grade.

Animal Treatments and Preparation of Microsomes. Three male beagle dogs from Hazleton Research Products Inc. (Cumberland, VA) were acclimatized in a controlled area maintained at 23 ± 2°C and 60 ± 10% relative humidity during 12-h light/dark cycles. The animals were fed laboratory chow CD-5 (Japan Clea Co., Ltd., Tokyo, Japan), and water was available ad libitum during acclimatization. Dogs 19 to 23 months of age were used in this study. The animals were sacrificed by exsanguination under anesthesia with pentobarbital. The livers were resected and washed with ice-cold saline and homogenized in 3 volume/liver weight of 1.15% KCl solution with a Waring Blender. The homogenate was centrifuged at 9000 g for 30 min at 4°C. To prepare liver microsomes, the 9000g supernatant was centrifuged at 105,000g for 60 min at 4°C. The microsomal pellet thus obtained was resuspended in 0.05 M phosphate buffer (pH 7.4), and the microsomes were stored at −80°C. The protein concentration was determined using Bio-Rad DC protein assay kits (Hercules, CA).

Assay of DM-210 Formation Activity. The reaction mixtures contained 0.1 M phosphate buffer (pH 7.4), 2.5 mM β-NADPH, DM-221 dihydrochloride, or DM-222 dihydrochloride, and dog microsomal protein (1 mg) in a final incubation volume of 0.5 ml. DM-221 dihydrochloride or DM-222 dihydrochloride was dissolved in methanol. The reaction was initiated by the addition of a cofactor and was carried out in air at 37°C in a shaking water bath for 30 min. Under the conditions used, the formation of DM-210 increased linearly with time (5–30 min) and with protein concentration (0.1–1 mg). In experiments to determine kinetic parameters for DM-210 formation, substrate concentrations of 0.25 to 10 mM were studied.

The effects of the following selective CYP inhibitors on the formation of DM-210 were examined: α-naphthoflavone for CYP1A1/2 (Tassaneeyakul et al., 1993), sulfaphenazole for CYP2C8/9 (Back et al., 1988), S-mephenytoin for CYP2C19 (Wrighton et al., 1993), quinidine for CYP2D6 (Otton et al., 1988), diethyldithiocarbamate for CYP2E1 (Guengerich et al., 1991), and troleandomycin for CYP3A (Watkins et al., 1985). The concentration of DM-221 dihydrochloride or DM-222 dihydrochloride was 5 μM. The inhibitors were preincubated with dog liver microsomes and cofactor for 15 min, and the reaction was initiated by the addition of the substrates. The concentrations of inhibitors ranged from 1 to 100 μM.

For all experiments the reaction was terminated by adding 0.5 ml of methanol. An aliquot (50 μl) of the supernatant obtained after centrifugation to pellet the denatured protein was analyzed by HPLC as described below.

Assay of DM-215 Formation Activity. The reaction mixtures contained 0.1 M phosphate buffer (pH 7.4), 2.5 mM β-NADPH, olanexidine monohydrochloride hemihydrate, and dog microsomal protein (0.25 mg) in a final incubation volume of 0.5 ml. Olanexidine monohydrochloride hemihydrate was dissolved in methanol. The reaction was initiated by the addition of a cofactor and was carried out in air at 37°C in a shaking water bath for 10 min. Under these conditions, the reaction rate was linear for at least 20 min at the
enzyme concentration. In experiments to determine the kinetic parameters for DM-215 formation, substrate concentrations of 2.5 to 75 \( \mu M \) were studied.

To examine the effects of selective CYP inhibitors on the formation of DM-215, a substrate concentration of 25 \( \mu M \) was selected. The inhibition studies were performed in the same manner described under Assay of DM-210 Formation Activity.

Reactions were terminated by adding 0.5 ml of methanol. An aliquot (50 \( \mu l \)) of the supernatant obtained after centrifugation to pellet the denatured protein was analyzed by HPLC.

**Assay of DM-221 or DM-222 Formation Activity.** The reaction mixtures contained 0.1 M phosphate buffer (pH 7.4), 2.5 mM \( \beta \)-NADPH, DM-215 dihydrochloride, and dog microsomal protein (1 mg) in a final incubation volume of 0.5 ml. DM-215 dihydrochloride was dissolved in methanol. The reaction was initiated by the addition of a cofactor and was carried out in air at 37°C in a shaking water bath for 30 min. Under these conditions, the reaction rate was linear for at least 60 min at the enzyme concentration. In experiments to determine the kinetic parameters for DM-221 or DM-222 formation, substrate concentrations of 5 to 250 \( \mu M \) were studied.

To examine the effects of selective CYP inhibitors on the formation of DM-221 or DM-222, a substrate concentration of 50 \( \mu M \) was selected. The inhibition studies were performed in the same manner described under Assay of DM-210 Formation Activity.

Reactions were terminated by adding 0.5 ml of methanol. An aliquot (50 \( \mu l \)) of the supernatant obtained after centrifugation to form a pellet from the denatured protein was analyzed by HPLC.

**HPLC.** The HPLC system consisted of two model 510 high-pressure HPLC pumps (Waters Associates, Milford, MA), a model 717 automatic sample processor (Waters), a model 486 UV detector (Waters), a model 680 automatic gradient controller (Waters), and a model C-R7A Chromatopac (Shimadzu, Kyoto, Japan).

In the assay for DM-210 production, a TSKgel ODS-80Ts column (4.6-mm i.d. \( \times \) 250-mm; Tosoh, Tokyo, Japan) was used at a flow rate of 1 ml/min with UV detection at 240 nm. The mobile phase used was a solution of 30% acetonitrile in water containing 1% acetic acid. The retention time for DM-215, DM-221, DM-210, and ketol (M-2) were 9.3, 10.1, 12.1, and 13.6 min, respectively. The calibration curves of DM-210 and M-2 were established by an absolute standard method, based on the peak area of DM-210 and M-2. The concentration of M-2 in the incubation was converted to the equivalent value of unchanged compounds (DM-210 or DM-222) because there was no authentic standard.

For measurement of DM-215 production, a TSKgel ODS-80Ts column (4.6-mm i.d. \( \times \) 250-mm; Tosoh, Tokyo, Japan) was used at a flow rate of 1 ml/min with UV detection at 240 nm. The mobile phase used was a solution of 36% acetonitrile in water containing 1% acetic acid. The retention time for DM-215 was 10.8 min. The calibration curve of DM-215 was established by an absolute standard method, based on the peak area of DM-215.

In the assay for DM-221 or DM-222 production, a TSKgel ODS-80Ts column (4.6-mm i.d. \( \times \) 150-mm; Tosoh) was used at a flow rate of 1 ml/min with UV detection at 240 nm. For the mobile phase, 10% acetonitrile in water containing 0.1% acetic acid was used as solution A and 80% acetonitrile in water containing 0.1% acetic acid as solution B. The metabolites were analyzed using a linear gradient developed from 10 to 35% solution B over a period of 30 min. The retention times for DM-221 and DM-222 were 17.0 and 18.1 min, respectively. The calibration curves of DM-210 and M-2 were established by an absolute standard method, based on the peak height of DM-210 and DM-222.

**Characterization of the M-1 and M-2 Metabolites.** The reaction mixture with DM-210 or DM-222 and dog liver microsomes was extracted by methanol, and the extract was analyzed by liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) to characterize the chemical structures of the metabolites.

HPLC analysis was carried out with a Waters HPLC system equipped with a model 600s controller, a model 616 FHU pump, a model 717P automatic sample processor, a model 717HC cooler, a model 486 UV Detector, and a model IL-DEGA in-line degasser. A YMC-Pack Pro C18 column (2.0-mm i.d. \( \times \) 150-mm; YMC Co., Ltd., Kyoto, Japan) was used at a flow rate of 0.2 ml/min. The metabolites were analyzed using a linear gradient developed from 0 to 50% solution B over a period of 45 min.

**MS/MS Analysis.** MS/MS analysis was performed using a Finnigan MAT (San Jose, CA) triple-stage quadrupole TSQ-7000 mass spectrometer equipped with an ESI source. The interface and mass spectrometer were operated under the following conditions: ion mode, positive; capillary temperature, 240°C; sheath gas pressure of nitrogen, 70 psi; auxiliary nitrogen gas flow rate, 10 U; electron multiplier voltage, 1.4 kV; collision gas of argon, about 2.0 mTorr; and collision energy, -24 eV.

**Kinetic Analysis.** The apparent \( \text{Km} \) and \( \text{Vmax} \) values were calculated from a nonlinear regression analysis with a computer program, WinNonlin Standard (version 2.1, Scientific Consulting, Inc., Apex, NC). Graphical analysis of Eadie-Hofstee plots was conducted for liver microsomal olanexidine oxidation. The intrinsic clearance (\( \text{CL}_{\text{intrinsic}} \) ) was calculated using the following equation: \( \text{CL}_{\text{intrinsic}} = \frac{\text{V}_{\text{max}}}{\text{K}_{\text{m}}} \).

**Results**

**DM-210 Formation Activity in Dog Liver Microsomes.** DM-221 or DM-222 incubated with dog liver microsomes in the presence of NADPH (2.5 mM) in 0.1 M phosphate buffer (pH 7.4) for 30 min. HPLC was performed with methanol extract from the incubation mixture as described under Materials and Methods.

**Characterization of the M-1 and M-2 Metabolites.** The reaction mixture with DM-210 or DM-222 and dog liver microsomes was extracted by methanol, and the extract was analyzed by liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) to characterize the chemical structures of the metabolites.

HPLC analysis was carried out with a Waters HPLC system equipped with a model 600s controller, a model 616 FHU pump, a model 717P automatic sample processor, a model 717HC cooler, a model 486 UV Detector, and a model IL-DEGA in-line degasser. A YMC-Pack Pro C18 column (2.0-mm i.d. \( \times \) 150-mm; YMC Co., Ltd., Kyoto, Japan) was used at a flow rate of 0.2 ml/min. The metabolites were analyzed using a linear gradient developed from 0 to 50% solution B over a period of 45 min.

**MS/MS Analysis.** MS/MS analysis was performed using a Finnigan MAT (San Jose, CA) triple-stage quadrupole TSQ-7000 mass spectrometer equipped with an ESI source. The interface and mass spectrometer were operated under the following conditions: ion mode, positive; capillary temperature, 240°C; sheath gas pressure of nitrogen, 70 psi; auxiliary nitrogen gas flow rate, 10 U; electron multiplier voltage, 1.4 kV; collision gas of argon, about 2.0 mTorr; and collision energy, -24 eV.

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**Results**

**DM-210 Formation Activity in Dog Liver Microsomes.** DM-221 or DM-222 incubated with dog liver microsomes in the presence of NADPH, and the methanol extract was analyzed by HPLC-UV and LC/ESI-MS/MS. In the reaction mixture, two metabolites, M-1 and M-2, were observed (Fig. 2).

M-1 was identified as DM-210 because the [M + H]\(^+\) ion in the precursor ion mass spectrum, the product ion mass spectrum, and
HPLC retention time were essentially the same as for the authentic standard. M-2 displayed a molecular ion of $m/z$ 402 [M+H] in the precursor ion mass spectrum, indicating this metabolite to have a molecular weight of 401, 2 less than that of the unchanged compound. In addition, the characteristic fragment ions at $m/z$ 218 and 202 were observed in the product mass spectrum (Fig. 3). M-2 would thus appear to be a ketol derivative of DM-221 or DM-222.

The formation of DM-210 was consistent with Michaelis-Menten kinetics, and Eadie-Hofstee plots of DM-210 formation activity demonstrated monophasic Michaelis-Menten kinetics (Fig. 4). The apparent $K_m$ and $V_{max}$ values for the formation of DM-210 were 2.42 $\mu$M and 26.6 pmol/min/mg in the oxidation of DM-221 and 2.48 $\mu$M and 30.2 pmol/min/mg in the oxidation of DM-222. The intrinsic clearance, calculated as $V_{max}/K_m$, was 11.0 $\mu$l/min/mg for DM-221 and 12.2 $\mu$l/min/mg for DM-222, indicating no stereoselectivity in the oxidation of diol derivatives.

Selective cytochrome P450 inhibitors were used in this study to determine the potential roles of cytochrome P450 isoforms on the metabolism of olanexidine (Fig. 5). Quinidine, a selective inhibitor of CYP2D subfamilies, inhibited DM-210 formation by approximately 30% at 10 $\mu$M. a-Naphthoflavone (an inhibitor of CYP1A1/2 subfamilies), sulfaphenazole (an inhibitor of CYP2C8/9 subfamilies), S-mephenytoin (an inhibitor of the CYP2C19 subfamily), diethyldithiocarbamate (an inhibitor of the CYP2E1 subfamily), and troleandomycin (an inhibitor of CYP3A subfamilies) had no inhibitory effect on DM-210 formation activity in male dog liver microsomes. Figure 6 shows the effects of selective cytochrome P450 inhibitors on the formation of M-2 (ketol). Quinidine, a-naphthoflavone, sulfaphenazole, and troleandomycin inhibited M-2 formation.

**DM-215 Formation Activity in Dog Liver Microsomes.** The biotransformation rate of olanexidine to 2-hydroxylated metabolite 2-octanol (DM-215) by dog liver microsomes was consistent with Michaelis-Menten kinetics, and Eadie-Hofstee plots of DM-215 formation activity demonstrated monophasic Michaelis-Menten kinetics (Fig. 7). The apparent $K_m$ and $V_{max}$ values for the formation of DM-215 were 20.8 $\mu$M and 737 pmol/min/mg, respectively. Quinidine, a specific inhibitor of CYP2D subfamilies, only inhibited the 2-hydroxylation of olanexidine (Table 1). Other CYP-specific inhibitors had no effect on this reaction.

**DM-221 and DM-222 Formation Activity in Dog Liver Microsomes.** The biotransformation rates of DM-215 to diol metabolites threo-2,3-octandiol (DM-221) and erythro-2,3-octandiol (DM-222) by dog liver microsomes were consistent with Michaelis-Menten kinetics, and Eadie-Hofstee plots of DM-221 or DM-222 formation activity demonstrated monophasic Michaelis-Menten kinetics (Fig. 8). The apparent $K_m$ and $V_{max}$ values for the formation of DM-221 and DM-222 were 36.5 $\mu$M and 20.8 pmol/min/mg, and 42.3 pmol/min/mg, respectively. Quinidine, a specific inhibitor of CYP2D subfamilies, only inhibited the hydroxylation of DM-215 (Table 2). Other CYP-specific inhibitors had no effect on the reaction.

**Discussion**

DM-210, a major metabolite of olanexidine, is produced via C-C bond cleavage following sequential oxidative reactions, but not $\beta$-oxidation, with possible involvement of cytochrome P450 systems (Umehara et al., 2000). However, there is no information identifying the enzyme(s) involved in the C-C bond cleavage of olanexidine or

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**Fig. 3.** ESI product ion mass spectrum of [M + H]$^+$ of metabolite M-2 at $m/z$ 402.

**Fig. 4.** Eadie-Hofstee plots for the formation of DM-210 by dog liver microsomes using DM-221 (A) or DM-222 (B) as substrate.

Each value of triplicate determinations using microsomes obtained from three different dog livers is shown.
the kinetics of metabolism. Characterizing the enzymes catalyzing
olaneoxide metabolism should contribute to clarification of the roles
and mechanism of oxidative C-C bond cleavage by cytochrome P450
in the metabolism of other drugs or endogenous compounds contain-
ing aliphatic chains. The present study shows the biotransformation of
olaneoxide to be catalyzed by male dog liver microsomes and char-
acterizes the enzyme(s) responsible for C-C bond cleavage reaction.

Olanexidine was initially monohydroxylated to DM-215, which
was subsequently oxidized to diol derivatives (DM-221 or DM-222).
Diols were further biotransformed to a ketol derivative and DM-210
(hexanoic acid derivative), an in vivo end product, in the incubation
with dog liver microsomes. The formation of DM-215, DM-221,
DM-222, and DM-210 was consistent with Michaelis-Menten kinet-
ics. Eadie-Hofstee analysis of the metabolite formation activity con-
ferred single-enzyme Michaelis-Menten kinetics, indicating that only
one cytochrome P450 isoform is involved in the metabolite formation.

The inhibition studies in dog liver microsomes using CYP isoform-
selective inhibitors indicated that these oxidative reactions are likely
mediated via the CYP2D subfamily. Several CYP inhibitors inhibited
ketol formation, indicating that cytochrome P450s are also involved
in the oxidation of diols to ketol.

Some cytochrome P450 enzymes integrated into biosynthetic path-
ways have catalytic activity not only in conventional hydroxylation
reactions but in C-C bond cleavage reactions as well (Akhtar et al.,
1993; Ortiz de Montellano, 1995). The C-20-C-22 bond of choles-
terol is cleaved by a mitochondrial P450 enzyme (CYP11A1) that
catalyzes three sequential oxidative steps, each consuming one mol-
ecule of oxygen and one of NADPH (Shikita and Hall, 1974; Byon
and Gutt, 1980; Ortiz de Montellano, 1995). The three steps are
22(R)-hydroxylation, 20(S)-hydroxylation, and severance of the
C-20-C-22 bond. There are two possible mechanisms for the
C-20-C-22 bond of cholesterol scission (Ortiz de Montellano, 1995).
In one, the activated oxygen complex ([Fe=O]) is intercepted by addition of a hydroxyl group of 20(R),22(R)-dihydroxycholesterol, followed by proton removal from the hydroxyl adjacent to the resulting hydroperoxide to initiate C-C bond cleavage. In the other mechanism, abstraction of the hydrogen from one of the two side chain hydroxyls by the activated oxygen complex could produce an alkoxy radical. Homolytic scission of the C-C bond in this species and electron transfer from the resulting carbon radical to the protonated iron-oxygen complex completes the reaction.

The 17α-hydroxylation of pregnenolone (or progesterone) and cleavage of the C-17-C-20 C-C bond in the 17α-hydroxysteroid to give dehydroepiandrosterone (or androstenedione) are catalyzed by CYP17 (Zuber et al., 1986; Barnes et al., 1991). Akhtar et al. (1993, 1994) propose that C-C bond cleavage reaction catalyzed by aromatase, lanosterol 14α-demethylase, and CYP17 occurs through the participation of the Fe\(^{3+}\).O-OH species produced as an intermediate in cytochrome P450 reactions and is trapped by the electrophilicity of the carbonyl compound to afford a peroxide adduct that fragments with consequent acyl-carbon cleavage.

In the case of olanexidine, the same mechanism involving the intermediate formation of a ferric peroxide complex (Fe\(^{3+}\).O-OH) for the bond cleavage catalyzed by CYP17 may be applicable to C-C bond cleavage of the octyl side chain of olanexidine, because the carbonyl compound [M-2 (ketol)] was observed in the reaction mixture in this study (Fig. 9, pathways A and B). Furthermore, the formation of DM-210 was not changed with inhibition of ketol formation (Figs. 5 and 6), suggesting that the same two mechanisms involving the intermediate formation of a high-valent oxoiron complex ([Fe=O]) for the bond cleavage catalyzed by CYP11A1 may be applicable to C-C bond cleavage of the octyl side chain of olanexidine (Fig. 9, pathways C and D). These oxidative reactions catalyzed by cytochrome P450s may be considered involved in the production of DM-212 and DM-213 in vivo.

The CYP2D subfamily is considered to be involved in the metabolism of the octyl side chain of olanexidine. The presence of some cytochrome P450s has been reported in dog, and characterization of cytochrome P450s by amino acid sequence has shown cytochrome P450s belonging to the CYP1A (Uchida et al., 1990), CYP2C (Uchida et al., 1990; Shiraga et al., 1994), CYP2D (Sakamoto et al., 1995), and CYP3A (Ciaccio et al., 1991) subfamilies to be present in dog liver. Catalytic activities using specific probes and inhibitors using specific
CYP-isoform inhibitors have been confirmed in dog (Sakamoto et al., 1995; Chauret et al., 1997). The canine CYP2D subfamily possesses enzymatic activities similar to human CYP2D6. Furthermore, quinidine and quinine, which are selective inhibitors of human CYP2D6 and rat CYP2D1, respectively, are both shown to be inhibitors of the CYP2D subfamily-catalyzed bufuralol 1-hydroxylase activity in dog liver microsomes with nearly equal potency (Roussel et al., 1998). The inhibition data in this study using quinidine are consistent with the proposed metabolism of olanexidine by the CYP2D subfamily. In the CYP2D subfamily-mediated catalytic reactions, it has been suggested that the CYP2D subfamily requires ion-pair formation between a substrate and the enzyme for effective catalytic activity (Smith, 1991; Smith and Jones, 1992) and, hypothetically, a positively charged basic nitrogen (pK_a 7.5) located 5 to 7 Å from the site of oxidation for typical CYP2D subfamily substrate (Strobl and Wolff, 1991). Olanexidine (pK_a 2.3 and 12) and its metabolites (DM-215, DM-221, and DM-222) have two basic nitrogen atoms in the biguanide structure, which is highly ionized at physiological pH. Therefore, they may be a typical substrate of the CYP2D subfamily. The stereoselectivity of oxidation is consistent for almost all CYP2D substrates (Smith and Jones, 1992). However, enantioselectivity was not apparent in DM-210 formation by dog liver microsomes. The apparent Michaelis-Menten constant K_m was found basically the same for the two enantiomers. The binding affinity of threo- and erythro-2,3-octandiol to CYP2D subfamily should thus not differ, and the site of oxidation rather than binding of the substrate may be under stereochemical control (Koymans et al., 1992).

As with the metabolic pathway of olanexidine, there are compounds whose metabolites contain side chains, in which odd and even numbers of carbon fragments have been removed by metabolism. The pentyl side chain of cannabidiol and its derivatives is reduced to two, three, or four carbon atoms by removal of even and odd numbers of carbon atoms (Harvey and Leuschner, 1985; Harvey, 1989, 1990; Samara et al., 1990). Sinz et al. (1997) found CI-976, a specific acyl coenzyme A:cholesterol acyltransferase inhibitor, to be metabolized to a 5- and 6-carbon cleavage metabolite in rat after oral administra-

### TABLE 2 Effects of various inhibitors on DM-221 and DM-222 formation with dog liver microsomes

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>DM-221 Formed</th>
<th>DM-222 Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.9 ± 0.1</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>α-Naphthoflavone</td>
<td>3.8 ± 0.1</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>4.1 ± 0.1</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>S-Mephenytoin</td>
<td>3.8 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>Quinidine</td>
<td>1.1 ± 0.0</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>4.2 ± 0.9</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>3.6 ± 0.0</td>
<td>3.8 ± 0.1</td>
</tr>
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Fig. 9. Possible mechanisms for the final step in the olanexidine side chain cleavage reaction.
tion. The removal of an even number of carbon fragments has been shown to result in β-oxidation in the metabolic pathways of these compounds (Harvey and Leuschner, 1985; Sinz et al., 1997). But the mechanism for removal of an odd number of carbon fragments still remains to be fully determined. The degraded metabolites with removal of an odd number of carbon fragments may possibly arise from pathways other than classical β-oxidation, and the metabolic routes may involve intermediates derived from monohydroxylation and dihydroxylation of the side chain (Harvey, 1989; Sumara et al., 1990; Woolf et al., 1990). Analogous with the production mechanism of C-C bond cleavage metabolites of oxanexidine, oxidative C-C bond cleavage by cytochrome P450s may be essential for the removal of an odd number of carbon fragments in the metabolism of these compounds.

In summary, the present in vitro study demonstrates that the metabolic C-C bond cleavage of the octyl side chain of oxanexidine is catalyzed by male dog liver microsomes with possible involvement of the CYP2D subfamily. Such aliphatic C-C bond cleavage by cytochrome P450s could play an important role in the metabolism of other drugs or endogenous compounds containing aliphatic chains.

References


Harvey DJ, Leuschner JTA (1985) Studies on the metabolic pathways of these compounds (Harvey and Leuschner, 1985; Sinz et al., 1997). But the mechanism for removal of an odd number of carbon fragments still remains to be fully determined. The degraded metabolites with removal of an odd number of carbon fragments may possibly arise from pathways other than classical β-oxidation, and the metabolic routes may involve intermediates derived from monohydroxylation and dihydroxylation of the side chain (Harvey, 1989; Sumara et al., 1990; Woolf et al., 1990). Analogous with the production mechanism of C-C bond cleavage metabolites of oxanexidine, oxidative C-C bond cleavage by cytochrome P450s may be essential for the removal of an odd number of carbon fragments in the metabolism of these compounds.

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