OXIDATIVE CLEAVAGE OF THE OCTYL SIDE CHAIN OF 1-(3,4-DICHLOROBENZYL)-5-OCTYLBIGUANIDE (OPB-2045) IN RAT AND DOG LIVER PREPARATIONS

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ABSTRACT:

The metabolism of 1-(3,4-dichlorobenzyl)-5-octylbiguanide (OPB-2045), a new potent biguanide antiseptic, was investigated using rat and dog liver preparations to elucidate the mechanism of OPB-2045 metabolite formation, in which the octyl side chain is reduced to four, five, or six carbon atoms. Chemical structures of metabolites were characterized by 1H NMR, fast atom bombardment/mass spectrometry, and liquid chromatography/electrospray ionization-tandem mass spectrometry. Three main metabolites were observed during incubation of OPB-2045 with rat liver S9: 2-octanol (M-1), 3-octanol (M-2), and 4-octanol (M-3). In the incubation of OPB-2045 with dog liver S9, eight metabolites were observed, seven of which being M-1, M-2, M-3, 2-octanone (M-4), threo-2,3-octandiol (M-5), erythro-2,3-octandiol (M-6), and 1,2-octandiol (M-7). M-5 and M-6 were further biotransformed to a ketol derivative and C-C bond cleavage metabolite (hexanoic acid derivative), an in vivo end product, in the incubation with dog liver microsomes. The reactions required NADPH as a cofactor and were significantly inhibited by the various inhibitors of cytochrome P450 (i.e., CO, n-octylamine, SKF 525-A, metyrapone, and a-naphthoflavone). The results indicate that the degraded products of OPB-2045 are produced by C-C bond cleavage after monohydroxylation, dihydroxylation, and ketol formation at the site of the octyl side chain with possible involvement of cytochrome P450 systems. This aliphatic C-C bond cleavage by sequential oxidative reactions may play an important role in the metabolism of other drugs or endogenous compounds that possess aliphatic chains.
abolic pathways leading to in vivo end product formation were further studied using in vitro metabolic intermediates as substrates.

Materials and Methods

Chemicals. OPB-2045, DM-210, M-1 (DM-215), M-2 (DM-218), M-3 (DM-217), M-4 (DM-219), M-5 (DM-221), M-6 (DM-222), and M-7 (DM-220) were provided by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). β-NADPH, β-NADH, β-NAD, β-NADP, and α-naphthoflavone were obtained from Sigma Chemical Co. (St. Louis, MO); glucose 6-phosphate and glucose 6-phosphate dehydrogenase (approx. 350 U/mg, 1 mg/ml) were purified from Research Biochemicals Inc. (Natick, MA); sodium phenobarbital and n-octylamine were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents and solvents were of analytical grade.

Animal Treatments and Preparation of 9000g Supernatant Fraction (S9) and Microsomes. Ten male Sprague-Dawley rats from Charles River Japan (Yokohama, Japan) were acclimatized in a controlled area maintained at 23 ± 2°C for temperature and 60 ± 10% for relative humidity during 12-h light/dark cycles. The animals were fed laboratory chow mouse food (MF) (Oriental Yeast Co., Ltd., Japan) and water was available ad libitum during acclimatization. Rats 12 weeks of age weighing 432 to 507 g were used in the experiments. The animals were fed laboratory chow CD-5 (Japan Clea Co., Ltd., Tokyo, Japan) and water was available ad libitum under acclimatization. Dogs 17 to 23 months of age were used in the experiments. The animals were sacrificed by decapitation and exsanguination under anesthesia with pentobarbital or phenobarbital. The rats were fasted on the final day of administration and sacrificed on the following day by decapitation and exsanguination. Laparotomy was immediately performed. The animals were fed laboratory chow mouse food (MF) and water was available ad libitum during acclimatization. Rats 12 weeks of age weighing 432 to 507 g were used in the experiments. The animals were sacrificed by decapitation and exsanguination under anesthesia with pentobarbital or phenobarbital. The reaction mixture was incubated for 60 min at 37°C. This procedure was carried out in duplicate and the reaction mixtures were combined and stored frozen at −80°C until use.

Incubation of OPB-2045 with Rat Liver S9. A NADPH-generating system (750 ml, 0.1 M Tris-HCl buffer, pH 7.4, containing 1 mM β-NADPH, 2 mM β-NADP, 16.9 mM glucose 6-phosphate, 0.75 ml of glucose 6-phosphate dehydrogenase (approx. 263 U), and 10 mM MgCl₂) and 5 ml of OPB-2045 in methanol solution at a concentration of 104 mg/ml (1.27 mM) were added to 250 ml of prepared rat liver S9. The mixture was incubated for 60 min at 37°C. This procedure was carried out in duplicate and the reaction mixtures were combined and stored frozen at −80°C until use.

Incubation of OPB-2045 with Dog Liver S9. The reaction mixtures contained 0.1 M phosphate buffer (pH 7.4), 10 mM β-NADPH, 245 μM OPB-2045, and 250 μl of dog S9 protein in a final incubation volume of 0.5 ml. OPB-2045 was dissolved in methanol. Reactions were carried out in air at 37°C in a shaking water bath for 45 min. The reaction was stopped by addition of 1 ml of methanol. The mixture was centrifuged at 140g for 10 min and the supernatant was evaporated to dryness. The residue was dissolved in 200 μl of methanol, 30 μl of which was analyzed by HPLC as described below.

Incubation of M-1 with Dog Liver S9. A NADPH-generating system (500 ml, 0.1 M Tris-HCl buffer, pH 7.4, containing 0.5 mM β-NADPH, 2 mM β-NADP, 20 mM glucose 6-phosphate, 1 ml of glucose 6-phosphate dehydrogenase (approx. 350 U), and 10 mM MgCl₂) and 0.5 ml of M-1 in methanol solution at a concentration of 200 mg/ml (362 μM) were added to 150 ml of prepared dog liver S9. The mixture was incubated for 3 h at 37°C. This procedure was carried out in duplicate and the reaction mixtures were combined and stored frozen at −80°C until use.

Isolation Procedure of In Vitro Metabolites. M-1, M-2, and M-3 were isolated from the reaction mixture with rat liver S9 and OPB-2045 by liquid-liquid extraction, solid-phase extraction, and HPLC. The reaction mixture was extracted by first adding 3 volumes of methanol. The methanol-soluble extract was evaporated to dryness and the residue was dissolved in 350 ml of methanol. Methylene chloride was added to the methanol solution to obtain a methylene chloride/methanol solution (9:1, v/v), which was then applied to 96 Sep-Pak Vac silica cartridges (20cc; Waters, Milford, MA). The cartridges were washed with the methylene chloride and methanol solution (9:1, v/v), and eluted with the methylene chloride and methanol solution (1:1, v/v). The eluent was dried under reduced pressure. The residue was suspended in 50 ml of acetic acid. Water was added to make a 10% aqueous acetic acid solution, which was applied to 48 Sep-Pak Vac C₁₈ cartridges (20cc; Waters). Elution was performed in a stepwise fashion using 1% acetic acid aqueous solution, 25% methanol in water containing 1% acetic acid, 50% methanol in water containing 1% acetic acid, 60% methanol in water containing 1% acetic acid, and 70% methanol in water containing 1% acetic acid. The resulting 70% methanol elution fraction was evaporated to dryness and dissolved in 1 ml of methanol to further purify the metabolites by HPLC. This fraction contained metabolites M-1, M-2, and M-3.

M-4, M-5, M-6, and M-7 were isolated from the reaction mixture with dog liver S9 and OPB-2045 by liquid-liquid extraction, solid-phase extraction, and HPLC. The reaction mixture (300 ml) was extracted by further adding 700 ml of methanol. The methanol-soluble extract was evaporated to dryness and the residue was dissolved in 20 ml of methanol. Methylene chloride was then added to the methanol solution to obtain a methylene chloride/methanol solution (9:1, v/v) solution, which was then applied to eight Sep-Pak Vac silica cartridges (20cc; Waters). The cartridges were washed with the methylene chloride and methanol solution (9:1, v/v), and eluted with the methylene chloride and methanol solution (1:1, v/v). The eluent was dried under reduced pressure. The residue was dissolved in 350 ml of acetic acid. Water was added to make a 10% aqueous acetic acid solution, which was applied to 48 Sep-Pak Vac C₁₈ cartridges (20cc; Waters). Elution was performed in a stepwise fashion using 1% acetic acid aqueous solution, 25% methanol in water containing 1% acetic acid, 50% methanol in water containing 1% acetic acid, 60% methanol in water containing 1% acetic acid, and 70% methanol in water containing 1% acetic acid. The resulting 70% methanol elution fraction was evaporated to dryness and dissolved in 1 ml of methanol to further purify the metabolites by HPLC. This fraction contained metabolites M-1, M-2, and M-3.

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1% acetic acid. The methylene chloride and methanol (85:15, v/v) elution fraction thus obtained was evaporated to dryness and dissolved in 0.5 ml of methanol to further purify the metabolites by HPLC. This fraction contained metabolite M-4. The methanol containing 1% acetic acid elution fraction was evaporated to dryness and dissolved in 1 ml of methanol to further purify the metabolites by HPLC; it contained metabolites M-5, M-6, and M-7.

**HPLC.** The HPLC system consisted of two model 510 high-pressure HPLC pumps (Waters), a model 717 automatic sample processor (Waters), a model U6K universal injector (Waters), model 484 or 486 UV detectors (Waters), a model 680 automatic gradient controller (Waters), and model C-R3A, C-R6A, or C-R7A Chromatopacs (Shimadzu, Kyoto, Japan).

In the analysis of metabolite production in the reaction mixtures with OPB-2045 and rat liver S9, 2 ml of the mixture was extracted by adding 6 ml of methanol, and 3 ml of the extract was evaporated to dryness. The residue was dissolved in 300 μl of methanol, 30 μl of which was injected into the HPLC system fitted with a column of TSKgel ODS-80TM (4.6 mm i.d. × 150 mm; Tosoh, Tokyo, Japan), with flow rate and detection wavelength set to 1 ml/min and UV-240 nm, respectively. 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. Elution was conducted with a 1-h linear gradient between solutions A and B.

In the analysis of metabolite production in the reaction mixtures with OPB-2045 and dog liver S9, 0.5 ml of mixture was extracted by adding 1 ml of methanol, and the extract was evaporated to dryness. The residue was dissolved in 200 μl of methanol, 30 μl of which was analyzed by HPLC. HPLC conditions were the same as above.

M-1, M-2, and M-3 were purified using HPLC equipped with a TSKgel ODS-80TM (7.8 mm i.d. × 300 mm; Tosoh) column. The flow rate was 2.5 ml/min and detection wavelength was 240 nm. The fraction containing the three metabolites was analyzed by isocratic elution of 27% solution B so as to bring about their separation. The retention times of M-1, M-2, and M-3 were 12.7, 13.5, and 16.1 min, respectively. HPLC purification was repeated by the same isocratic elution to isolate M-1, M-2, and M-3. The metabolites were analyzed using HPLC using isocratic elutions of 30% and 33% solution B, respectively.

M-4, M-5, M-6, and M-7 were purified using HPLC with a TSKgel ODS-80TM (7.8 mm i.d. × 300 mm; Tosoh) column, at a flow rate of 2.0 ml/min and a detection wavelength of 240 nm. The fraction containing the four metabolites was analyzed using a linear gradient developed from solution A to B over a period of 30 min. The retention times of M-4 and the mixture of M-5, M-6, and M-7 were 18.7 and 16.6 min, respectively. The fraction containing M-5, M-6, and M-7 was further analyzed using a linear gradient developed from 10 to 100% solution B over a period of 60 min. The retention times of M-5, M-6, and M-7 were 17, 19, and 20 min, respectively. Each fraction containing M-5, M-6, and M-7 was analyzed using a linear gradient developed from solutions A to B over a period of 60 min, once or twice.

**3H NMR and Mass Spectral Analysis.** The 3H NMR spectra were measured with a Bruker AC-200 NMR spectrometer (Karlsruhe, Germany) with tetramethylsilane as the internal standard. All samples were dissolved in DMSO-d6. The FAB mass spectrum was measured with a JMS-SX102A mass spectrometer and LMA-DA-6000 data system (JEOL, Tokyo, Japan), with a direct inlet using glycerol as the matrix.

**In Vitro Metabolism of M-5 and M-6 with Dog Liver Microsomes.** The reaction mixtures contained 0.1 M phosphate buffer (pH 7.4), 5 mM β-NADPH, 5 mM β-NADH, 210 μM M-5 or M-6, and 250 μM of dog microsomal protein in a final incubation volume of 0.5 ml. M-5 or M-6 was dissolved in methanol. Reactions were carried out in air at 37°C in a shaking water bath for 60 min. The reaction mixtures were extracted by adding 1 ml of methanol, and the extract was evaporated to dryness. The residue was dissolved in 200 μl of methanol, and an aliquot (10–20 μl) was analyzed by HPLC-UV and LC/ESI-MS/MS for characterization of the chemical structures of the metabolites.

**HPLC-UV analysis.** HPLC analysis was carried out with the same Waters HPLC as above. A TSKgel ODS-80TM column (4.6 mm i.d. × 150 mm; Tosoh) was used at a flow rate of 1 ml/min with detection at 240 nm. The mobile phase used was a solution of 10% acetonitrile in water containing 0.1% acetic acid 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 0 to 50% solution B over a period of 45 min.

**Results**

In Vitro Metabolism of OPB-2045 by Rat Liver S9. A representative HPLC chromatogram of OPB-2045 metabolites produced by rat liver S9 in the presence of NADPH is shown in Fig. 2. Detected UV peaks were primarily found for three types of metabolites in the reaction mixture: M-1, M-2, and M-3. These metabolites were further isolated and purified, and chemical structures were determined from...
**In Vitro Metabolism of OPB-2045 by Dog Liver S9.** A representative HPLC chromatogram of OPB-2045 metabolites produced by dog liver S9 is shown in Fig. 3. Detected UV peaks were primarily found for eight types of metabolites in the reaction mixture: M-1, M-2, M-3, M-5, M-6, M-7, M-8, and M-9. The UV peaks were characterized by their retention times and absorbance maxima. The FAB/MS spectra of the metabolites were recorded to confirm their structures. The 1H NMR spectra of the metabolites were also recorded to determine their chemical shifts and proton resonances.

**Materials and Methods.**

The UV peaks were identified by comparing their retention times and absorbance maxima with those of the standards. The FAB/MS spectra were recorded using a matrix of ammonium acetate and an accelerating voltage of 7 kV. The 1H NMR spectra were recorded using a Bruker Avance 400 spectrometer at a frequency of 400 MHz. The samples were dissolved in DMSO-d$_6$ and referenced to TMS as an internal standard.

**Fig. 3. HPLC chromatogram of the extract obtained from the incubation mixture of OPB-2045 with dog liver S9.**

OPB-2045 (245 μM) was incubated at 37°C with dog liver S9 in the presence of 10 mM NADPH in 0.1 M phosphate buffer (pH 7.4) for 45 min. HPLC was performed with methanol extract from the incubation mixture as described under Materials and Methods.
M-7. The FAB/MS spectrum of M-7 exhibited a protonated molecular ion $[M+H]^+$ at $m/z$ 404 (28% abundance). The $^1$H NMR ($\delta$ in ppm) of M-7 in DMSO-$d_6$ was as follows: 1.04 to 1.52 (m, 13H), 2.93 (br. s, 2H), 4.27 (br. s, 2H), 7.26 (br. d, $J = 6.2$ Hz, 1H), 7.52 (br. s, 1H), and 7.56 (d, $J = 6.2$ Hz, 1H). M-7 has a molecular weight of 403, which is 16 greater than that of the unchanged M-1. It was thus thought to be the monohydroxylated form of M-1, as in the case of M-5 or M-6. The $^1$H NMR spectrum showed the disappearance of a doublet proton signal ($\delta = 1.01$) for the terminal methyl group on the M-1 alkyl chain. This suggests that M-7 is a metabolite in which the alkyl chain of M-1 is hydroxylated at the 8 position. DM-220, 8-[5-(3,4-dichlorobenzyl)-1-biguanidino]-1,2-octandiol, appeared on the cochromatogram with M-7 in HPLC analysis. FAB mass and $^1$H NMR spectra of metabolite were identical with those of the prepared standard, DM-220.

In Vitro Metabolism of M-5 and M-6 by Dog Liver Microsomes. M-5 was incubated with dog liver microsomes in the presence of NADPH and NADH, and the methanol extract was analyzed by HPLC-UV and LC/ESI-MS/MS. In the reaction mixture, three metabolites were observed: M-9, M-10, and M-11 (Fig. 4).

M-9 and M-10 were identified as M-6 and DM-210, respectively, because their $[M+H]^+$ ions in the precursor ion mass spectra, the product ion mass spectra (Fig. 5), and HPLC retention time were in good agreement with those for the authentic standards. M-11 displayed molecular ions of $m/z$ 402 $[M+H]^+$ in the precursor ion mass spectrum, indicating this metabolite to have a molecular weight of 401, this being 2 smaller than that of the unchanged M-5. In addition, the characteristic fragment ions at $m/z$ 218 and 202 were observed in the product mass spectrum (Fig. 6). M-11 would thus appear a ketol derivative of M-5, whereas it is not clear which hydroxy group of M-5 is oxidized to a ketone. On using M-6 as substrate in the same reaction system, M-5, DM-210, and a ketol derivative of M-6 (M-11) were obtained.

The cofactor requirement for microsomal formation of DM-210 was examined (Table 1). The reactions required NADPH for maximal activity. NADH was much less effective for the catalytic activity, and NAD and NADP were ineffective as the cofactor. The reaction was significantly inhibited by the addition of CO gas and n-octylamine, typical inhibitors of the cytochrome P450 system (Minato et al., 1999) (Table 2). Both SKF 525-A and metyrapone, known to be inhibitors of phenobarbital-inducible cytochrome P450 isozymes (Lu et al., 1972; Buening and Franklin, 1974), completely inhibited the DM-210 formation. $\alpha$-Naphthoflavone, an inhibitor of CYP1A1/2 (Chang et al., 1994; Newton et al., 1995), inhibited this reaction by 60% at 1 mM.
in the incubation of these compounds with rat liver microsomes (data not shown). The DM-210 formation required NADPH as a cofactor, and it was inhibited by the cytochrome P450 inhibitors; this indicates that this reaction is catalyzed by the cytochrome P450 system. The ketol may possibly be M-8 found in the incubation of OPB-2045 or M-1 with dog liver S9, although this remains to be confirmed. In the reaction mixture, the conversion of M-5 to M-6 or vice versa was observed. These conversions may occur via a ketol derivative and may be catalyzed by ketone reductase located in the microsomes. Thus, these experiments have shown that degraded products of OPB-2045 can arise from C-C bond cleavage after sequential oxidative reactions at the octyl side chain of OPB-2045.

There are two possible mechanisms for C-C bond cleavage in biotransformation: one is β-oxidation after hydroxylation and oxidation at the terminal carbon atom of the chain, and the other is oxidative cleavage by cytochrome P450 (Shikita and Hall, 1973a,b; Watabe and Akamatsu, 1975; Takikawa et al., 1978; Kashiwagi et al., 1980; Nakajin et al., 1981; Nakajin and Hall, 1981; Akhtar et al., 1993). β-Oxidation, as well known in fatty acid metabolism, conducts C-C bond cleavage of successive two-carbon fragments starting from the carboxy-terminal, leading to fatty acids with an even number of carbon atoms in the methylene chain. In contrast, OPB-2045 metabolites biotransformed at the octyl side chain have both odd- and even-carbon chains, and a metabolite of a compound with octanoic acid cannot be detected in the excreta of rats and dogs (data not shown). In addition, 1-octanol was not observed in the incubation of OPB-2045 with rat or dog liver S9. These evidences indicate that β-oxidation is not responsible for the metabolic degradation of OPB-2045.

Some cytochrome P450 systems have been reported to have catalytic activity not only in conventional hydroxylation reactions but also in the oxidation of an alcohol to a carbonyl compound (Tyndale et al., 1991) and is also involved in C-C bond cleavage (Akhtar et al., 1993). Removal of the pregnenolone side chain to produce dehydroisoandrosterone is mediated by the cytochrome P450 system (Corina et al., 1991; Miller et al., 1991). Watabe and Akamatsu (1975) have shown that sequential steps of the oxidative reaction by rabbit liver microsomes are involved in the cleavage of ethylenic double bonds of stilbene. Stilbenes are converted by rabbit liver microsomes to glycols via epoxides and the resultant glycols are oxidized to benzoin ketol and benzil diketone. C-C bonds of the ketol or diketone are enzymatically cleaved with benzoic acid as an end product. Cytochrome P450 in microsomes may possibly be involved in the metabolism of stilbene because NADPH is essential for metabolic conversion of the compound (Watabe and Akamatsu, 1972, 1974, 1975).

DM-210 was found to be produced by sequential oxidative reactions, indicating a C-C bond cleavage mechanism similar to that in stilbene metabolism to likely be involved in DM-210 production (Fig. 7). Namely, OPB-2045 is initially monohydroxylated to M-1 and then oxidized to the ketol (M-8) via formation of the diol (M-5 or M-6). The ketol is biotransformed to the end product, DM-210, by C-C bond cleavage. These sequential oxidative reactions may be considered involved in the production of DM-212 and DM-213 in vivo (Fig. 7). The enzymes in this process are considered to be cytochrome P450s. CYP4A, CYP1A, CYP2C, CYP2E, and CYP3A isoforms, which catalyze the metabolism of fatty acids (Kimura et al., 1989; Falck et al., 1990; Tanaka et al., 1990; Yokotani et al., 1991; Laethem et al., 1993), may be involved in the oxidation. Further investigation concerning cytochrome P450 isoform(s) involved in C-C bond cleavage of the octyl side chain of OPB-2045 is now in progress.
cleavage reaction occurs through the participation of the Fe⁺³-O-OH species that is produced as an intermediate in cytochrome P450 reactions and is trapped by the electrophilicity of the carbonyl compound to afford a peroxy adduct that fragments with consequent acyl-carbon cleavage. The same mechanism may be applicable to C-C bond cleavage of the octyl side chain of OPB-2045, because the carbonyl compound was observed here in the reaction mixture.

As with the metabolic pathway of OPB-2045, 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; Harvey and Mechoulam, 1990; Samara et al., 1990). Sinz et al. (1997) have shown that CI-976, a specific acyl coenzyme A/cholesterol acyltransferase inhibitor, is metabolized to a 5- and 6-carbon cleavage metabolite in rats after oral administration. The metabolic route of these compounds to the degraded metabolites with removal of an even number of carbon fragments has been shown to be \( \beta \)-oxidation after hydroxylation and oxidation at the terminal carbon atom of the chain (Harvey and Leuschner, 1985; Sinz et al., 1997). On the other hand, the mechanism for removal of an odd number of carbon fragments remains only partially characterized, but the metabolites of these compounds may possibly arise from pathways other than classical \( \beta \)-oxidation and involve intermediates derived from hydroxylation of the side chain (Harvey, 1989; Samara et al., 1990; Wolff et al., 1990). Oxidative C-C bond cleavage by sequential oxidative reactions may thus be essential for removal of an odd number of carbon fragments.

In summary, this study demonstrates that the degraded products of OPB-2045 are produced by C-C bond cleavage after sequential oxidative reactions, but not \( \beta \)-oxidation, with possible involvement of cytochrome P450 systems. Such aliphatic C-C bond cleavage by sequential oxidative reactions could play an important role in the metabolism of other drugs or endogenous compounds containing aliphatic chains.

References


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