Short Communication

N-OXYGENATION OF CLOZAPINE BY FLAVIN-CONTAINING MONOOXYGENASE

ABSTRACT:
The involvement of FMO in the N-oxygenation of CLZ was investigated by use of purified FMOs and human liver microsomes that contained the mean amount of immunoreactive FMO3 relative to other human liver microsomal preparations in a liver bank. In the microsomal preparation the involvement of FMO was indicated through enzyme inhibition by methimazole, heat inactivation, and protection against heat inactivation by NADPH. Also the Michaelis-Menten kinetic constant; \( K_m \) determined for CLZ N-oxygenation catalyzed by purified human FMO3 (324 \( \mu \)M) was very similar to the mean value obtained in these laboratories for the microsomal preparations of seven human livers.

CLZ,\(^1\) an antipsychotic drug with a novel mode of action, undergoes extensive hepatic metabolism in humans (1). The major metabolites in human include CLZ N-oxide and N-desmethyl-CLZ that result from oxidation of the tertiary amine N-methyl group (fig. 1). Many other metabolites resulting from phase I and phase II reactions have been identified in humans and rats (2, 3). Ongoing in vitro studies in these laboratories,\(^2\) and studies reported in the literature (4–6), indicate that CYP enzymes—including CYP1A2 and CYP3A4—play a major role in the metabolism of CLZ to both the N-oxide and N-desmethyl metabolites. In comparison with CYP-mediated biotransformations in humans, far less is known concerning the involvement of adult human liver FMO-dependent oxygenation, albeit a number of studies have shown that adult human liver microsomes are capable of tertiary amine N-oxygenation (7, 8). The microsomal FMO enzyme family is comprised of five isoforms: FMO1–FMO5 (9). Currently, there is evidence for several FMOs in human liver; however, FMO3 seems to be the dominant isozyme expressed in adult human liver microsomes (10). Recently, it was observed that human FMO3 catalyzes the N-oxygenation of the structurally related drug olanzapine (11). Also, the FMO-mediated N-oxygenation of N-methyl-N-substituted piperazine side chains of xenobiotics occurs preferentially at the N-methyl nitrogen atom (12). The objective of the current investigation was to examine the involvement of FMO in the metabolism of CLZ, with particular reference to the N-oxygenation and N-desmethylation metabolic pathways.

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1 Abbreviations used are: CLZ, clozapine; CYP, cytochrome P450; FMO, flavin-containing monooxygenase; TCA, trichloroacetic acid.

2 M. Tugnait et al., unpublished observations.


Send reprint requests to: Dr. Edward M. Hawes, College of Pharmacy and Nutrition, University of Saskatchewan, 110 Science Place, Saskatoon, SK, S7N 5C9, Canada.

Fig. 1. Chemical structures of (I) CLZ, (II) N-desmethyl-CLZ, and (III) CLZ N-oxide.

Materials and Methods
CLZ was kindly supplied by Sandoz, Canada, Inc., whereas the N-oxide and N-desmethyl metabolites were synthesized in these laboratories by established methods (13). Both metabolites were identified and characterized based on comparison of the melting point, TLC, HPLC, \(^1\)H-NMR, \(^1\)C-NMR, FAB/MS, and ES/MS data to that of the authentic samples. NADPH, methimazole, and clomipramine hydrochloride were purchased from Sigma-Aldrich Canada Ltd. (Mississauga, Ontario, Canada), whereas \( N \), \( N \)-dimethylclozapine was from Aldrich Chemical Co., Inc. (Milwaukee, WI). Prochlorperazine dimeinate was supplied by SmithKline French (lot 10-OPDM). HPLC-grade acetanilide and dichloromethane were purchased from BDH Chemicals (Vancouver, British Columbia, Canada). All other chemicals and reagents were of reagent-grade quality. The KIMBLE glass culture tubes (16 mm \( \times \) 125 mm) used in these studies were purchased from VWR, Canada. All glassware used for sample preparation was silanized.

Liver and Enzyme Preparations. The human liver microsomes HL112 were prepared and assessed for FMO viability according to the method of Sadeque et al. (14). These microsomes were found to contain the mean amount of immunoreactive FMO3 relative to other human liver microsomal preparations in the Liver Bank established at the Department of Medicinal Chemistry, University of Washington (Seattle, WA) (14). CYP content was determined by the method of Estabrook et al. (15). Specific FMO enzymes (minipig FMO1, rabbit FMO2, and human FMO3) were purified according to the methods of Rettie et al. (16) and Haining et al.\(^3\). The FAD concentrations were determined...
by the method of Faeder and Siegel (17). The microsomes and purified enzymes were stored at −70°C until used.

**Investigations of the Involvement of FMO in the Metabolism of CLZ: Incubations of CLZ with Purified FMOs.** Incubations with minipig FMO1, rabbit FMO2, and human FMO3 were conducted in a volume of 1 ml. A typical incubation mixture contained 25 mM potassium pyrophosphate, 100 mM glycine (pH 8.5), 50 pmol of the purified FMO, and 0.5 mM NADPH. The mixture was equilibrated in a shaking water bath for 5 min, after which the reaction was initiated by addition of CLZ (500 μM final concentration in the incubation volume of 1 ml). Control incubations were conducted in the absence of FMO and/or NADPH. The reaction was terminated after 20 min by the addition of 75 μl of 1 M TCA with cooling on ice. Incubated samples were spiked with 40 μl of the internal standard, clomipramine (2 μg) and 30 μl of prochlorperazine (3 μg) serving as a carrier. The pH of the incubation mixture was adjusted to 9.5, with 500 μl of 0.65 M sodium carbonate and extracted with 5 ml of dichloromethane for 15 min. After centrifugation for 10 min, the aqueous layer was aspirated to waste, and the organic layer evaporated to dryness under a gentle stream of N₂ at 35°C. Residues were reconstituted in the mobile phase, and an aliquot of 100 μl was injected onto the column.

**Kinetic Studies of CLZ Metabolism by Purified Human FMO3.** Preliminary experiments were performed in human liver microsomes to establish the linearity of CLZ N-oxide metabolite formation with respect to time of incubation and amount of enzyme added (data not shown). Incubations were conducted at 37°C in a final volume of 250 μl. Time dependency was determined from incubations of 25, 250, and 750 μM CLZ with 200 μg microsomal protein for 1–60 min. Formation of CLZ N-oxide was linear up to at least 60 min. Protein dependency was determined from incubations of 25, 250, and 750 μM CLZ for 30 min, with 25–500 μg microsomal protein. Formation of CLZ N-oxide was linear up to 400 μg microsomal protein. For the kinetic study, a typical incubation mixture consisted of 0.5 M Tris buffer (pH 7.8), 84 pmol of human FMO3, 6 mM MgCl₂, and CLZ added, in the concentration range of 1–1000 μM (i.e., 1, 2, 5, 10, 20, 50, 75, 100, 250, 500, 750, and 1000 μM). The reaction was initiated by the addition of NADPH at a final concentration of 5 mM in the incubation volume and stopped after 30 min by the addition of 75 μl of 1 M TCA with cooling on ice. Incubated samples were spiked with 40 μl of the internal standard, clomipramine (2 μg) and 30 μl of prochlorperazine (3 μg) serving as a carrier. Samples were extracted and reconstituted as described herein before analysis by HPLC.

**Incubations of CLZ with Human Liver Microsomes HL112.** To investigate further the involvement of FMO in the metabolism of CLZ, experiments were conducted with human liver microsomes HL112. In the heat inactivation experiment, the time of addition of NADPH to the incubation mixture containing 200 μg microsomal protein differed from that described previously. While NADPH was added to one set of samples, it was omitted from the other set. Both sets of samples (with or without NADPH) were then incubated in a shaking water bath at 45°C. After exactly 5 min, all samples were placed on ice, and CLZ (750 μM) was added to each sample. Those samples without NADPH were reconstituted with the cofactor, and all samples were incubated at 37°C for 30 min. A control was included in the heat experiment such that the N-oxidation of CLZ at 37°C was determined in the absence of any preincubation. The effect of methimazole on N-oxidation of CLZ was investigated in the same microsomal preparation HL112 (200 μg microsomal protein) using three substrate concentrations (100, 350, and 500 μM CLZ) and six inhibitor concentrations (0, 10, 25, 50, 100, and 150 μM methimazole), with an incubation time of 30 min. All samples were extracted and reconstituted in the mobile phase as described previously before analysis by HPLC.

**HPLC Assay Conditions for Quantifying CLZ Metabolites.** The HPLC system consisted of a Dell Optiplex computer system 466/6L using Millenium software (Waters Canada Ltd., Mississauga, Ontario, Canada), a solvent delivery pump (Waters model 590), a Shimadzu SCL-10A autosampler equipped with a 200 μl loop, and a variable-wavelength UV absorbance detector (Waters model 480) set at 260 nm with a sensitivity range of 0.005 absorbance unit full scale. Aliquots of 100 μl from the incubations described herein were injected on to a Spherisorb CN (Phase Separations, Inc., Waters Canada Ltd.) column (5 cm × 4.6 mm i.d., 3-μm particle size). The mobile phase consisted of 70% buffer (10 mM ammonium acetate adjusted to pH 5.0 with acetic acid) and 30% acetonitrile, and N-N-dimethylectylamine (200/500 ml mobile phase), and was delivered at a flow rate of 0.8 ml/min. The mobile phase was degassed

before use (HVLP-type membrane filters; Millipore Canada Ltd., Mississauga, Ontario, Canada). Under these chromatographic conditions, CLZ N-oxide, N-desmethyl-CLZ, CLZ, clomipramine, and prochlorperazine eluted at 4.7, 9.6, 12.0, 22.1, and 34.2 min, respectively.

**Calculation of Vₘₐₓ, K_M.** Kinetic parameters for the formation of CLZ N-oxide were determined using models for the participation of one and two enzymes through the use of a nonlinear regression analysis data program Enzfitter (Biosoft, Cambridge, UK), and user-defined equation for the two-enzyme model using nonlinear regression analysis (Delta Graph, DeltaPoint, Inc., Monterey, CA).

**Results and Discussion.**

The potential contribution of FMO to the metabolism of CLZ was investigated in vitro following incubations of CLZ with purified preparations of minipig FMO1, rabbit FMO2, and human FMO3. Results revealed extensive N-oxidation of CLZ (respective turnovers of 57.6, 19.5, and 15.3 nmol/nmol enzyme/min with minipig FMO1, rabbit FMO2, and human FMO3); however, no N-desmethylation was observed (fig. 2), suggesting that FMO can catalyze the oxidation of CLZ to the N-oxide but not to the N-desmethyl metabolite. The Michaelis-Menten kinetic constants for CLZ N-oxidation catalyzed by purified FMO3 indicated a Vₘₐₓ of 324 μM and a Vₘₐₓ of 12.8 nmol/nmol enzyme/min (76.9 nmol/mg protein/min) (fig. 3). The K_M for CLZ N-oxidation catalyzed by purified human FMO3 was of the same order of magnitude as that obtained in the microsomal fraction of human livers (mean K_M = 336 ± 61.7 μM; N = 7; range = 270–453 μM). However, as would be expected, the Vₘₐₓ for the purified enzyme was far greater than that obtained for these human
liver preparations (mean \( V_{\text{max}} = 0.36 \pm 0.23 \ \text{nmol/mg protein/min}; \ N = 7; \ \text{range} = 0.09–0.57 \ \text{nmol/mg protein/min}). The Eadie-Hofstee plots (not shown) from data obtained in human liver microsomal incubations suggested the involvement of two enzymes in the formation of CLZ \( N \)-oxide. However, when data were fit to the one- and two-enzyme models for all preparations examined, the single enzyme model resulted in a better fit of the data.

Relative contributions of the CYP and FMO families in CLZ \( N \)-oxidation were further investigated in a human liver microsomal preparation HL112 using metabolism inhibitors. A decrease in CLZ \( N \)-oxide formation to 17% of the control caused by preincubation at 45°C for 5 min in the absence of NADPH, suggests a heat-mediated inactivation of the FMO that was protected when NADPH was included (table 1). The extent of CLZ \( N \)-demethylation was measured in the same experiment to ensure that the heat inactivation did not compromise CYP activity (18, 19). The retention of 60.2% activity for \( N \)-demethylation of CLZ under these conditions is comparable with that reported for similar control experiments. CLZ \( N \)-oxide formation was inhibited in the presence of methimazole, an alternate-substrate competitive inhibitor of FMO (table 1). Formation of \( N \)-desmethyl-CLZ showed a similar profile albeit a much lower degree of substrate inhibition that is compatible with the fact that methimazole can also inhibit CYP enzymes in addition to FMO (20). Methimazole inhibition data also gave a two-enzyme profile for CLZ \( N \)-oxide formation in microsomes: one with a high sensitivity to the inhibitor and the other with a much lower affinity supporting the previously described biphasic Michaelis-Menten kinetics observed for CLZ \( N \)-oxide in human liver microsomes. In summary, the results of this study demonstrate clearly that human FMO3 has the capacity to form CLZ \( N \)-oxide. Microsomal data indicating the involvement of FMO in the \( N \)-oxidation of CLZ contradicts the findings of PirMohamed et al. (5).

A possible explanation for this discrepancy may be the lack of FMO viability in the microsomal preparations used in the latter studies (5). Although it is clear from the present study that FMO plays a role in the formation of CLZ \( N \)-oxide in vitro, further studies are required to clarify the situation in vivo. A major reason for this is that the \( K_M \) value observed for human FMO3 (324 \( \mu \)M) is 2 orders of magnitude greater than the therapeutic CLZ plasma concentrations (~1 \( \mu \)M) encountered in patients (21).

The involvement of both FMO and CYP-dependent monooxygenases is observed frequently in the metabolism of xenobiotics; however, the lack of an inhibitor selective for FMOs remains a major impediment to definitive studies on the relative role of these enzymes in the metabolism of xenobiotics. The involvement of specific CYP isozymes in particular metabolic pathways is probed by using a wide array of in vitro techniques. Comparable approaches for evaluating the contribution of FMO include the determination of kinetics using the purified enzyme to complement well established characteristics of FMO-mediated reactions in microsomal preparations, including enzyme inhibition by methimazole, heat inactivation, and protection against heat inactivation by NADPH (22). The use of these techniques in the present study gave indication that FMO is involved in the \( N \)-oxidation of CLZ and can be extended to evaluate the relative contributions of CYP and FMO in the metabolism of other drugs that can potentially be metabolized by both enzyme systems.

**References**


