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, 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, 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 isoform 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-demethylation 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.


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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, $^{13}$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-dimethylcylamine was from Aldrich Chemical Co., Inc. (Milwaukee, WI). Prochlorperazine dimaleate was supplied by SmithKline & French (lot 12-OPDM), HPLC-grade acetone 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 0.1 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 micromolar concentration 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 micromolar 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 supplemented 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 micromolar preparation HL112 (200 µg micromolar 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 Deltaplex 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 x 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), 30% acetonitrile, and N, N-dimethylethylamine (200/500 ml mobile phase), and was delivered at a flow rate of 0.8 ml/min. The mobile phase was degassed before use (HVL-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 $V_{\text{max}}$, $K_M$. 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 $K_M$ of 324 µM and a $V_{\text{max}}$ 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 micromolar fraction of human livers (mean $K_M = 336 ± 61.7$ µM; $N = 7$; range = 270–453 µM); however, as would be expected, the $V_{\text{max}}$ for the purified enzyme was far greater than that obtained for these human
greater than the therapeutic CLZ plasma concentrations (~1 µM) 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


TABLE 1
Incubations of CLZ with human liver microsomes HL112: evidence for FMO involvement in CLZ N-oxide formation

<table>
<thead>
<tr>
<th>CLZ Incubation Conditions</th>
<th>CLZ N-oxidation</th>
<th>CLZ N-demethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td>pmol/mg protein/min</td>
<td>pmol/mg protein/min</td>
</tr>
<tr>
<td>750</td>
<td>Control</td>
<td>368.1</td>
</tr>
<tr>
<td>750</td>
<td>Heat with NADPH</td>
<td>369.2 (100.3)</td>
</tr>
<tr>
<td>750</td>
<td>Heat without NADPH</td>
<td>62.7 (17.0)</td>
</tr>
<tr>
<td>500</td>
<td>Control</td>
<td>242.6</td>
</tr>
<tr>
<td>500</td>
<td>Methimazole (10 µM)</td>
<td>197.1 (81.2)</td>
</tr>
<tr>
<td>500</td>
<td>Methimazole (25 µM)</td>
<td>163.1 (67.2)</td>
</tr>
<tr>
<td>500</td>
<td>Methimazole (50 µM)</td>
<td>142.7 (58.8)</td>
</tr>
<tr>
<td>500</td>
<td>Methimazole (100 µM)</td>
<td>120.8 (49.8)</td>
</tr>
<tr>
<td>500</td>
<td>Methimazole (150 µM)</td>
<td>105.9 (43.7)</td>
</tr>
</tbody>
</table>

* Incubations were conducted in quadruplicate for the heat inactivation experiment and in duplicate for the methimazole inhibition experiments. Each value represents the mean of these determinations. In parentheses is the percentage of control.
* Microsomal protein was preincubated with CLZ for 5 min at 45°C in the presence of NADPH, cooled on ice for 2 min, and followed by a normal assay procedure.
* Comparable data at 100 and 350 µM CLZ are not shown.


