Metabolism of clozapine by rat brain: the role of flavin-containing monooxygenase (FMO) and cytochrome P450 enzymes

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SUMMARY

The atypical antipsychotic clozapine has been reported to be metabolised mainly to its N-oxide and N-demethylated products. Brain, the target organ of clozapine, is known to contain numerous drug-metabolising enzymes which could alter the local concentrations of the drug. The metabolism of clozapine was, therefore, studied in rat brain preparations. Clozapine N-oxide was the major metabolic pathway in rat brain.

We characterised the N-oxygenation of clozapine by rat brain preparations. The $K_m$ and $V_{max}$ values were found to be 319.6 μM and 28.1 pmol/min/mg protein, respectively. The formation of clozapine N-oxide was shown to be inhibited by thiourea (a flavin-containing monooxygenase inhibitor) but not by ketoconazole, quinidine or furafylline. This finding suggests prominent involvement of FMO in the N-oxygenation of clozapine in rat brain. This conclusion was further confirmed by the observation that the formation of clozapine N-oxide is sensitive to heat treatment of the brain preparation and can be partially protected from thermal degeneration by the presence of an NADPH generating system. It was further observed that the rate of clozapine N-oxygenation was much higher at pH 8.5 than at pH 7.4. Taken together, the data suggest that N-oxygenation is the major metabolic pathway catalysed by rat brain and this reaction is catalysed mainly by FMO.

As significant interindividual differences have been observed in brain FMO activities, these differences may contribute to the interindividual differences in patient response to clozapine.

INTRODUCTION

Numerous cytochrome P450 (CYP) and flavin-containing monooxygenase (FMO) hepatic enzymes involved in drug metabolism have been shown to be present in the human and rat brain (1–4). Although levels of these enzyme systems are low in the brain as compared to those in the liver, they can have a significant impact on the local concentrations of drugs in the brain. With regard to psychotropic drugs, their metabolism in the brain, the target organ of their therapeutic effects, can have important effects on their local levels and those of their metabolites (5). These effects may not be detected by blood monitoring. Therefore, it is important to understand their disposition in the brain, the target organ of psychotropic drugs.
Despite the approximately 3% risk of granulocytopenia or agranulocytosis in patients treated with clozapine (6,7), clozapine has been widely used for otherwise treatment-resistant schizophrenia patients. It has also been shown to be effective against both positive and negative symptoms of schizophrenia and apparently causes fewer extrapyramidal side-effects than those experienced with typical neuroleptics (8,9).

Clozapine is extensively metabolized, mainly by N-oxygenation and N-demethylation (Fig. 1) (10). Large interindividual variations in bioavailability, steady state plasma concentrations and clearance have been observed (11–13). The variation in plasma steady-state concentrations can be related, in part, to the gender and age of the recipient and to whether or not the patient is a smoker (14,15).

Clozapine concentrations in rat brain average 24-fold higher than in plasma (16). Interestingly, brain clozapine levels averaged only 7.2 times higher than in serum when clozapine levels were equal to or less than 5 μg/g. This tendency of the brain/blood ratio of clozapine to rise with dose was suggested to be due to some sequestration of drug in brain tissue to limit its metabolism and clearance (16).

There is no information available on the metabolism of clozapine in the brain. In the current study, we investigated systematically the enzymes involved in the metabolism of clozapine in rat brain.

MATERIALS AND METHODS

Materials

Clozapine, clozapine N-oxide and N-desmethylclozapine were obtained from Research Biochemicals International (Natick, MA, USA). All other chemicals were of analytical grade.

Preparation of brain fractions

Brain fraction was prepared as follows. Male Sprague-Dawley rat brains were homogenised in 5 volumes of 0.32 M sucrose and centrifuged at 3500 g for 1.2 h. The pooled supernatants were further centrifuged at 21,000 g for 1.5 h. The pellets were then resuspended in phosphate buffer (0.1 M, pH 7.4). Protein contents were assayed by the method of Lowry et al. (17).

Enzymatic studies

Incubation procedures were as follows: 0.1 ml reaction mixtures containing 10 μl brain preparation, a cofactor-generating system consisting of β-nicotinamide adenine dinucleotide phosphate (1.3 mM), glucose 6-phosphate (3.3 mM), glucose-6-phosphate dehydrogenase (0.4 U/ml), MgCl₂ (3.3 mM) and appropriate concentrations of substrates in phosphate buffer (0.1 M, pH 7.4) were incubated at 37°C for 5–60 min. Control incubations contained heat-inactivated brain preparations. Biological reactions were terminated by the addition of perchloric acid (1 N, 20 μl) and denatured proteins were removed by centrifugation. The clear supernatants were subjected to HPLC analysis.

HPLC analysis

Clozapine, clozapine N-oxide and N-desmethylclozapine were analysed using an HPLC method described previously (18). The HPLC system comprised a Waters Model 510 solvent delivery system, a WISP 710B and a Lambda-Max Model 481 LC spectrophotometer set at 254 nm. A Hewlett Packard 3392A integrator was used to record, store and analyse chromatograms. The HPLC method used a 5 μm Hypersil CN column (4.6 x 250 mm). The mobile phase was a combination of acetonitrile (50%) and ammonium acetate buffer (10 mM) adjusted to pH 5.4 with acetic acid. The solvent was delivered at a flow rate of 1 ml/min.

Inhibition studies

To elucidate the enzymes responsible for the metabolism of clozapine in the brain, metabolic incubations were carried out in the presence of inhibitors of CYP and FMO: ketoconazole (1–6 μM), quinidine (1–6 μM), furafylline (1–6 μM) or thiourea (30–2000 μM). Clozapine (300 μM) was used as substrate and the incubation carried out for 60 min. These concentrations of inhibitors are those that usually have inhibitory effects on the CYPs and FMOs. Sample preparation and analysis were as described above.

Effects of thermal inactivation and pH

Rat brain preparations were pre-incubated in the presence of the NADPH generating system at 45°C for 5 min before the addition of 300 μM clozapine. The incubations were carried out for a further 60 min at 37°C. To determine the possible protective effect of NADPH on the thermal degeneration of FMO, rat brain preparations were pre-incubated without the presence of the NADPH system at 45°C for 5 min before the addition of the NADPH system and clozapine (300 μM). The incub-
Enzyme kinetic studies

Enzyme kinetic parameters for the formation of clozapine N-oxide and N-desmethylclozapine were estimated by incubating clozapine (25–400 μM, 6 concentrations) with rat brain preparations (20 μl/incubation) for 20 min. Sample preparation and analysis were as described above. $K_m$ and $V_{max}$ for the formation of N-desmethylclozapine and clozapine N-oxide were estimated using a non-linear regression analysis by fitting the data to equations representing one enzyme system as follows (GraphPad Prism).

$$V = V_{max} \times \frac{S}{(K_m + S)}$$  \hspace{1cm} \text{Eq. 1}

RESULTS

Clozapine N-oxide was the major metabolic pathway detected in whole brain preparations. No metabolites were observed in the control incubations with heat-inactivated brain preparations. This indicates that the metabolites observed were produced enzymatically. The clozapine N-oxide/N-desmethylclozapine ratio was much higher in the brain than in liver (18).

We further characterised the N-oxygenation of clozapine by rat brain preparations. The rate of formation of clozapine N-oxide was investigated using clozapine concentrations up to 400 μM to determine apparent kinetic parameters. Determination of the kinetic parameters for the formation of clozapine N-oxide was accomplished using non-linear regression analysis. Equations representing one-enzyme or two-enzyme systems (GraphPad Prism) were used. The one-enzyme system gave the best fit for the data (Fig. 2). The $K_m$ and $V_{max}$ values were 319.6 μM and 28.1 pmol/min/mg protein, respectively.

All the inhibitors were used at concentrations at which they usually inhibit the selected enzymes (Fig. 3). Thiourea, an FMO inhibitor, was the only one that inhibited the formation of clozapine N-oxide. Ketoconazole (a CYP3A4 inhibitor), quinidine (a CYP2D6 inhibitor) and furafylline (a CYP1A2 inhibitor) did not inhibit the formation of clozapine N-oxide. Inhibitors of these CYP enzymes were used because of their suggested involvement in the metabolism of clozapine in human liver (18,19). This finding suggests the prominent involvement of FMO in the N-oxygenation of clozapine in rat brain.

As FMO is known to be sensitive to thermal degeneration and NADPH can partially protect FMO from inactivation, rat brain preparations were pre-incubated at 45°C for 5 min in the presence or absence of an NADPH generating system before starting the usual metabolic incubation. It was found that a large percentage of clozapine N-oxygenation activity (75%) was lost following thermal treatment and that its thermal inactivation could be reduced to 52% by the inclusion of the NADPH system during heat treatment (Table I). FMO is also known to exhibit higher activities at pH 8.5 than at pH 7.4.
Ketoconazole, quinidine and furafylline are selective inhibitors of CYP3A4, CYP2D6 and CYP1A2, respectively, in human microsomes. However, these inhibitors have been shown to have different enzyme selectivity in rat liver microsomes. Eagling et al. (21) compared inhibitory selectivity of ketoconazole and furafylline between human and rat liver towards phenacetin O-de-ethylation (CYP1A2-mediated in the human), tolbutamide 4-hydroxylation (CYP2C9-mediated in the human), chlorzoxazone 6-hydroxylation (CYP2E1-mediated in the human) and testosterone 6α-hydroxylation (CYP3A-mediated in the human). Ketoconazole was shown to inhibit not only testosterone 6α-hydroxylation but also inhibits all the other three reactions at concentrations below 5 μM. Furafylline was shown to inhibit phenacetin O-de-ethylation as well as tolbutamide 4-hydroxylation. Thus, ketoconazole and furafylline can be considered as non-selective CYP inhibitors in the rat. Quinidine was suggested to be a relatively selective inhibitor for the CYP2D family in both human and rat (22). Lack of inhibition by quinidine, ketoconazole and furafylline indicate the lack of involvement of CYP enzymes in the formation of clozapine N-oxide. Taken together, these data suggest the prominent involvement of FMO in the N-oxygenation of clozapine in rat brain.

A major difference between rat brain and liver in their metabolism of clozapine seems to be that rat brain produces far more clozapine N-oxide relative to N-desmethyloclozapine (18). As we have shown that FMO is mainly responsible for the production of clozapine N-oxide, this seems to indicate that there is relatively more FMO activity in rat brain than liver as compared to CYP activity that is also responsible for the metabolism of clozapine. This observation seems to reinforce the notion that, when a number of enzymes are capable of catalysing a particular metabolic reaction, the relative contribution of a particular CYP enzyme will be determined by enzyme kinetic factors, such as the relative abundance and affinity of each enzyme.

**DISCUSSION**

We have shown previously that, in human liver microsomes, both FMO and CYP3A4 are important in the N-oxygenation of clozapine (18). Pirmohamed et al. (20) also showed that the formation of clozapine N-oxide in human liver microsomes was inhibited by ketoconazole (CYP3A4 inhibitor) but not by furafylline (CYP1A2 inhibitor). Thus, while in human liver both FMO and CYP3A4 are responsible for the N-oxygenation of clozapine, in rat liver and brain FMO seems to be the primary enzyme for catalysing the N-oxygenation of clozapine.

A number of inhibitors selective for human CYP enzymes and FMO were used to delineate enzymes responsible for the metabolism of clozapine (Fig. 3). Thiourea, an FMO inhibitor, was the only one that inhibited the formation of clozapine N-oxide. Ketoconazole, quinidine and furafylline did not inhibit the formation of clozapine N-oxide. Therefore, the above experiments were carried out in phosphate buffers of each pH value. Brain preparations exhibited much higher activity at pH 8.5 than pH 7.4 for the N-oxygenation of clozapine (Table I). It is, therefore, clear that the behaviour of brain clozapine N-oxidase responds to thermal inactivation and the pH value of the environment, similar to what is known for FMO.

**Table I : Effect of heat treatment on the metabolism of clozapine by rat brain preparations**

<table>
<thead>
<tr>
<th>Pretreatment of brain preparations</th>
<th>Formation of clozapine N-oxide (pmole/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td>pH 8.5</td>
</tr>
<tr>
<td>Heated 5 min at 45°C with NADPH</td>
<td>1.33</td>
</tr>
<tr>
<td>Heated 5 min at 45°C without NADPH</td>
<td>0.67</td>
</tr>
<tr>
<td>No heating</td>
<td>2.74</td>
</tr>
<tr>
<td></td>
<td>9.13</td>
</tr>
</tbody>
</table>

Data are means of two independent duplicate experiments. See text for experimental details.

**Fig. 3 : Inhibition of the formation of clozapine N-oxide by rat brain preparations by selective inhibitors of FMO and CYPs.** Clozapine (300 μM) was incubated with brain preparations (20 μl) in the presence of various concentrations of the inhibitors for 60 min. Sample preparations and HPLC assay conditions were as described in the text. Closed circles, thiourea; open circles, furafylline; closed triangles, ketoconazole; open triangles, quinidine.
There also seems to be considerable interspecies differences in the N-oxygenation of clozapine between rat and human, at least in liver microsomes. Microsomal preparations from control rats and rats previously treated with inducers of CYP enzymes (clofibrate, isoniazid, β-naphthoflavone, dexamethasone or phenobarbital) were used in metabolic studies of clozapine (18). The formation of clozapine N-oxide was not affected to a significant degree in any induced microsomal preparations compared with the results obtained with control microsomes. This seems to indicate the lack of involvement of CYP enzymes, as the CYP enzymes capable of catalysing clozapine N-oxygenation \textit{(i.e.} CYP1A and CYP3A) can be induced by some of the above-mentioned inducers. We have also carried out a preliminary inhibition study with rat liver microsomes (data not shown). It was found that the response of rat liver microsomes is similar to rat brain preparations, \textit{i.e.} thiourea is the only inhibitor capable of reducing their activities towards clozapine N-oxygenation.

Whole brain preparations were used in the current study. Since differences in CYP metabolism occurring among brain regions have been observed, use of a whole brain preparation may underestimate or miss highly localised enzyme activities. A detailed study using different brain regions will be needed to determine whether the metabolic activities for clozapine are localised in specific regions.

Taken together, the data from the above inhibition and thermal inactivation studies suggest that N-oxygenation is the major metabolic pathway catalysed by rat brain and this reaction is mainly catalysed by FMO.

As very significant interindividual differences have been observed in brain FMO activities (4), these differences may contribute to the interindividual differences in patient response to clozapine.

**CONCLUSIONS**

In summary, the present studies have shown that clozapine N-oxide is the major metabolite of clozapine catalysed by rat brain preparations. In contrast to liver microsomes, N-desmethylclozapine is a minor metabolite of clozapine produced by brain preparations. Through inhibition and thermal-lability studies, we have found that the enzymes mainly responsible for the N-oxygenation of clozapine in rat brain preparations are members of the FMO family.

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**REFERENCES**


