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Pharmacokinetics of clozapine and its metabolites in hippocampal HT22 cells

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Abstract

Up to now, it is not yet clear whether and how clozapine and its metabolites are metabolized in neuronal cells. The interconversion of clozapine and its metabolites, clozapine-*N*-oxide and norclozapine, was studied in the hippocampal neuronal in vitro system of HT22 cells. Clinically relevant concentrations of clozapine (200 + 400 ng/ml) and its metabolites (100 + 200 ng/ml) were used for the examination of the metabolizing effects after short- (4 h) and long- (24 h) term incubation. Two-way analysis of variance revealed a significant decrease of clozapine ($P < 0.01$) and norclozapine ($P < 0.01$) levels in the supernatants of HT22 cells after the treatment procedures. Student–Newman–Keuls tests showed a significant decrease of clozapine 400 after 24 h of incubation ($P = 0.01$) as well as of all concentrations of norclozapine. No significant treatment effects were found for the clozapine-*N*-oxide degradation. Using semi-quantification by reverse transcriptase-polymerase chain reaction methods, we could show a significant increase of cytochrome *P*450 (CYP) 1A2 mRNA levels ($P < 0.05$) after clozapine treatment with 200 ng/ml. The results of the present study strongly suggest that clozapine and norclozapine are metabolized in hippocampal neuronal HT22 cells by CYP1A2, whereas the levels of clozapine-*N*-oxide were not affected. Moreover, CYP1A2 mRNA levels were significantly changed by incubation with clozapine 200.

Keywords: Clozapine; Metabolite; Metabolism; Hippocampus

1. Introduction

The atypical antipsychotic clozapine is mainly metabolized by the hepatic microsomal cytochrome *P*450 (CYP) system. It is demethylated to norclozapine (= desmethylclozapine) by the CYP1A2 and other CYP-enzymes, whereas the *N*-oxidation of clozapine to clozapine-*N*-oxide is conducted by CYP3A4 with additional involvement of the flavin-containing monooxygenase 3 enzyme (Aitchison et al., 2000; Eiermann et al., 1997; Fang et al., 1998; Linnet and Olesen, 1997; Prior et al., 1999). Regarding the CYP system, it has been shown that clozapine may inhibit the activity of CYP2C9 and CYP2C19, and may induce CYP1A, CYP2B and CYP3A (Prior et al., 1999). A possible link between the CYP2D6 genotype and the treatment response to clozapine was found independently from serum concentrations in the treatment course of schizophrenic patients (Dettling et al., 2000). However, Arranz et al. (1995) found no correlation between CYP2D6 alleles and the response to clozapine in schizophrenic patients and concluded that CYP1A2, rather than CYP2D6, is the major enzyme responsible for the metabolism of clozapine.

Clozapine and its metabolites are actively transported into cells. This has been described for cells of blood origin (Henning et al., 2002a) and for artificial cells of neuronal and glial origin (including HT22 cells) (Henning et al., 2002b). The rank order of drug transport was norclozapine>clozapine>clozapine-*N*-oxide. The active transport was saturable, energy- and temperature-dependent.

There are some results which indicate that norclozapine is the neurobiologically active metabolite of clozapine (Guitton et al., 1998): Norclozapine—but not clozapine-*N*-oxide—has the same dopamine D_2 receptor affinity as clozapine (Odou et al., 1996). Regarding 5-HT_2 receptor affinity, clozapine shows about the same affinity as norclozapine. Norclozapine has even a higher affinity to the 5-

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HT_{2C} receptor, if compared to clozapine (for review, see Seeman, 1993). Norclozapine significantly increases Fos protein expression in the medial prefrontal cortex and nucleus accumbens, but not in the dorsolateral striatum, thus mirroring the effects of the parent compound (Young et al., 1998). Additionally, norclozapine plasma concentrations correlated positively with granulocyte counts in schizophrenic patients (Combs et al., 1997).

An important brain region in terms of dysfunction in schizophrenia is the hippocampus (Dean et al., 1996; Grace, 2000; Joyce et al., 1993; Scarr et al., 2001), a part of the limbic system. Many structural and functional alterations have been found in the hippocampus of schizophrenic patients (Naylor et al., 1996; Scarr et al., 2001) which is important for the organization of declarative memory, especially with regard to context and episodic memory as well as memory consolidation (Kuperberg and Heckers, 2000; Nadel et al., 2000).

In the human hippocampus, the CYP2D6 mRNA and its protein as well as CYP11A1 (P450scc) and CYP19 mRNA (aromatase) are expressed (Siegle et al., 2001; Stoffel-Wagner et al., 1999; Watzka et al., 1999). CYP21 and CYP2E are expressed in both, the human and rodent hippocampus (Upadhya et al., 2000; Beyenburg et al., 2001), whereas CYP1A2 and several other CYP enzymes are expressed in the hippocampus of the rat (Huang et al., 2000; Morse et al., 1998; Norris et al., 1996; Riedl et al., 2000; Schoedel et al., 2001; Tindberg and Ingelman-Sundberg, 1996). A novel cytochrome *P*450 enzyme, CYP7B1, is strongly expressed in the hippocampus of rodent brain (Martin et al., 2001). Alcohol did not alter the CYP2B1/2 protein expression in the hippocampus of rats (Schoedel et al., 2001), whereas 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin significantly increased CYP1A1 mRNA in the hippocampus of rats (Huang et al., 2000).

Up to now, it is not yet clear whether and how clozapine and its metabolites are metabolized in neuronal cells. To study this aspect, we used immortalized hippocampal HT22 cells as a neuronal hippocampal model system and evaluated the uptake and interconversion of clozapine and its metabolites. CYP1A2 is the enzyme which is the major determinant of clozapine clearance (Aitchison et al., 2000). In addition to the metabolism of clozapine and its metabolites, we examined the gene-expression of this enzyme and its regulation by clozapine in HT22 cells under defined in vitro conditions excluding the interference of other factors derived from other brain areas or other tissues of the organism.

2. Material and methods

2.1. Preparation and incubation of HT22

HT22 cells were received from Dr. C. Behl (Max Planck Institute of Psychiatry, Munich, FRG) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Taufkirchen, Germany), supplemented with 10% fetal calf serum (Sigma-Aldrich), 2% sodium pyruvate and 1% antibiotic–antimycotic solution.

For the experiments, 4×10^6 HT22 cells were incubated in Petri dishes with 10 ml START V medium (Seromed, Berlin, Germany) for 4 and 24 h, respectively. Different amounts of clozapine (400 + 200 ng/ml), norclozapine (200 + 100 ng/ml), and clozapine-*N*-oxide (200 + 100 ng/ml) (Novartis, Nürnberg, Germany) were added to the culture media. After the incubation period, the supernatant was sampled and cells for the determination of mRNA were dissolved in Trizol (Life Technology, Frederick, USA). All specimens were subsequently frozen in − 80 °C.

2.2. Determination of clozapine and its metabolites

Clozapine and its metabolites clozapine-*N*-oxide and *n*-desmethyl-clozapine (= norclozapine) were assessed from the incubation medium in advance and after the incubation of the substances with the cells. Sample preparation was performed by protein precipitation using the precipitation reagent from Chromsystems (Munich, Germany). After precipitation, a 50-μl aliquot was used for high-pressure liquid chromatography (HPLC) analysis. A HPLC method with electrochemical detection after RP-Select B column separation was used as described elsewhere in detail (Schulz et al., 1995).

2.3. Extraction and quantification of mRNA by reverse transcriptase-polymerase chain reaction methods

After defrosting the samples, the mRNA was extracted according to standard methods (Peqlab extraction kit, Peqlab, Erlangen, Germany). The amounts of extracted mRNA were quantified by established optical methods at A_{260}/A_{280} (Genequant II, Pharmacia Biotech, Freiburg, Germany) and structural integrity was determined by agarose gel electrophoresis (1.0% agarose; Gibco/BRL, Dreieich, Germany). Equivalent amounts of mRNA were used for the reaction with reverse transcriptase (RT) (Superscript II RNase H Reverse Transcriptase, Gibco/BRL, Eggenstein, Germany) and reaction products specifically amplified to detect transcripts of rat CYP1A2 mRNA. The primers for CYP1A2

5′primer: 5′-GAG ATG GAG AAA CAG GGA CC-3′;
3′primer: 5′-TCT TCC TGG AAT CAA TGT GG-3′

were used in a final concentration of 10 μM and the primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

5′primer: 5′-CGT CTT CAC CAC CAT GGA GA-3′;
3′primer: 5′-CGG CCA TCA CGC CAC AGT TT-3′

in a final concentration of 20 μM (both received from MWG Biotech, Ebersberg, Germany). Samples were amplified in

parallel in a polymerase chain reaction (PCR) cycler (Biometra Trio, Göttingen, Germany) for CYP1A2 (42 cycles, 63 °C) and GAPDH (31 cycles, 62 °C) and amplification products subjected to gel electrophoresis in 1.5% agarose gels. Semiquantitative determination was achieved by digitization of gels with a Polaroid video system (Rothaar and Schroeder, Heidelberg, Germany) and further densitometric evaluation with the BIO-1D program (Vilbert Lourmat; Marne La Vallée, Cedex, France). To ensure linear conditions for the amplification of RT products, different concentrations of CYP1A2- and GAPDH-RT products were quantified in the same manner. The results are expressed in percentage of the respective control condition.

Initially, the samples were analysed in parallel for GAPDH to correct for variations in the PCR amplification and the quantification process (Vedder et al., 1999). No treatment effects were detectable for the GAPDH mRNA levels.

2.4. Statistical analysis

All results are expressed as mean values ± standard error of the mean (S.E.M.). To simultaneously evaluate the effects of treatment and time, two-way analysis of variance (ANOVA) was used in order to discriminate the differences between the treatment groups at random. In case of significance (significance level: $P<0.05$), Student–Newman–Keuls tests were performed as post hoc tests. The respective degrees of freedom (*df*), *F* statistics (*F*), and *p*-values are presented. The statistical evaluation war carried out using SigmaStat software (Jandel Scientific, Erkrath, Germany).

3. Results

3.1. Clozapine metabolism in HT22 cells

ANOVA revealed significant treatment effects (ANOVA: $df=39$; $F=0.047$; $P<0.01$) which were due to a decrease of clozapine (200 and 400) levels in HT22 cells after treatment for 4 and 24 h, respectively (Fig. 1).

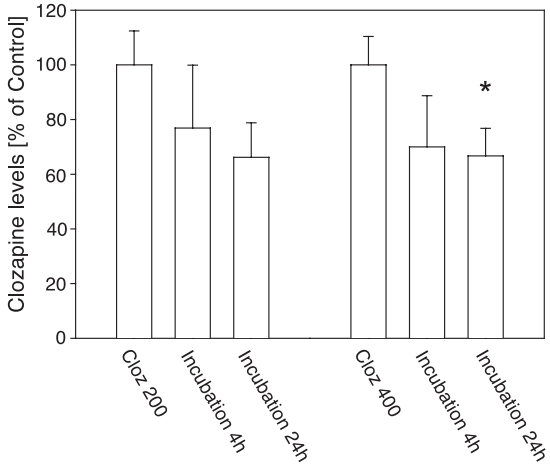

Fig. 1. Clozapine levels (mean ± S.E.M.) of clozapine-treated HT22 cells (clozapine = Cloz) (*$P<0.05$ compared to control).

The post hoc tests exhibited the following results:

- Clozapine 200 before incubation (211.4 ± 21.5 ng/ml) vs. clozapine levels after 4 h of incubation (162.6 ± 37.3 ng/ml): $P=0.29$
- Clozapine 200 before incubation (207.6 ± 29.8 ng/ml) vs. clozapine levels after 24 h of incubation (137.0 ± 17.3 ng/ml): $P=0.07$
- Clozapine 400 before incubation (432.6 ± 55.1 ng/ml) vs. clozapine levels after 4 h of incubation (303.0 ± 56.6 ng/ml): $P=0.14$
- Clozapine 400 before incubation (433.0 ± 34.3 ng/ml) vs. clozapine levels after 24 h of incubation (289.0 ± 29.1 ng/ml): $P=0.01$.

The values for norclozapine and clozapine-*N*-oxide were below 10 ng/ml in the course of this part of the experiments.

No significant interaction was detectable between 4 and 24 h of incubation.

3.2. Norclozapine metabolism in HT22 cells

ANOVA revealed significant treatment effects (ANOVA: $df=39$; $F=0.4$; $P<0.01$). There was a significant decrease of all norclozapine (100 and 200) levels in HT22 cells after treatment for 4 and 24 h, respectively (Fig. 2).

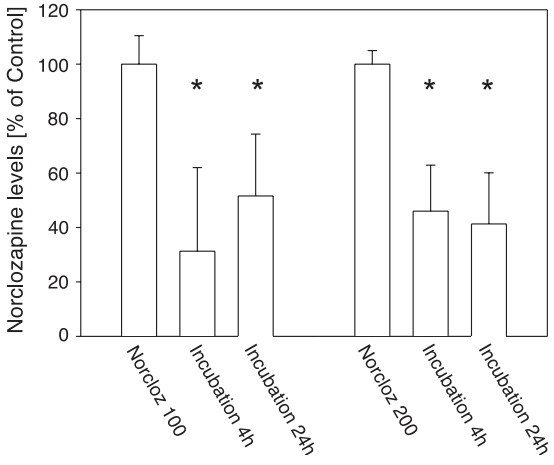

Fig. 2. Norclozapine levels (mean ± S.E.M.) of norclozapine-treated HT22 cells (norclozapine = Norcloz) (*$P<0.05$ compared to control).

The post hoc tests exhibited the following results:

- Norclozapine 100 before incubation (92.0 ± 12.9 ng/ml) vs. norclozapine levels after 4 h of incubation (29.0 ± 8.9 ng/ml): $P<0.01$
- Norclozapine 100 before incubation (98.2 ± 7.9 pmol) vs. norclozapine levels after 24 h of incubation (51.0 ± 11.6 pmol): $P<0.01$

- Norclozapine 200 before incubation (211.0 ± 7.6 ng/ml) vs. norclozapine levels after 4 h of incubation (97.8 ± 16.6 ng/ml): $P < 0.01$
- Norclozapine 200 before incubation (227.4 ± 14.0 ng/ml) vs. norclozapine levels after 24 h of incubation (94.0 ± 17.7 ng/ml): $P < 0.01$

The values for clozapine and clozapine-*N*-oxide were below 10 ng/ml in the course of this part of the experiments.

No significant interaction was seen between 4 and 24 h of incubation.

3.3. Clozapine-N-oxide metabolism in HT22 cells

ANOVA revealed no significant treatment effects for the clozapine-*N*-oxide metabolism (ANOVA: $df = 39$; $F = 0.78$; $P = 0.51$) (Fig. 3).

The values for clozapine and norclozapine were below 10 ng/ml in the course of this part of the experiments.

3.4. Quantification of CYP1A2 mRNA

For the CYP1A2 mRNA levels (Fig. 4), a significant influence could be demonstrated for the treatments (ANOVA: $df = 2$; $F = 6.242$; $P = 0.004$). The post hoc tests showed that these results were due to a significant higher value of clozapine 200 (24 h) compared to clozapine 400 (4 h) as well as to both control groups.

No significant differences were found for the different incubation times of the experiments (ANOVA: $df = 1$; $F = 1.074$; $P = 0.306$).

GAPDH levels did not differ significantly during this experiment (ANOVA: $df = 47$; $F = 0.19$; $P = 0.83$) (control: 100 ± 5%, clozapine 200: 103 ± 6%, clozapine 400: 106 ± 9%).

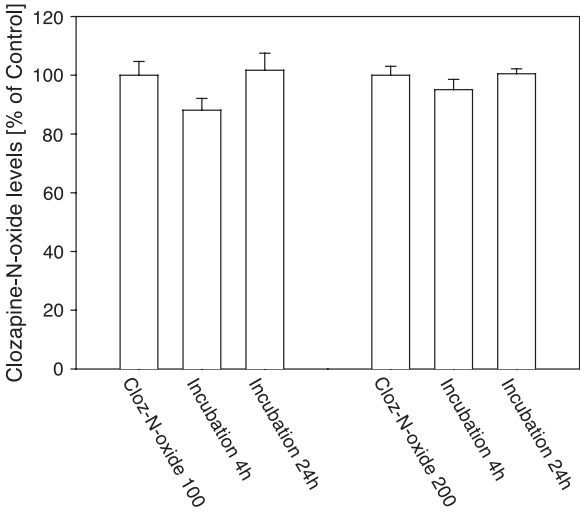

Fig. 3. Clozapine-*N*-oxide levels (mean ± S.E.M.) of clozapine-*N*-oxide-treated HT22 cells (clozapine-*N*-oxide = Cloz-*N*-oxide) (*$P < 0.05$ compared to control).

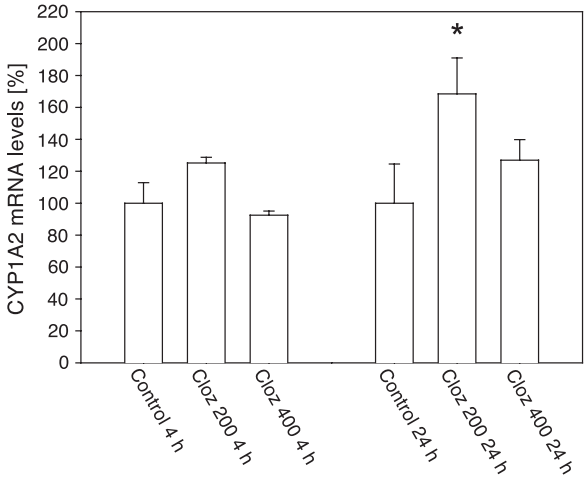

Fig. 4. CYP1A2 mRNA levels (mean ± S.E.M.) of clozapine-treated HT22 cells (clozapine = Cloz) (*compared to Cloz 400 4 h as well as to both controls).

4. Discussion

In the present study, we used an in vitro approach to investigate the metabolism of different concentrations of clozapine and its metabolites in hippocampal HT22 cells. Data from the literature confirm the hippocampal origin and the neuronal nature of these cells (Morimoto and Koshland, 1990). Using this system, we excluded other relevant influences on the changes induced for example by innervation, release of neurotransmitters from other brain regions or by other biochemical factors from various types of surrounding cells or biochemical factors.

The concentrations of clozapine and its metabolites were derived from clinical practice, since serum levels of clozapine and its metabolites are usually found in the range of 100–400 ng/ml under standard clinical conditions (Aymard et al., 1997; Remschmidt et al., 1994; Schulz et al., 1997). We therefore used clozapine in concentrations of 200 and 400 ng/ml, and clozapine-*N*-oxide/norclozapine in concentrations of 100 and 200 ng/ml for our study and additionally controlled these concentrations by HPLC methods.

The metabolism of clozapine and its metabolites clozapine-*N*-oxide as well as norclozapine were studied after short- (4 h) and long- (24 h) term incubation.

ANOVA revealed a significant decrease of clozapine ($P < 0.01$) and norclozapine ($P < 0.01$) levels in the media after treatment. Student–Newman–Keuls tests showed a significant decrease of clozapine 400 after 24 h of incubation ($P = 0.01$) as well as of all concentrations of norclozapine. No significant treatment effects were found for the clozapine-*N*-oxide metabolism. The reason for these results could be the rank order of drug transport into cells (norclo-

zapine>clozapine>clozapine-*N*-oxide) as described by Henning et al. (2002b).

Since clozapine-*N*-oxide is metabolized by CYP3A4 with additional involvement of the flavin-containing monooxygenase 3 enzyme (Eiermann et al., 1997; Fang et al., 1998; Linnet and Olesen, 1997; Prior et al., 1999), the latter results strongly suggest that HT22 cells do not contain these enzymes. This conclusion is in line with data from the Gene Expression Atlas (http://www.expression.gnf.org) that shows only low activity of those two enzymes in the brain.

With regard to the changes in clozapine and norclozapine, several data from the literature suggest that the enzyme CYP1A2 might be responsible for the metabolization of these compounds.

In a therapeutic drug monitoring study, clozapine plasma concentrations were obtained from 23 patients and the results suggested that the CYP1A2 activity is the major factor, which determines clozapine clearance and that the norclozapine/clozapine ratio could constitute a valuable measure of the CYP1A2 activity (Dailly et al., 2002). The same results were obtained in CYP1A2-null mice. These results indicate that CYP1A2 is the major determinant of clozapine clearance, contributes significantly to the demethylation of clozapine, and has almost no contribution to the *N*-oxidation to clozapine-*N*-oxide (Aitchison et al., 2000).

We therefore examined CYP1A2 activity in HT22 cells with a RT-PCR approach. Our results show that the CYP1A2 mRNA is indeed present in these cells. Moreover, the selective decrease of clozapine and clozapine-*N*-oxide suggests that the protein is also functionally active in the cultured neuronal cells.

Additionally, a significant influence of the treatment on CYP1A2 mRNA levels could be demonstrated. In vivo data from the literature showed, that clozapine induces protein levels of CYP1A2, CYP2B1, and CYP3A in rats after treatment for 14 days with 114 mg/kg/day (Rane et al., 1996), also demonstrating an effect of clozapine on the CYP enzyme levels. The differences in the results may be due to the different detection levels (mRNA vs. protein), the in vitro vs. the in vivo situation or the longer time course of incubation.

Clinically, Ozdemir et al. (2001a) described a patient with a very high CYP1A2 activity who only reached therapeutic serum levels of clozapine by coadministration of fluvoxamine (a potent CYP1A2 inhibitor) while the intake of grapefruit juice (an inhibitor of intestinal CYP3A4) as an alternative treatment had no effect. It was also found, that the patient was homozygous for the CYP1A2*1F allele.

In a study with 18 patients treated with clozapine, CYP1A2 activity was measured using the caffeine metabolic ratio in overnight urine. A significant negative association was found between the caffeine metabolic ratio and the dose-corrected clozapine and norclozapine concentrations (Ozdemir et al., 2001b). In contrast, three prospective studies did not find changes in clozapine concentration after treatment with potent CYP3A4 inhibitors in vivo (Hagg et al., 1999; Raaska and Neuvonen, 1998; Taylor et al., 1999).

In accordance with these data from the literature, the results of the present study strongly suggest that clozapine and norclozapine are metabolized in the hippocampal neuronal in vitro system of HT22 cells, whereas clozapine-*N*-oxide was not affected. The mRNA of the main metabolizing enzyme of clozapine, CYP1A2, was present in the cells and also affected by the treatment, further enforcing the functional relevance of the data and the in vitro system applied.

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