

A Comparison of the Oxidation of Clozapine and Olanzapine to Reactive Metabolites and the Toxicity of these Metabolites to Human Leukocytes

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Received December 19, 1997; Accepted February 24, 1998

This paper is available online at <http://www.molpharm.org>

ABSTRACT

Olanzapine was shown to be oxidized to a reactive intermediate by HOCl, which is the major oxidant produced by activated neutrophils. A mass spectrum obtained using a flow system in which the reactants were fed into a mixing chamber and the products flowed directly into a mass spectrometer revealed a reactive intermediate at m/z 311. This is 2 mass units less than the protonated molecular ion of parent olanzapine and suggests that the reactive intermediate is a nitrenium ion. The reactive intermediate could be trapped with glutathione or *N*-acetylcysteine to produce two conjugates. These data are analogous to results we reported previously with the structurally related atypical antipsychotic agent clozapine. However, the clozapine and olanzapine reactive metabolites showed differences in their ability to cause toxicity to human neutrophils. Toxicity to neutrophils was observed only at high concentra-

tions of clozapine ($>50 \mu\text{M}$) when HOCl was used to generate reactive metabolite. In contrast, concentration-dependent toxicity ($p < 0.05$) was observed when neutrophils were incubated with clozapine ($0-20 \mu\text{M}$) and H_2O_2 to generate clozapine reactive metabolite. No toxicity was observed with clozapine alone (at concentrations of $> 50 \mu\text{M}$). Similar results were observed in monocytes and HL-60 cells. Olanzapine reactive metabolite only seemed to cause slight toxicity at the highest concentrations tested ($20 \mu\text{M}$), even when the reactive metabolite was generated using H_2O_2 . Neutrophils from two patients with a history of clozapine-induced agranulocytosis seemed to be more sensitive to the toxic effects of the clozapine reactive metabolite; however, the numbers are too small to draw any definite conclusions.

Clozapine (Fig. 1) is an atypical antipsychotic agent that is more effective than standard neuroleptic drugs in the treatment of refractory schizophrenia. Unfortunately, the use of clozapine has been limited because it causes agranulocytosis in almost 1% of patients treated with the drug (Safferman *et al.*, 1992; Alvir *et al.*, 1993; Atkin *et al.*, 1996). The mechanism of clozapine-induced agranulocytosis is unknown. A possible mechanism involving a toxic serum factor with characteristics of an antibody (Pisciotta *et al.*, 1992) has been proposed; however, there are limitations to this hypothesis (Liu and Uetrecht, 1995).

Fischer *et al.* (1992) reported indirect evidence for the formation of a free radical after oxidation of clozapine by horseradish peroxidase and MPO. In addition, they identified two GSH conjugates formed from incubations of clozapine with horseradish peroxidase. It is unclear how the clozapine

free radical would lead to formation of a GSH conjugate because it would be more likely to abstract a hydrogen atom from GSH, producing a glutathionyl radical and regenerating parent compound. More recently, we identified a reactive metabolite of clozapine that covalently binds to activated human neutrophils (Liu and Uetrecht, 1995), and others have demonstrated that it binds to bone marrow cells (Maggs *et al.*, 1995). Formation of this reactive metabolite could lead to drug-induced agranulocytosis, either by covalently modifying critical cellular proteins, leading to cell death, or perhaps by acting as a hapten and eliciting an immune response. Although the evidence for an antibody-mediated immune response against clozapine is weak, it is conceivable that hapten formation could trigger a cell-mediated immune response (Liu and Uetrecht, 1995). Furthermore, recent work from the laboratory of Park has shown that exposure of human neutrophils to the clozapine reactive metabolite *in vitro* results in toxicity (Williams *et al.*, 1998).

Regardless of the mechanism of clozapine-induced agranulocytosis, the seriousness of this problem has led to at-

This work was supported by Grant MT-13478 from the Medical Research Council of Canada. I.G. was a recipient of a Postdoctoral Fellowship from the Pharmaceutical Manufacturers Association of Canada and the Medical Research Council of Canada.

ABBREVIATIONS: MPO, myeloperoxidase; GSH, glutathione; HBSS, Hanks' balanced salt solution (without phenol red); NAC, *N*-acetylcysteine; LC, liquid chromatography; MS, mass spectrometry; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline.

tempts to find new therapeutic agents that share the unique pharmacological activity of clozapine without causing drug-induced agranulocytosis. One such agent is olanzapine, which is a thienobenzodiazepine derivative (Fig. 1). Despite the structural similarity between clozapine and olanzapine, no cases of agranulocytosis have been reported with the clinical use of olanzapine (Fulton and Goa, 1997). In fact, olanzapine has been used safely to treat patients who have had clozapine-induced agranulocytosis (Fulton and Goa, 1997). In this study, we evaluated the ability of olanzapine to form a reactive intermediate in a reaction analogous to that occurring with clozapine. In addition, we investigated the toxicity of clozapine and olanzapine reactive metabolites toward human leukocytes and the promyelocytic HL-60 cell line.

Experimental Procedures

Materials. Clozapine was provided by Sandoz Canada (now Novartis, Dorval, Quebec, Canada). Hydrogen peroxide (H_2O_2) was purchased from ACP Chemicals (Montreal, Quebec, Canada). Sodium hypochlorite ($NaOCl$) was purchased from Aldrich Chemical (Milwaukee, WI). Ketoprofen and dapsone were purchased from Sigma Chemical (Oakville, Ontario, Canada). MPO was obtained from Cortex Biochemical (San Leandro, CA). One unit of MPO activity is defined as the amount of enzyme that would decompose 1 μ mol of H_2O_2 /min at 25° and pH 6. HBSS was obtained from Media Services, University of Toronto.

Preparation of olanzapine. Olanzapine was isolated from tablets (7 10-mg tablets, Zyprexa; Eli Lilly, Indianapolis, IN). The tablets were crushed and extracted with methanol (10 ml). The methanolic solution was centrifuged ($500 \times g$, 5 min) to remove insoluble material. This was repeated three times, the methanolic solutions were combined, and the solvent was removed under reduced pressure. The residue was dissolved in water and applied to an LC-18 solid-phase extraction column (Supelco; Supelco Inc., Bellefonte, PA). The solid-phase extraction column was washed sequentially

with water and 5% methanol, and the olanzapine was eluted with methanol. The drug was shown to be >99% pure by HPLC.

Analytical studies. HPLC was performed using a Shimadzu system (LC-600 pump; SPD-6A UV spectrophotometer set at 254 nm, and a C-R6A integrator; Shimadzu, Kyoto, Japan). An Ultracarb ODS 30 column (2×100 mm, 5 μ m; Phenomenex, Torrance, CA) equipped with a 2×30 mm guard column was used for the chromatography. The mobile phase consisted of water/acetonitrile/acetic acid (79:20:1, v/v/v) containing 2 mM ammonium acetate unless stated otherwise and was degassed before use.

LC/MS and LC/MS/MS were carried out on a Sciex API III mass spectrometer (Perkin-Elmer, Sciex, Thornhill, Ontario, Canada) equipped with an Ion Spray interface. Analyses were performed using an ionizing voltage of 5 kV, and the orifice voltage was 60 V. Collision-induced dissociation of selected precursor ions was performed in the RF-only quadrupole region, and argon was used as target gas at an energy of 26 eV. The mobile phase flow rate was 0.2 ml/min, and a postcolumn splitter decreased the flow through the mass spectrometer to ~ 20 μ l/min. 1H NMR spectra (dimethylsulfoxide- D_6) were recorded at 500 MHz with a Varian Unity Plus 500 Spectrometer (Varian Associates, Palo Alto, Ca.).

Oxidation of olanzapine by hypochlorous acid. A Hewlett Packard diode-array spectrophotometer (HP 8452A; Hewlett Packard, Palo Alto, CA) was used to determine the rate of oxidation of olanzapine by hypochlorite. Scanning of the reaction mixture was initiated immediately after the addition of $NaOCl$ (40 μ l, 5 mM aqueous solution) to a solution of the drug [2 ml, 100 μ M in phosphate buffer (0.1 M, pH 6.0)] with rapid stirring. A Hi-Tech stopped-flow spectrophotometer (Stopped-Flow SHU; Hi-Tech Scientific, Salisbury, UK; dead time, 2 msec) was used to obtain accurate kinetic data on the oxidation of olanzapine and clozapine by $HOCl$. Concentrations of drug and $NaOCl$ were 500 and 50 μ M, respectively. Reactions were performed in PBS (137 mM sodium chloride, 8 mM disodium hydrogen phosphate, 1.5 mM potassium dihydrogen phosphate, and 2.7 mM potassium chloride, pH 6.0) or in phosphate buffer (0.1 M, pH 6.0), and the reactive intermediates were monitored at 540 nm (olanzapine) or 460 nm (clozapine).

Mass spectra of the reactive intermediate of olanzapine were obtained using a flow system coupled to the mass spectrometer. An olanzapine solution (250 μ M in water adjusted to pH 6.0 with acetic acid) and a solution of $NaOCl$ (62.5 μ M aqueous solution) were fed into an Mixing Tee (dead volume, 3.1 μ l) (Upchurch Scientific, Concord, Ontario, Canada). The flow rate was 50 μ l/min for olanzapine and 100 μ l/min for hypochlorite. From the mixing chamber, the products flowed through a fused silica capillary to the mass spectrometer in ~ 10 sec with a splitter just before the mass spectrometer inlet decreasing the flow rate to 15 μ l/min.

Trapping of the olanzapine reactive intermediate with glutathione and NAC. $NaOCl$ (250 μ l, 1 M aqueous solution) was added to olanzapine (5 ml, 20 mM in an aqueous solution of 60% ethanol with the pH adjusted with 20 μ l of acetic acid) with rapid stirring. The solution immediately became dark red, and GSH or NAC (2 ml, 0.2 M aqueous solution) was quickly added to the solution. The reaction products were analyzed by LC/MS. The mobile phase consisted of water/acetonitrile/acetic acid (79:20:1, v/v/v) containing 2 mM ammonium acetate.

Purification of NAC adducts of the olanzapine reactive intermediate. After reaction of the olanzapine reactive intermediate with NAC (as described above), the sample was concentrated under reduced pressure. The pH was adjusted to 9.0 with $NaHCO_3$, and the solution was extracted with ethyl acetate (three times 15 ml). The aqueous layer was adjusted to pH 6.0 with 1 N HCl and applied to an LC-18 solid-phase extraction column (Supelco; Supelco Inc.). The solid-phase extraction column was washed sequentially with water and 5% methanol (v/v), and then the NAC conjugates were eluted with methanol. The methanolic solution was separated by normal-phase thin layer chromatography (100% methanol mobile phase), resulting in five bands. Two bands (retention time, 0.2 and

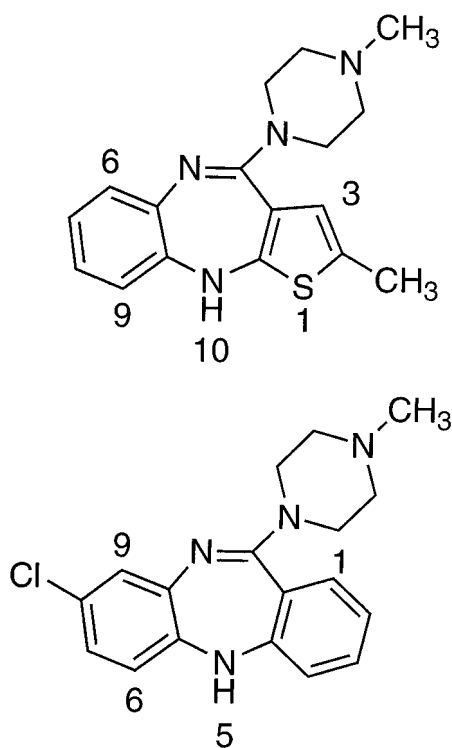


Fig. 1. Chemical structures of olanzapine (top) and clozapine (bottom).

0.4) contained the olanzapine-NAC conjugates. The olanzapine-NAC conjugates were purified further by preparative HPLC (Ultracarb 5 ODS 30, 150 × 10 mm; Phenomenex, Torrance, Ca) using a mobile phase of water/acetonitrile/acetic acid (84:15:1, v/v/v) at a flow rate of 4 ml/min.

Oxidation of olanzapine by human MPO. Olanzapine (100 μ M, in 1 ml PBS, pH 7.4) was incubated with MPO (1 unit) in the presence of H_2O_2 (100 μ M). After incubation for 30 min at 37°, the reaction was stopped by cooling the sample on ice, and the mixture was analyzed by HPLC or LC/MS. GSH (1 mM), NAC (1 mM), or *N*-acetyllysine (1 mM) was included in some incubations. Control experiments involved the omission of olanzapine, H_2O_2 , or MPO from the reaction mixture.

Human neutrophils (5×10^6 /ml, in 1 ml of HBSS) were incubated at 37° in the presence of olanzapine (100 μ M) and GSH (1 mM). The cells were activated with 40 ng of phorbol-12-myristate-13-acetate (in 10 μ l of dimethylsulfoxide) and incubated for an additional 40 min. The neutrophils were pelleted by centrifugation (1000 × *g*, 5 min), and the supernatant was analyzed by LC/MS.

Human leukocyte isolation. Neutrophils and mononuclear cells were isolated from venous blood of healthy volunteers by differential centrifugation over Ficoll-Paque as described in detail previously (Liu and Uetrecht, 1995). Peripheral blood mononuclear cells (2×10^6 cells/ml) were resuspended in RPMI 1640 (Media Services, University of Toronto) containing 10% heat-inactivated fetal bovine serum, 4 mM glutamine, 60 μ g/ml penicillin, and 100 μ g/ml streptomycin and then aliquoted onto 12-well plates (1 ml/well; Corning Glassworks, Corning, NY). The plates were incubated for 2 hr at 37° in an incubator (5% CO_2) to isolate monocytes (adherent cells) from T and B lymphocytes (nonadherent).

Cells were stained with 0.1% (w/v) trypan blue and counted with a hemocytometer. Trypan blue exclusion showed the initial viability to be >96% for all preparations. For some neutrophil preparations, cytospin slides were prepared. Light microscopy, after staining of cytospin slides with Wright's stain, confirmed that >98% of the cells had characteristic neutrophil morphology.

Neutrophils also were isolated from the peripheral blood of two patients who had had clozapine-induced agranulocytosis. Patient 1 was a woman on a clozapine dose of 300 mg/day. After 14 weeks of therapy, her neutrophil count dropped to 0, and the drug was stopped; 12 days later, the neutrophil count had recovered. Patient 2 was a woman receiving clozapine (75 mg/day); 12 weeks after starting therapy, her neutrophil count dropped to 0, and she was hospitalized. She received granulocyte colony-stimulating factor and was discharged 2 weeks later with a normal neutrophil count. The experiments in this report were performed 21 and 15 months after agranulocytosis occurred for patients 1 and 2, respectively.

Toxicity of the reactive intermediates of olanzapine and clozapine to neutrophils. Three different protocols were used to produce reactive metabolites. First, reactive metabolite was produced chemically by allowing clozapine or olanzapine to react with HOCl. Drug was dissolved in PBS (100 μ l, pH 7.4), and 0.9 molar equivalent of NaOCl (dissolved in PBS, pH 6.0, 100 μ l) was added. This results in the formation of an orange (clozapine) or red (olanzapine) reactive metabolite. Immediately, neutrophils (4×10^6 in 1 ml of HBSS) were added to the reactive metabolite solution. Assuming complete reaction between drug and NaOCl, the concentrations of reactive metabolite produced were 0.18, 1.8, or 18 μ M. Given the rapid reaction between hypochlorite and drug (Liu and Uetrecht, 1995), the use of molar excess of drug ensures that the neutrophils are not exposed to NaOCl, which may be directly toxic to the cells. In the second protocol, 4×10^6 neutrophils were incubated in 1 ml of HBSS containing drug and MPO (1 unit), and the reaction was started by the addition of 200 μ l of PBS containing H_2O_2 . In the final 1.2-ml volume, the concentration of drug was 0, 0.2, 2, or 20 μ M, and the H_2O_2 concentration was 10 mM. In the third protocol, 4×10^6 neutrophils were incubated in 1 ml of HBSS containing drug, and the reaction was started by the addition of 200 μ l of HBSS containing

H_2O_2 . In the final 1.2-ml volume, the concentration of drug was 0, 0.2, 2, or 20 μ M, and the H_2O_2 concentration ranged from 0 to 10 mM. After initiation of the reaction by one of the protocols outlined above, cells were incubated at 37° for 2 hr in a shaking water bath. At the end of this period, the cells were counted using a hemocytometer, and cell viability was assessed by trypan blue exclusion. In some experiments, the time course of clozapine toxicity was investigated. In these experiments, 100- μ l aliquots were removed at 0, 15, 30, 60, 90, and 120 min. At each time point, cells were counted using a hemocytometer, and cell viability was assessed by trypan blue exclusion. In a series of additional experiments, neutrophils were incubated in the presence of clozapine (20 μ M) and H_2O_2 in the presence of either 10 or 100 μ M dapsone or ketoprofen.

Toxicity of the reactive intermediates of olanzapine and clozapine to HL-60 cells and monocytes. HL-60 (human promyelocytic) cells were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 4 mM glutamine, 60 μ g/ml penicillin, and 100 μ g/ml streptomycin and passaged twice a week. Cell culture was carried out at 37° in a humidified atmosphere containing 5% CO_2 . The number of cells was determined using a hemocytometer, and cell viability was determined by trypan blue exclusion. Initial viability of the cells used in these experiments was always >95%. For exposure to drug reactive metabolites, cells were washed three times in HBSS, resuspended to 2×10^6 cells/ml, and then exposed to reactive metabolites as described above for neutrophils.

Purified monocyte preparations were washed three times in HBSS (1 ml) to remove cells that were not adherent to the tissue culture plates and then incubated with HBSS containing 0, 0.2, 2, or 20 μ M concentration of drug. To the solution, we added H_2O_2 (10 mM) to act as a cofactor for reactive metabolite generation. The total volume used per well was 1.2 ml. The cells then were incubated for 2 hr at 37°. At the end of this period, the HBSS was removed, and the percentage of viable cells was assessed using a 0.1% trypan blue solution.

Statistical analysis. Statistical tests were performed using In-Stat 2.01 (GraphPAD Software, San Diego, CA). Unless otherwise stated statistical significance was tested by one-way analysis of variance with Student-Newman-Keuls post-test. Values of *p* < 0.05 were considered statistically significant.

Results

Oxidation of olanzapine by hypochlorous acid. Olanzapine reacted with hypochlorous acid to produce an unstable intermediate with a λ_{max} of 540 nm (Fig. 2). Stopped-flow spectrophotometer measurements revealed that under the conditions used, the reactive intermediates of clozapine and olanzapine had formation rate constants (*K*, 1/sec) of 225 ± 0.7 and 213 ± 20 (mean \pm standard deviation; three or four determinations), respectively. The olanzapine reactive intermediate had a half-life of 35.5 ± 0.7 sec. The disappearance of the clozapine reactive intermediate was best described by a biexponential equation. The two half-lives were 5.1 ± 0.7 and 45.0 ± 3.5 sec (mean \pm standard deviation; three determinations). The longer half-life is equivalent to the value reported previously for the disappearance of the clozapine reactive intermediate (Liu and Uetrecht, 1995). In the absence of chloride anion, the disappearance of the clozapine reactive intermediate was described by a biexponential equation with half-lives of 5.06 and 93.52 sec.

The major ions observed when olanzapine was oxidized with HOCl in the flow system interfaced with a mass spectrometer had *m/z* values of 311 (100%) and 313 (83%). The ion at *m/z* 313 corresponds to the protonated molecular ion of olanzapine, whereas the ion at 311 corresponds to the olan-

zoline reactive metabolite (Fig. 3). When reduced GSH was added to the reactive intermediate formed when olanzapine reacts with HOCl, two olanzapine-GSH conjugates, with protonated molecular ions at m/z 618, were detectable by LC/MS (data not shown). The products were formed in the ratio 1:5. The reactive intermediate of olanzapine also could be trapped with NAC. When analyzed by LC/MS, two olanzapine-NAC conjugates were detected with retention times of 1.4 and 2.2 min and protonated molecular ions at m/z 474. The two conjugates were formed in a ratio of 1:6. LC/MS/MS of the conjugate with a retention time of 1.4 min showed major peaks at m/z 474 (MH^+ , 36%), 345 (olanzapine + S, 84%), 314 (17%), 288 (100%), 254 (22%), and 84

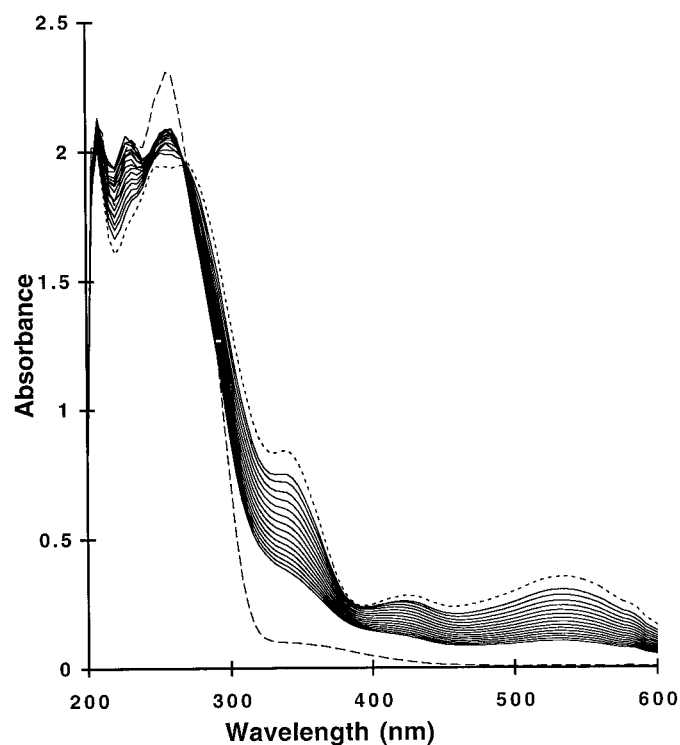


Fig. 2. Repetitive absorption spectra from the oxidation of olanzapine by HOCl. Cycle time, integration time, and total run time were 2, 0.5, and 30 sec, respectively. Drug and NaOCl concentrations were 100 μM in PBS, pH 6.0. Dashed line, UV absorbance spectrum of parent olanzapine. Dotted line, initial spectrum recorded after the addition of NaOCl.

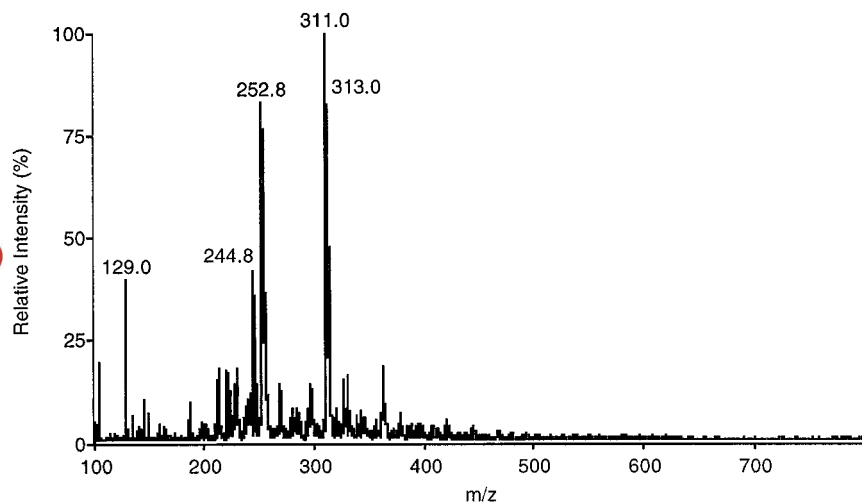


Fig. 3. Mass spectrum of the reactive intermediate in the reaction of olanzapine with HOCl obtained with a flow system coupled to a Sciex API III mass spectrometer.

[$CH_2CHN(CH_3)CH_2CH_2^+$, 53%]. LC/MS/MS of the conjugate with a retention time of 2.2 min showed major peaks at m/z 474 (MH^+ , 48%), 345 (olanzapine + S, 95%), 288 (100%), 245 (19%), and 84 [$CH_2CHN(CH_3)CH_2CH_2^+$, 56%]. This olanzapine-NAC conjugate was characterized further by 1H NMR spectroscopy. In the aromatic region, four signals, each integrating for a single proton, were observed: 6.88 p.p.m. (1 H, d, $J = 7.8$ Hz), 6.73 (1 H, t, $J = 7.8$ Hz), 6.42 (1 H, d, $J = 6.8$ Hz), and 6.34 (1 H, s). The 1H NMR of parent olanzapine had aromatic proton signals at 6.76–6.84 p.p.m. (3H, m), 6.68 (1 H, d, $J = 7.33$ Hz), and 6.33 (1 H, s). The olanzapine-NAC conjugate has one less aromatic proton than olanzapine. Because the signal at 6.33 p.p.m. (which is characteristic of a thiophene proton and we found exchanges in protic solvents) is preserved in the olanzapine-NAC conjugate, the NAC moiety must be attached to the phenyl ring of olanzapine. Although it is not possible to assign unequivocally the position at which NAC is attached, the fact that the olanzapine-NAC conjugate 1H NMR contains aromatic proton signals for a doublet, a triple, and a doublet indicates that substitution has occurred at either position 6 or 9 (Fig. 1).

Oxidation of olanzapine by the MPO/ H_2O_2 /Cl $^-$ system. When olanzapine was incubated with MPO and H_2O_2 in the presence of chloride, three species with MH^+ ions at m/z 329 were detected, which corresponds to the addition of oxygen to olanzapine. The three species had retention times of 1.5, 2.2, and 5.5 min. LC/MS/MS analysis revealed that the conjugate with a retention time of 1.5 min showed major peaks at m/z 329 (24%), 272 (loss of $CH_2=CHNHCH_3$, 48%), 242 (40%), 229 (loss of methylpiperazine, 100%), 213 (52%), 188 (63%), and 84 [$CH_2CHN(CH_3)CH_2CH_2^+$, 37%]. LC/MS/MS of the conjugate with a retention time of 2.2 min showed major fragments at m/z 329 (17%), 242 (35%), 229 (100%), 213 (60%), and 188 (37%). LC/MS/MS of the conjugate with a retention time of 5.5 min showed major fragments at m/z 329 (26%), 159 (19%), 146 (79%), 133 (100%), and 84 (38%). Inclusion of GSH in the incubation resulted in the formation of two olanzapine-GSH conjugates (with protonated molecular ions at m/z 618), which were detectable by LC/MS. The conjugates had retention times of 1.4 and 2.2 min. In the absence of MPO, no GSH conjugates were observed, suggesting that there is no direct reaction between olanzapine and H_2O_2 leading to the formation of a reactive intermediate. The corresponding NAC conjugates were ob-

served when NAC was included in the incubation, but no olanzapine conjugates were detectable when *N*-acetyllysine was included in the incubation (data not shown).

When human neutrophils were activated with phorbol-12-myristate-13-acetate in the presence of olanzapine and GSH, the same two olanzapine-GSH conjugates were formed (data not shown).

Toxicity of olanzapine and clozapine reactive intermediates toward human leukocytes and HL-60 cells. The toxicity of clozapine toward human neutrophils varied with the method used to generate reactive metabolites (Fig. 4). Although no toxicity was observed when NaOCl was used (Fig. 4A), significant toxicity was observed at clozapine concentrations of 2 and 20 μM when H_2O_2 (10 mM) was included

in the incubations (Fig. 4C). The toxicity was found to be dependent on both clozapine (Fig. 4C) and H_2O_2 concentration (Fig. 5). The addition of exogenous MPO (1 unit) to the reaction mixtures in the presence of H_2O_2 (10 mM) had little effect on the amount of toxicity observed, and under both conditions, the major effect of clozapine was to produce a decrease in the number of neutrophils with few blue-stained cells being observed. Time course studies revealed that toxicity was not detectable at 15 min after the initiation of reactive metabolite generation (Fig. 6). By 30 min, statistically significant toxicity was observed, and the number of viable neutrophils continued to decrease until the experiment was ended (120 min). No toxicity was observed with clozapine alone at concentrations up to 50 μM (data not shown). Although it seemed that there might be some toxicity at the highest concentration of olanzapine tested, this did not reach statistical significance.

Similar results to those obtained in neutrophils were observed in HL-60 cells (data not shown) and in human monocytes (Fig. 7). Exposure of human monocytes to the high concentration of H_2O_2 used in this *in vitro* system resulted in some toxicity toward the monocytes in the absence of clozapine (Fig. 7).

Dapsone, but not ketoprofen, was able to prevent the H_2O_2 -mediated clozapine-dependent toxicity observed in human neutrophils (Fig. 8).

Toxicity of clozapine reactive intermediates toward neutrophils from patients who have had clozapine-induced agranulocytosis. When neutrophils from patients who had clozapine-induced agranulocytosis were incubated with HOCl-generated clozapine reactive intermediate at concentrations up to 20 μM , no toxicity was observed (data not shown). When neutrophils from these patients were incubated with clozapine in the presence of H_2O_2 , the cells seemed to be more sensitive to the toxic effects than those from normal control subjects; however, the difference was

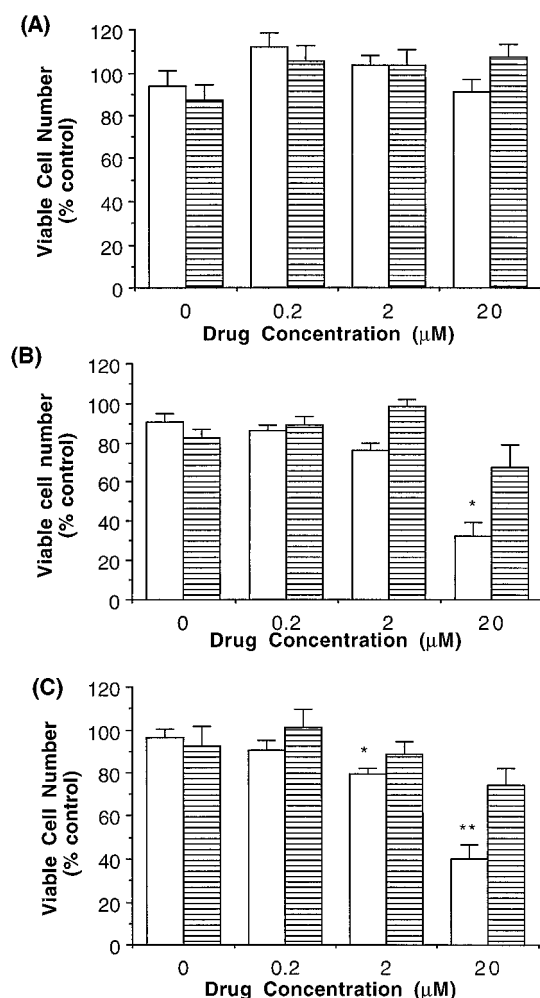


Fig. 4. Toxicity of clozapine (open bars) or olanzapine (striped bars) reactive intermediates to human neutrophils. Drug concentration varied between 0 and 20 μM as indicated. Three methods were used to generate the reactive intermediates: (A) reactive metabolite was produced chemically by reacting drug with NaOCl; (B) neutrophils were incubated with drug and MPO (1 unit), and the reaction was started by the addition of H_2O_2 (10 mM); (C) neutrophils were incubated with drug, and the reaction was started by the addition of H_2O_2 (10 mM). Cells not receiving drug (0) were treated with (A) 20 μM NaOCl, (B) 1 unit of MPO and H_2O_2 (10 mM), or (C) H_2O_2 (10 mM). Cells then were incubated at 37° for 2 hr. Results are expressed as percentage of the number of viable cells present in untreated cell samples incubated at 37° for 2 hr. Values are mean \pm standard error of 5 (olanzapine) or 6–13 (clozapine) individual experiments. *, $p < 0.05$; **, $p < 0.001$. Statistical differences compared with values in the absence of drug (0).

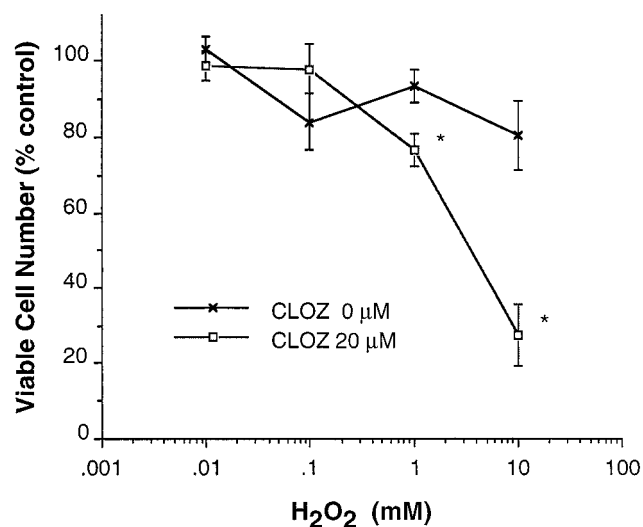


Fig. 5. Influence of H_2O_2 concentration on clozapine-induced toxicity toward neutrophils. Neutrophils were incubated at 37° for 2 hr with clozapine (CLOZ) (0 or 20 μM) and H_2O_2 (0.1–10 mM) as described in the text. Values are mean \pm standard error of four individual experiments. The number of viable cells is expressed relative to the number of cells in control incubations (without clozapine or H_2O_2). *, $p < 0.05$, Statistically different (Student's *t* test) from cells incubated with the equivalent H_2O_2 concentration in the absence of clozapine.

small and did not allow clear differentiation from control cells (Fig. 9).

Discussion

The results of these experiments demonstrate that like the structurally related antipsychotic drug clozapine, olanzapine is oxidized to a reactive intermediate by HOCl and by the MPO/H₂O₂/Cl⁻ system. It was possible to trap the reactive olanzapine metabolite using soft nucleophiles, such as GSH and NAC, to form two olanzapine-nucleophile conjugates. However, the olanzapine reactive intermediate did not react with the harder nucleophile, *N*-acetyllysine. The two olanzapine-NAC conjugates had very similar mass spectra, suggest-

ing that they have similar chemical structures. The observation that the same GSH conjugates were formed when olanzapine was incubated with activated neutrophils provides strong evidence that the reactive intermediate was a true metabolite.

Using a flow system, the molecular ion of the reactive olanzapine intermediate was observed to be *m/z* 311, which is 1 mass unit less than that of the parent compound. It is probable that the only hydrogen that could be lost to form an intermediate of this mass and react with NAC or GSH to form the observed conjugates is the N10 hydrogen (Fig. 1). Because the reactive species observed in the mass spectrometer has a positive charge, it is formally a nitrenium ion; however, the positive charge must be highly delocalized. The formation of the reactive metabolite from olanzapine described in this report is entirely analogous to the formation of a clozapine reactive metabolite we reported previously (Liu and Uetrecht, 1995).

Although the use of clozapine has been associated with a relatively high incidence of agranulocytosis (0.8%), no cases of agranulocytosis have been reported after the administration of olanzapine. Because the two compounds produced similar reactive metabolites, we wanted to determine whether exposure of human neutrophils and monocytes and the promyelocytic HL-60 cell line to the reactive metabolites of clozapine and olanzapine resulted in toxicity. Three different methods were used to produce the reactive metabolite. The toxicity of clozapine and olanzapine reactive metabolites toward target cells was found to vary both with the drug used and with the method of reactive metabolite generation. With clozapine, no toxicity was observed when NaOCl was added to drug to produce the reactive intermediate that was then added to the cells (Fig. 4A). However, when neutrophils were incubated with clozapine and H₂O₂ (in the presence or absence of exogenous myeloperoxidase), significant toxicity toward human neutrophils was observed (Fig. 4). No toxicity was observed in the absence of H₂O₂. The most likely explanation of this finding is that the H₂O₂ enters the cell and is used as a cofactor by intracellular enzymes (presumably MPO) to generate the cytotoxic clozapine reactive metabolite. It was not possible to detect oxidation of clozapine after mixture of the drug with H₂O₂, suggesting there is no direct chemical reaction between the two substances (Liu and Uetrecht, 1995). Cytotoxicity also was observed when HL-60 cells and monocytes were exposed to clozapine and H₂O₂ (in the presence or absence of exogenously added myeloperoxidase). Because both HL-60 cells (Koeffler *et al.*, 1985; Murao *et al.*, 1988) and monocytes (Winterbourn, 1989) are known to express MPO, these results are consistent with the MPO-catalyzed conversion of clozapine to a cytotoxic metabolite. Further support for this hypothesis comes from the data showing that dapsone can inhibit the toxicity produced by clozapine (Fig. 8). Previous studies have shown that dapsone is both an inhibitor (Kettle and Winterbourn, 1991) and a substrate for human MPO (Uetrecht *et al.*, 1993). Although it is not possible to discount the involvement of other pharmacological effects of dapsone in the inhibition of clozapine induced toxicity, it is of interest that ketoprofen, an inhibitor of cyclooxygenase, was unable to prevent the clozapine-induced toxicity.

We cannot be certain why only the H₂O₂-based methods of reactive metabolite production should cause toxicity, but

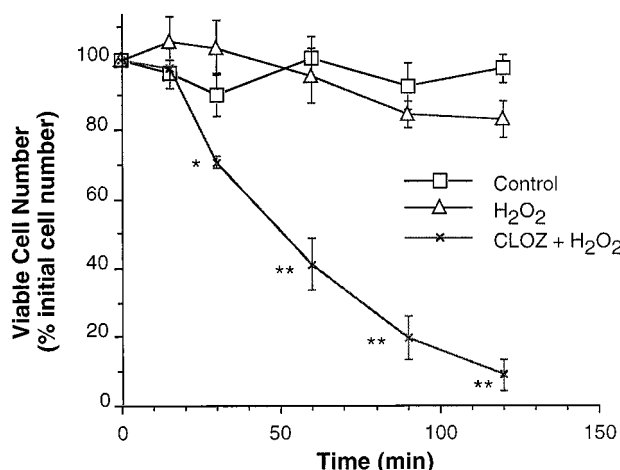


Fig. 6. Time course of clozapine toxicity toward human neutrophils. Neutrophils were incubated at 37° for various time periods with clozapine (CLOZ) (0 or 20 μ M) and H₂O₂ (10 mM) as described in the text. Control cells were incubated in HBSS alone. Values are mean \pm standard error of three individual experiments. The number of viable cells is expressed relative to the initial value (time 0) determined for each experiment. *, $p < 0.05$; **, $p < 0.001$. Statistically different from the viable cell number at time 0.

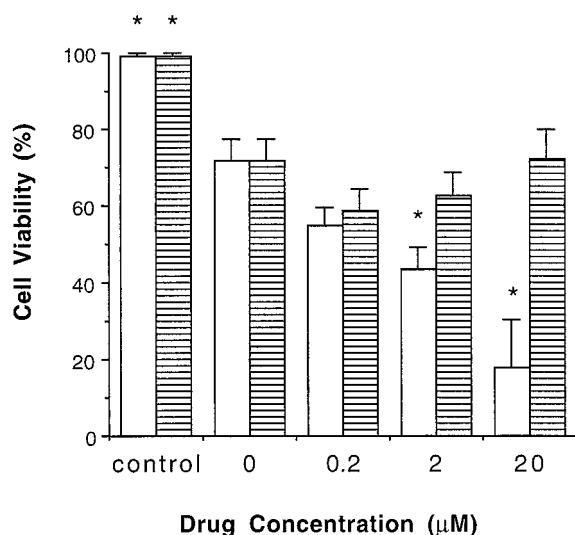


Fig. 7. Toxicity of clozapine (open bars) and olanzapine (striped bars) toward human monocytes. Monocytes were incubated at 37° for 2 hr with drug concentrations of 0–20 μ M and H₂O₂ (10 mM). Toxicity was assessed by trypan blue exclusion. Control cells were incubated in HBSS alone. Values are mean \pm standard error of three or four individual experiments. *, $p < 0.01$. Statistically different from those of cells receiving H₂O₂ alone.

some explanations can be proposed. One possibility is that the toxicity may be related to the length of time to which the cells are exposed to drug reactive metabolite. In the HOCl method, exposure of the cells to reactive intermediate occurs for only a short initial period of time. In the H_2O_2 methods, the reactive metabolite presumably is generated over a longer time period; hence, the duration of exposure of the cells to reactive metabolite is longer. A second possible explanation is that when cells are exposed to HOCl-generated reactive metabolite, the reactive metabolite is not able to reach critical sites inside the cells, but when H_2O_2 is added, the reactive metabolite can be generated intracellularly. Support for this hypothesis comes from studies showing that extracellular GSH can inhibit covalent binding of HOCl-generated clozapine reactive intermediate to neutrophils but does not inhibit covalent binding of H_2O_2 -generated clozapine reactive metabolite (Gardner *et al.*, 1998). These two hypotheses are not mutually exclusive.

Although incubation of clozapine and H_2O_2 with neutrophils produced toxicity, no statistically significant toxicity was observed when olanzapine and H_2O_2 (or HOCl) were incubated with human neutrophils, monocytes, or HL-60 cells. Thus, although both olanzapine and clozapine produce reactive nitrenium ions, these metabolites seem to have differing abilities to cause cytotoxicity. This difference was surprising because the two nitrenium ions seem to have similar chemical reactivities. In particular, both clozapine and olanzapine reactive metabolites preferentially bind to *S*-nucleophiles, with little reactivity toward harder nucleophiles such as *N*-acetyllysine being observed. However, although the reaction to simple nucleophiles *in vitro* was similar, the covalent binding of the two drugs to neutrophil proteins under the same conditions that led to clozapine toxicity in these studies was significantly different (Gardner *et al.*, 1998). This difference in covalent binding of reactive metabolite to cellular protein may explain the differences in toxicity.

The observation that the reactive metabolite of clozapine is toxic to neutrophils, as well as HL-60 cells, and that of olanzapine is not under the same conditions could be taken

as evidence that clozapine-induced agranulocytosis is due to the cytotoxicity of the reactive metabolite. In addition, the observation that the clozapine metabolite seems to be more toxic to neutrophils from patients with a history of clozapine-induced agranulocytosis supports this hypothesis. However, if direct cytotoxicity were the mechanism, it would be somewhat difficult to explain the idiosyncratic nature of clozapine-induced agranulocytosis and the long delay between starting the drug and the onset of toxicity. Furthermore, we have been unable to induce neutropenia in animals despite treatments with high doses of clozapine and additional treatments to increase the formation of the reactive metabolite in bone marrow (Utrecht JP, unpublished observations). Phenytoin, carbamazepine, and sulfamethoxazole are examples of other drugs in which the reactive metabolite of the drug seems to be more toxic to cells from patients with a history of a severe idiosyncratic reaction to that drug, but the adverse reaction does not seem to be a simple cytotoxic reaction (Shear and Spielberg, 1988; Rieder *et al.*, 1989). It may be that cytotoxicity plays a role in such reactions by leading to an increase in the phagocytosis of drug-modified cells, which may in turn help to stimulate an immune reaction leading to a severe idiosyncratic reaction. However, there is no strong evidence that clozapine-induced agranulocytosis is immune mediated, and it is difficult to explain the long delay (6 weeks in several cases) in the onset of clozapine-induced agranulocytosis on reexposure of patients with a previous history of clozapine-induced agranulocytosis if the mechanism were a simple immune reaction against drug-modified cells (Safferman *et al.*, 1992).

This *in vitro* test system showed an interesting difference in the ability of clozapine and olanzapine to induce toxicity toward human neutrophils, but the methods used have some limitations. Induction of toxicity required exposure of neutrophils to clozapine in the presence of high concentrations of H_2O_2 . The neutrophils were relatively resistant to short term exposure to H_2O_2 , but a variable amount of toxicity was observed (loss of up to 25% of cells present in control incubations) in the absence of clozapine (Fig. 9). This background toxicity means that the method is not very sensitive toward a

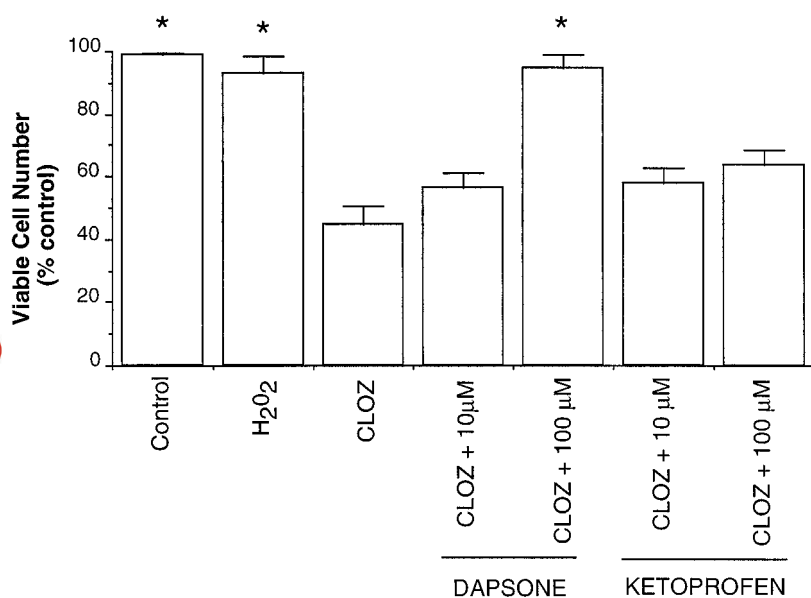


Fig. 8. Effect of dapsone and ketoprofen on clozapine toxicity toward human neutrophils. Neutrophils were incubated at 37° for 90 min with 20 μ M clozapine, 10 mM H_2O_2 (CLOZ), and dapsone (10 or 100 μ M) or ketoprofen (10 or 100 μ M) as indicated. H_2O_2 , cells incubated with H_2O_2 in the absence of clozapine. Control, neutrophils incubated in the absence of both clozapine and H_2O_2 . Values are mean \pm standard error of four individual experiments. *, $p < 0.01$. Statistically different from those of cells incubated with clozapine and H_2O_2 .

small amount of drug-induced toxicity. In monocytes, which have lower levels of MPO (Klebanoff, 1990), statistically significant toxicity was observed in the absence of clozapine; in monocyte-depleted lymphocytes, it was not possible to observe toxic effects of clozapine because ~50% of cells died after exposure to H_2O_2 (10 mM) in the absence of clozapine (data not shown). This value is similar to a published LC_{50} value for H_2O_2 toward human lymphocytes (O'Donnell-Tormey et al., 1985).

In summary, we demonstrated that like clozapine, olanzapine is oxidized to a reactive nitrenium ion by HOCl , the major oxidant produced in activated neutrophils, and by an $\text{MPO}/\text{H}_2\text{O}_2/\text{Cl}^-$ system. However, the olanzapine reactive metabolite has a lower propensity to cause toxicity toward human neutrophils, monocytes, and HL-60 cells than the reactive clozapine nitrenium ion in an *in vitro* cytotoxicity assay. The lower toxic potential of the olanzapine reactive metabolite in conjunction with the lower therapeutic plasma concentrations of olanzapine (Aravagiri et al., 1997) compared with clozapine (Weigmann and Hiemke, 1992) (0.1 μM versus 2 μM) may help to explain why compared with clozapine, olanzapine is not associated with agranulocytosis in humans. Furthermore, understanding the mechanism of toxicity of clozapine in this model system may provide new insights into the mechanism of clozapine-induced agranulocytosis, which currently is undefined.

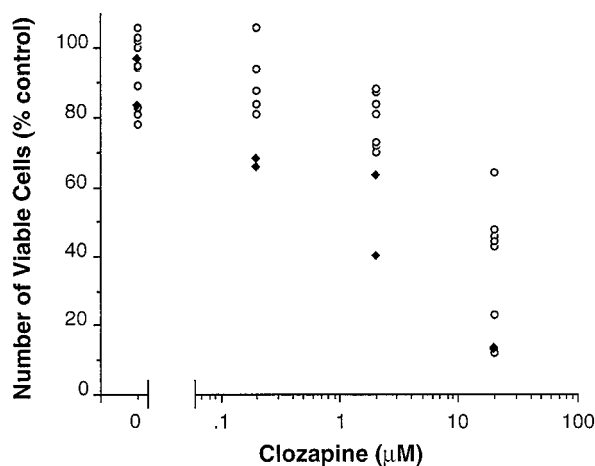


Fig. 9. Neutrophils from healthy volunteers (○) (5–10) or patients who had had clozapine-induced agranulocytosis (◆) (2) were incubated with clozapine (0–20 μM) and H_2O_2 (10 mM) for 2 hr at 37°. The results are expressed as the percentage of the number of viable cells present in untreated cell samples incubated at 37° for 2 hr.

Acknowledgments

We thank Dr. Ian Guest for drawing the blood samples, Cynthia Ju for the stopped-flow kinetic data, and Doreen Wen for help with the LC/MS analysis.

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