

# A Comparison of the Covalent Binding of Clozapine and Olanzapine to Human Neutrophils *In Vitro* and *In Vivo*

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## ABSTRACT

Covalent binding of a reactive metabolite of clozapine to neutrophils or their precursors is thought to play a role in the development of clozapine-induced agranulocytosis. Immunoblotting studies with an anti-clozapine antiserum detected covalent binding of clozapine to human neutrophils *in vitro* when HOCl was used to generate clozapine reactive metabolite (major clozapine adducts of 31, 49, 58, 78, 86, 126, 160, and 204 kDa). In addition, incubating neutrophils with clozapine and H<sub>2</sub>O<sub>2</sub> (major clozapine adducts of 49 and 58 kDa) or clozapine, H<sub>2</sub>O<sub>2</sub>, and human myeloperoxidase (major clozapine adducts of 31, 49, 58, and 126 kDa) also resulted in covalent binding of clozapine to the neutrophils. The covalent binding of clozapine to neutrophils was inhibited by extracellular glutathione when HOCl, but not H<sub>2</sub>O<sub>2</sub> was used to generate reactive metabolite. We found that the antiserum against clozapine also recognized

olanzapine, an antipsychotic drug that forms a similar reactive metabolite to clozapine but has not been associated with induction of agranulocytosis. Repeating the *in vitro* experiments with olanzapine revealed that the major olanzapine-modified polypeptides had molecular masses of 96, 130–170, and 218 kDa. Only relatively low levels of 31, 49, and 58 kDa adducts were observed. Clozapine-modified polypeptides also were detected in neutrophils from patients being treated with clozapine. A major 58-kDa clozapine-modified polypeptide was detected in all patients tested. In contrast, no drug-modified polypeptides were detected in neutrophils from patients taking olanzapine. The differences in covalent binding exhibited by the two compounds and, in particular, the lack of olanzapine binding to human neutrophils *in vivo* may help to explain the difference in toxicity of these two drugs.

Clozapine (Fig. 1) is an atypical antipsychotic agent that has been shown to be effective in the treatment of refractory schizophrenia. Therapeutically, the use of clozapine is limited because it has been shown to cause agranulocytosis in ~0.8–1.0% of patients treated with the drug (Alvir *et al.*, 1993; Krupp and Barnes, 1989). The mechanism underlying clozapine-induced agranulocytosis is at present undefined, but toxic (Veys *et al.*, 1992; Williams *et al.*, 1998) and immunological mechanisms (Pisciotta *et al.*, 1992) have been proposed. In addition, clozapine-induced agranulocytosis may have a genetic component (Corzo *et al.*, 1995; Lieberman *et al.*, 1990).

Recent *in vitro* studies have demonstrated that clozapine is metabolized to a reactive nitrenium ion by activated human neutrophils (Liu and Uetrecht, 1995) and bone marrow cells (Maggs *et al.*, 1995), which subsequently are covalently modified by this clozapine metabolite. Covalent modification of

neutrophil/bone marrow proteins by clozapine could lead to agranulocytosis, either by a direct toxic or an immunological mechanism. For instance, covalent binding of clozapine to proteins that are key for survival of the cell could alter the function of the protein and lead to cell death, as has been suggested for covalent binding of acetaminophen in the liver (reviewed by Pumford and Halmes, 1997). Alternatively, clozapine-modified proteins could be recognized by the immune system, leading to an antibody- or a cell-mediated response against the cells expressing the modified neoantigens and to their destruction, as seems to be the case in halothane-induced hepatitis (Kenna and Neuberger, 1995).

The problems associated with the use of clozapine have led to efforts to develop related antipsychotic agents that have a similar therapeutic profile to clozapine but do not cause agranulocytosis. One such agent is olanzapine (Fig. 1), which although structurally very similar to clozapine, does not seem to cause agranulocytosis (Fulton and Goa, 1997). Recently, we demonstrated that like clozapine, olanzapine can be metabolized to a reactive nitrenium ion, but that although the clozapine reactive metabolite induces toxicity in human

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**ABBREVIATIONS:** PBMC, peripheral blood mononuclear cell; ECL, enhanced chemiluminescence; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; ELISA, enzyme-linked immunosorbent assay; HBSS, Hanks' balanced salt solution (without phenol red); KLH, keyhole limpet hemocyanin; NAC, *N*-acetylcysteine; RSA, rabbit serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

neutrophils at concentrations close to those used therapeutically (2  $\mu$ M), the olanzapine reactive metabolite did not cause toxicity toward human neutrophils at concentrations up to 20  $\mu$ M (Gardner *et al.*, 1998). The bases for the difference in toxicity of these two structurally similar reactive metabolites currently are unclear.

In an effort to gain further insight into the molecular mechanisms involved in clozapine-induced agranulocytosis, we developed sensitive immunochemical techniques and used these techniques to compare the pattern of covalent binding of clozapine and olanzapine to human neutrophils *in vitro*. An additional unresolved issue is whether clozapine is metabolized to reactive metabolites by human bone marrow and/or neutrophils *in vivo*. In this study, we also used the immunochemical techniques to demonstrate that in patients treated with clozapine, neutrophils, but not PBMCs, are covalently modified by drug.

## Experimental Procedures

**Materials.** Clozapine, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), sodium hypochlorite (NaOCl), and MPO were obtained from the sources given previously (Gardner *et al.*, 1998). Casein, RSA, thimerosal, *N*-hydroxysuccinimide, methylumbelliferyl phosphate, and EDC were purchased from Sigma Chemical (St Louis, Mo). Stock acrylamide solution (40%) was purchased from BioRad (Mississauga, Canada). Nitrocellulose and ECL film were purchased from Amersham Canada (Oakville, Ontario, Canada). Supersignal ECL detection reagents were purchased from Pierce Chemical (Rockford, IL). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L chains) was purchased from Zymed (San Francisco, CA). Alkaline phos-

phatase-conjugated goat anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Isolation of olanzapine.** Olanzapine was isolated from tablets (Zyprexa; Eli Lilly, Indianapolis, IN) as described in the companion article (Gardner *et al.*, 1998).

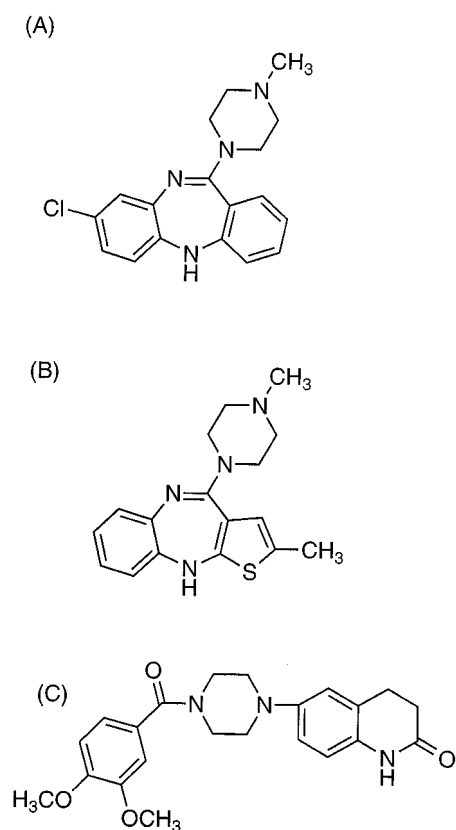
**Synthesis of clozapine-NAC-modified KLH and RSA.** Clozapine (10 mM) was dissolved in 60% ethanol (10 ml, pH 4.0) and treated with NaOCl (10 mM). NAC (20 mM, 10 ml) was added immediately, and the mixture was stirred for 10 min. Ethanol was removed under reduced pressure, and solid  $\text{NaHCO}_3$  was added to the acidified solution until most of the unreacted clozapine precipitated out (pH 8.0). Precipitated clozapine was removed by filtration, and the filtrate then was extracted with ethyl acetate (three times 10 ml). The aqueous solution of clozapine-NAC was adjusted to pH 6.0 with glacial acetic acid and applied to an LC-18 solid phase extraction column (Supelco; Supelco Inc., Bellefonte, PA). The column was washed with  $\text{H}_2\text{O}$  followed by a 7.5%  $\text{CH}_3\text{CN}$  solution (containing 1%  $\text{CH}_3\text{COOH}$ , 2 mM ammonium acetate) to remove unreacted NAC and NAC disulfide. Finally, the clozapine-NAC conjugate was eluted with 25%  $\text{CH}_3\text{CN}$  (containing 1%  $\text{CH}_3\text{COOH}$ , 2 mM ammonium acetate).  $\text{CH}_3\text{CN}$  was removed under reduced pressure, and the clozapine-NAC conjugate was applied to an LC-18 solid-phase extraction column, washed with water to remove ammonium acetate, and then eluted with methanol. Formation of the clozapine-NAC conjugate was confirmed by liquid chromatography-mass spectrometry.

Clozapine-NAC conjugate (10 mM) was dissolved in a 1:1 mixture of dimethylformamide (dried over  $\text{Na}_2\text{CO}_3$ ) and acetonitrile (1 ml). To the solution, we added solid *N*-hydroxysuccinimide (15 mM final concentration) followed by solid EDC. The formation of clozapine-NAC-hydroxysuccinimide active ester (retention time, 12.4 min) was monitored using high performance liquid chromatography, and small aliquots of EDC were added until all of the clozapine-NAC had reacted. High performance liquid chromatography was carried out with an Ultracarb 5 ODS 30 column (150  $\times$  10 mm; Phenomenex, Torrance, CA) and a mobile phase of 2 mM ammonium acetate/ $\text{CH}_3\text{CN}/\text{CH}_3\text{COOH}$  (74:25:1, v/v) at a flow rate of 1 ml/min.

The clozapine-NAC active ester was placed under a stream of nitrogen until the acetonitrile and most of the dimethylformamide had evaporated. The mixture then was dissolved in ethyl acetate (2 ml) and extracted with dilute  $\text{NaHCO}_3$  (one times 2 ml) followed by  $\text{H}_2\text{O}$  (two times 2 ml). This helped to remove excess hydroxysuccinimide, EDC, and the urea product of EDC. The ethyl acetate solution was evaporated to dryness under nitrogen, and the clozapine-NAC active ester was dissolved in  $\text{CH}_3\text{CN}$  (100  $\mu$ l). The clozapine-NAC active ester was added dropwise with stirring to either KLH (8 mg) or RSA (8 mg) dissolved in 1 ml of potassium phosphate buffer (0.1 M; pH 8.0). The mixture was stirred for 1 hr at room temperature, dialyzed extensively against water, and then lyophilized.

**Human leukocyte isolation.** Neutrophils and PBMCs were isolated from venous blood of healthy volunteers as described previously (Liu and Uetrecht, 1995). Trypan blue exclusion showed the initial viability to be >98% for all preparations. For some neutrophil preparations, cytospin slides were prepared and stained with Wright's stain. The use of light microscopy confirmed that >95% of the cells had characteristic neutrophil morphology.

**Exposure of neutrophils and PBMCs to drug reactive metabolites.** As described in the companion article (Gardner *et al.*, 1998), three different protocols were used to produce drug reactive metabolites, and the neutrophils ( $5 \times 10^6/\text{ml}$ ) were incubated at 37° for 2 hr in a shaking water bath. In some experiments, the ability of glutathione (1 mM) to inhibit covalent binding of clozapine reactive metabolite to neutrophils was examined. At the end of the incubation period, cells were pelleted by centrifugation (500  $\times$  g, 5 min) and resuspended in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% Triton X-100; 200  $\mu$ l). An aliquot of the sample was taken for measurement of protein concentration. An equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/ml) was



**Fig. 1.** Chemical structures of clozapine (A), olanzapine (B), and vesnarinone (C).

added to the remainder of the lysed cell sample. The samples were boiled at 100° for 10 min before loading onto the gel.

To examine the selectivity of clozapine reactive metabolite for covalent binding to neutrophils and PBMCs, a mixture of equal numbers of neutrophils and PBMCs ( $5 \times 10^6$  of each/ml) were exposed to HOCl-generated clozapine reactive metabolite (18  $\mu$ M) and incubated at 37° for 2 hr. At the end of this incubation period, the cell suspension was underlaid with Ficoll-Paque, and neutrophils and PBMCs were separated by centrifugation ( $500 \times g$ , 25 min). The neutrophils and PBMCs were washed twice in PBS and then resuspended in cell lysis buffer. An aliquot of the sample was taken for measurement of protein concentration. An equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/ml) was added to the remainder of the lysed cell sample. The samples were boiled at 100° for 10 min before loading onto the gel.

**Covalent binding of clozapine and olanzapine to human MPO.** Clozapine or olanzapine (0, 0.2, 2, 20, or 200  $\mu$ M; 0.1 ml) was incubated with MPO (1 or 5 units) in the presence of  $H_2O_2$  (100  $\mu$ M). After incubation for 30 min at 37°, the reaction was stopped by cooling the sample on ice, and an equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/ml) was added. The samples were boiled at 100° for 10 min before analysis by SDS-PAGE.

**Experiments to detect drug-modified neutrophil or PBMC proteins in patients taking clozapine or olanzapine.** Blood samples (20 ml) were drawn from patients taking clozapine (four patients) or olanzapine (four patients; patient details are outlined in Table 1). Neutrophils and PBMCs were isolated by centrifugation over Ficoll-Paque as described previously (Liu and Uetrecht, 1995). After washing cells twice in HBSS, the neutrophils and PBMCs were resuspended at a concentration of  $1 \times 10^6$  cells/ $10 \mu$ l cell lysis buffer. An equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/ml) was added to the sample, which then was boiled at 100° for 10 min before analysis by SDS-PAGE.

**Production of anti-clozapine-NAC-KLH antiserum.** Polyclonal anti-clozapine-NAC-KLH antibodies were raised in a 2-kg, male, pathogen-free New Zealand White rabbit (Charles River, Quebec, Canada) housed in the animal care facility at The Hospital for Sick Children, Toronto. After preimmune serum was obtained, each animal was immunized with the clozapine-NAC-KLH conjugate (1 mg in 0.5 ml of PBS emulsified with an equal volume of Freund's complete adjuvant) subcutaneously at multiple sites. Injections with 500  $\mu$ g of clozapine-NAC-KLH in Freund's incomplete adjuvant divided into six to eight subcutaneous sites were repeated 4, 6, 8, and 12 weeks after the initial immunization. Exsanguination of the animal while it was under pentobarbital anesthesia was conducted 10 days after the final immunization. Blood was allowed to clot overnight at 4° and then centrifuged at  $400 \times g$ . The serum was recovered and heat-inactivated at 56° for 30 min before being placed in aliquots and stored at -20°.

TABLE 1  
Characteristics of patients taking clozapine and olanzapine

Patient	Sex	Dosage mg/day	Treatment duration months
Clozapine			
1	F	125	24
2	M	100	36
3	M	600	48
4	M	500	66
Mean $\pm$ standard deviation		331 $\pm$ 256	43.5 $\pm$ 17.9
Olanzapine			
1	M	5	6
2	M	10	7
3	F	10	3
4	M	5	2
Mean $\pm$ standard deviation		7.5 $\pm$ 2.9	4.5 $\pm$ 2.4

**ELISA.** Clozapine-NAC-RSA or RSA (100  $\mu$ l of a 15  $\mu$ g/ml solution) was incubated overnight in flat-bottom 96-well plates (Costar, Cambridge, MA) at 4°. The following morning, plates were emptied and washed with ELISA wash buffer [10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 0.5% (w/v) casein, and 0.02% (w/v) thimerosal]. The plates then were emptied and washed an additional three times. After the last wash, the plates were tapped dry, and various dilutions of the anti-clozapine-NAC-KLH antiserum (100  $\mu$ l in PBS) were added to the plates. The ELISA plates were incubated at room temperature for 3 hr. Plates subsequently were washed four times with ELISA wash buffer and tapped dry. Alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:5000 in PBS) was added to each well of the plate (100  $\mu$ l/well). The ELISA plates were incubated at room temperature for 2 hr. Plates subsequently were washed four times with ELISA wash buffer and two times with PBS. A stock solution of methyl umbelliferyl phosphate (10 mg/ml in dimethylsulfoxide, kept at -20°) was diluted 1:100 in PBS, and this solution was added to the ELISA plates (100  $\mu$ l/well). Plates were incubated at room temperature for 10 min before fluorescence was measured with a Fluorescence Concentration Analyzer (Pandex, Mundelein, IL) set at 365/450 nm (excitation/emission).

**SDS-PAGE and immunoblotting.** Samples for analysis by SDS-PAGE were solubilized by boiling for 10 min in sample buffer [8% (w/v) SDS, 20% (v/v) glycerol, 0.002% bromphenol blue, 125 mM Tris-HCl, pH 6.8] containing dithiothreitol (6 mg/ml). SDS-PAGE was performed using a minigel system (Mini-PROTEAN II; BioRad, Mississauga, Ontario) and the discontinuous buffer system described by Laemmli (1970). Stacking and resolving gels were 4% and 10% acrylamide, respectively. Gels were run at 200 V until the dye front reached the bottom of the resolving gel (~45 min). Electrophoretic transfer of resolved proteins to nitrocellulose was carried out using a buffer of 15.7 mM Tris, 120 mM glycine, pH 8.3, containing 20% (v/v) methanol, for 75 min at 100 V using a mini Trans-Blot transfer cell (BioRad). Nitrocellulose was either stained for protein for 5 min using 0.1% amido black 10B in 45% (v/v) methanol and 10% (v/v) acetic acid and then destained using 70% (v/v) methanol and 2% (v/v) acetic acid or used for antibody development.

The subsequent steps were conducted at room temperature with constant shaking. Before exposure to antiserum, the nitrocellulose was blocked by incubation in a solution containing 10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 2.5% (w/v) casein, and 0.02% (w/v) thimerosal for 2 hr. The blocked nitrocellulose was incubated for 15 hr with anti-clozapine-NAC-KLH antiserum diluted in ELISA wash buffer [10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 0.5% (w/v) casein, and 0.02% (w/v) thimerosal]. Unbound antibodies were removed by washing the nitrocellulose in wash buffer (3  $\times$  10 min). The nitrocellulose then was incubated for 2 hr with horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L chain) antiserum (diluted 1:10,000 in wash buffer). The nitrocellulose was washed in wash buffer (3  $\times$  10 min) followed by washing with 50 mM Tris-HCl, pH 7.4, and 154 mM NaCl (3  $\times$  5 min). The nitrocellulose sheets were incubated in Super Signal ECL reagent for 5 min, and bound antibodies were visualized by exposing the nitrocellulose to ECL film under safe-light conditions.

In experiments to determine the specificity of the anti-clozapine-NAC-KLH antiserum, the antiserum (1:3000 final dilution) was incubated with varying concentrations of clozapine, clozapine-NAC, olanzapine, or vesnarinone for 30 min before the addition of the primary antiserum to the nitrocellulose membrane. The washing and subsequent development of the immunoblots were performed as outlined above.

**Dosing of rats with clozapine *in vivo*.** Female Lewis rats (200 g) were obtained from Charles River and housed in standard cages with free access to water and powdered lab chow. After a 1-week acclimation period, during which food intake was monitored, the rats were either continued on the powdered lab chow diet (control) or switched to a diet in which clozapine was mixed with the powdered lab chow such that the rats had an intake of 40 mg of clozapine/kg/day (treated). Rats were fed control or clozapine-containing diets for

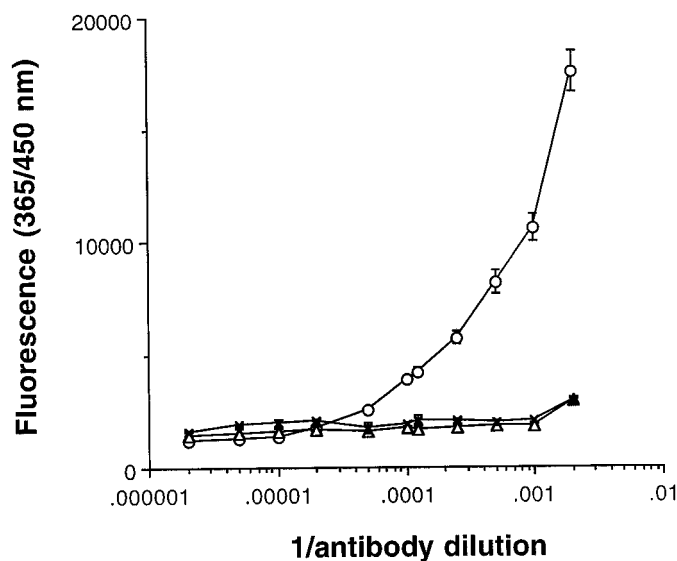


a period of 6 weeks. At the end of the study, rats were killed by cervical dislocation, and blood was collected in heparinized syringes by cardiac puncture. The plasma was collected after centrifugation at  $1000 \times g$  for 20 min and stored at  $-20^\circ$  until clozapine concentrations were measured. The femurs were removed, and the bone marrow was collected into RPMI 1640 culture medium (University of Toronto, Media Services). The bone marrow was suspended by passage (five times) through a 1-ml automatic pipette tip. After washing the cells twice in RPMI 1640 and once in HBSS, contaminating red blood cells were lysed by incubation in ammonium chloride (0.16 M)/Tris (17 mM; pH 7.2) buffer for 10 min. The bone marrow cells then were washed an additional two times in HBSS and counted using a hemocytometer. Bone marrow cells were lysed in cell lysis buffer, protein concentration was determined, and the samples were diluted to give a protein concentration of 3 mg/ml; then, an equal volume of SDS-PAGE sample buffer containing dithiothreitol (6 mg/ml) was added. The sample was boiled at  $100^\circ$  for 10 min before analysis by SDS-PAGE.

**Other methods.** Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.  $IC_{50}$  values for inhibition of antiserum recognition were calculated from image analysis of the ECL films using an image analysis system from Image Research (St. Catherine's, Ontario, Canada) (Tyndale *et al.*, 1997). Cross-reactivity of the antiserum for clozapine and olanzapine was calculated using the formula: cross-reactivity = ( $IC_{50}$  clozapine/ $IC_{50}$  olanzapine)  $\times$  100%. Clozapine concentration in rat plasma was measured according to the method of Weigmann and Hiemke (1992).

## Results

**Characterization of the antiserum.** ELISA analysis (Fig. 2) demonstrated that the anti-clozapine-NAC-KLH antiserum recognized clozapine-NAC-RSA but not RSA alone. Hapten inhibition studies demonstrated that in immunoblot experiments, the recognition of clozapine reactive metabolite-modified neutrophil proteins by the antiserum could be inhibited by preincubation of the antiserum with clozapine, olanzapine, or clozapine-NAC conjugate but not by the structurally unrelated compound vesnarinone (Fig. 3). Detailed analysis revealed that the  $IC_{50}$  value for inhibition of the antiserum binding was 1.0 nM for clozapine (mean of three



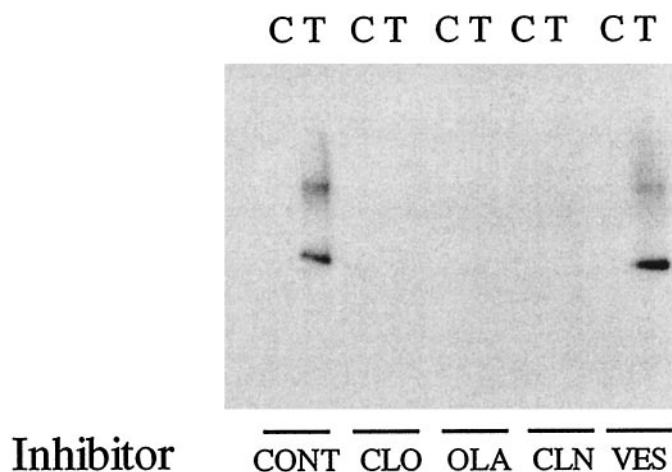
**Fig. 2.** ELISA analysis showing binding of the anti-clozapine-NAC-KLH antiserum to wells of microtiter plates coated with clozapine-NAC-RSA conjugate (O), RSA ( $\Delta$ ), or water (X).

determinations) and 9.7 nM for olanzapine (mean of three determinations) but  $>100 \mu\text{M}$  for vesnarinone. The cross-reactivity between clozapine and olanzapine was 10.3%.

**Immunoblotting of neutrophils and PBMCs exposed to reactive metabolites of clozapine and olanzapine *in vitro*.** Covalent binding of clozapine to human neutrophils was found to be highly dependent on the method of reactive metabolite generation (Fig. 4). When HOCl was used to generate reactive metabolite, a wide range of neutrophil proteins with molecular masses of 31–200 kDa were covalently modified. The major clozapine-modified polypeptides had molecular masses of 31, 49, 58, 78, 86, 126, 160, and 204 kDa.

In contrast, when  $\text{H}_2\text{O}_2$  was added as the cofactor for reactive metabolite formation, a lower amount of covalent binding occurred with the formation of major clozapine-polypeptide adducts of 49 and 58 kDa and minor adducts of 31, 96, and 126 kDa (Fig. 4) being observed. In one of the six subjects studied, the major adduct formed was 31 kDa with relatively less of the 49- and 58-kDa proteins (data not shown). When  $\text{H}_2\text{O}_2$  and MPO were used to generate reactive metabolite, the signal intensity was intermediate between that seen with HOCl and  $\text{H}_2\text{O}_2$  alone; again, major clozapine-modified polypeptides of 31, 49, and 58 kDa were observed. Prolonged development of the immunoblots (Fig. 4B) showed that when  $\text{H}_2\text{O}_2$  alone or in combination with MPO was used to produce reactive metabolite, covalent binding could be detected at clozapine concentrations as low as  $0.2 \mu\text{M}$ . At this concentration, the 49-kDa clozapine-protein adduct was the only detectable clozapine-modified polypeptide. Regardless of the method of reactive metabolite generation, it was possible to inhibit the immunoblot signal by incubating the primary antiserum with clozapine ( $10 \mu\text{M}$ ) before adding it to the nitrocellulose (data not shown).

Inclusion of glutathione (1 mM) in the incubations led to a complete inhibition of reactive metabolite binding when HOCl was used to generate reactive metabolite (Fig. 5). In contrast, when  $\text{H}_2\text{O}_2$  was used to generate reactive metabolite, covalent binding of clozapine to neutrophils was not

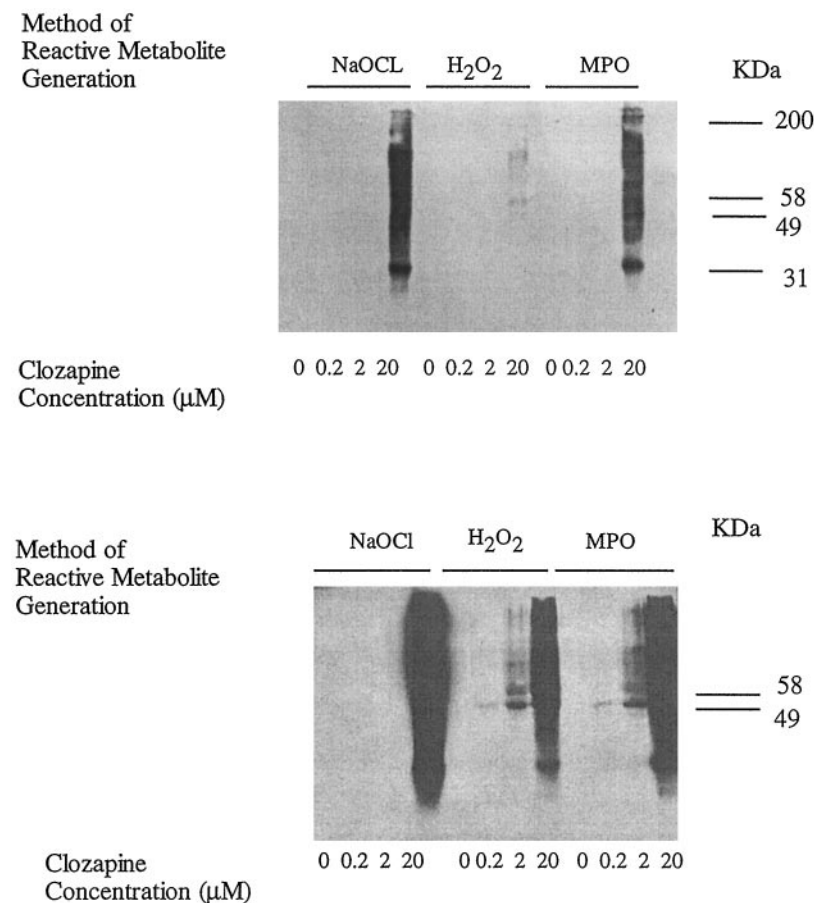


**Fig. 3.** Effect of preincubation of anti-clozapine-NAC-KLH antiserum with various chemical inhibitors on the binding of the antiserum to proteins from human neutrophils exposed to clozapine ( $20 \mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  (10 mM, T) or  $\text{H}_2\text{O}_2$  (10 mM) alone (C). The inhibitors used were none (CONT), clozapine ( $100 \mu\text{M}$ , CLO), olanzapine ( $100 \mu\text{M}$ , OLA), clozapine-NAC ( $100 \mu\text{M}$ , CLN), or vesnarinone ( $100 \mu\text{M}$ , VES). Protein loading was  $6 \mu\text{g}/\text{lane}$ , primary antiserum was used at a final dilution of 1:3000, and the nitrocellulose was exposed to film for 30 sec.

affected by the presence of glutathione. When a combination of MPO and  $H_2O_2$  was used to generate reactive metabolite, glutathione partially inhibited covalent binding of clozapine to the neutrophils, resulting in a pattern of binding that resembled that seen when  $H_2O_2$  alone was used to generate clozapine reactive metabolite. Similar results were obtained using NAC (1 mM) instead of glutathione (data not shown).

**Covalent binding of clozapine to human PBMCs *in vitro*.** Clozapine reactive metabolite (generated by addition of HOCl) covalently bound to human PBMCs as well as to neutrophils (data not shown). In fact, when a mixture of neutrophils and PBMCs was exposed to clozapine reactive metabolite, generated extracellularly by HOCl and then separated by centrifugation through Ficoll-Paque, the PBMC proteins were shown to have more covalently bound clozapine than the neutrophil proteins (data not shown).

**Immunoblotting of neutrophils exposed to reactive metabolites of olanzapine *in vitro*.** Using the anti-clozapine-NAC-KLH antiserum, it also was possible to detect the covalent binding of olanzapine to human neutrophils. The pattern of covalent binding of olanzapine to human neutrophils *in vitro* was different than that seen with clozapine (Fig. 6). Compared with clozapine, olanzapine bound to polypeptides with a higher molecular mass, and there were relatively small amounts of the 49- and 58-kDa adducts. As with clozapine, the intensity of olanzapine covalent binding was greatest when HOCl was used to generate reactive metabolite and least when  $H_2O_2$  was used to generate reactive metabolite. With MPO and  $H_2O_2$ , the binding of reactive metabolite was intermediate between these two extremes.



**Fig. 4.** Immunoblotting experiment showing covalent binding of clozapine reactive metabolite to human neutrophil proteins *in vitro*. Reactive metabolites were generated by directly reacting clozapine (0–20  $\mu M$ ) with NaOCl or incubating neutrophils with clozapine (0–20  $\mu M$ ) and  $H_2O_2$  (10 mM) in the absence ( $H_2O_2$ ) or presence of 1 unit MPO. Protein loading was 8  $\mu g$ /lane, the primary antiserum was used at a final dilution of 1:3000, and the film was exposed to the nitrocellulose sheet for either 30 sec (A) or 5 min (B). This result is representative of results obtained with neutrophils from five different individuals.

When aliquots of neutrophils from the same subject were exposed to either clozapine or olanzapine reactive metabolites and analyzed on the same immunoblot, the intensity of signal was much lower in the olanzapine-treated neutrophils (Fig. 7). The amount of covalent binding in neutrophils exposed to HOCl-generated olanzapine reactive metabolite was similar to that seen when  $H_2O_2$  was used to generate clozapine reactive metabolite (Fig. 7). Due to the large difference in signal intensity seen between the binding of the HOCl-generated clozapine reactive metabolite and the  $H_2O_2$ -generated olanzapine reactive metabolite, it was not possible to observe both signals on the same immunoblot. However, neutrophils do generate the reactive metabolite of olanzapine in the presence of  $H_2O_2$ , but the amount of binding of the olanzapine metabolite is less than that of clozapine, and the proteins to which it binds are different (Fig. 8).

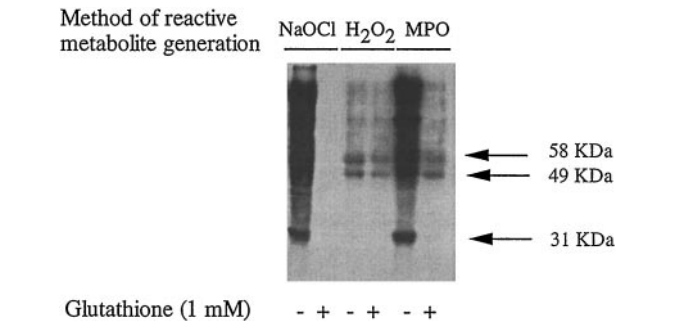
**Immunoblotting of neutrophils and PBMCs isolated from the peripheral blood of patients taking clozapine or olanzapine.** In neutrophils isolated from patients taking clozapine, it was possible to detect clozapine-modified polypeptides (Fig. 9A). A major 58-kDa clozapine-modified polypeptide was detected in each patient tested. In addition, neutrophils from some of the patients had other clozapine-modified polypeptides of molecular mass 63, 96, or 106 kDa. In contrast, no drug-modified polypeptides were observed in neutrophils isolated from patients taking olanzapine. In one patient (olanzapine #3) who had been switched from clozapine to olanzapine 3 months before withdrawal of the blood sample, no clozapine-modified polypeptides were detected. It was possible to inhibit the binding of the antiserum to pro-

teins from neutrophils isolated from patients treated with clozapine by preincubating the antiserum with 10  $\mu\text{M}$  clozapine before adding the antiserum to the nitrocellulose (Fig. 9B). In some experiments, clozapine (2  $\mu\text{M}$ ) was added to blood from control individuals before isolation of neutrophils; in this case, no covalent binding of drug to neutrophils was observed (data not shown), indicating that these results are not an artifact from activation of neutrophils, in the presence of therapeutic levels of clozapine, during the isolation procedure. In two patients taking clozapine, both neutrophils and PBMCs were isolated. Although clozapine was found to covalently bind to neutrophils, no such binding was observed in PBMCs from these patients (Fig. 10).

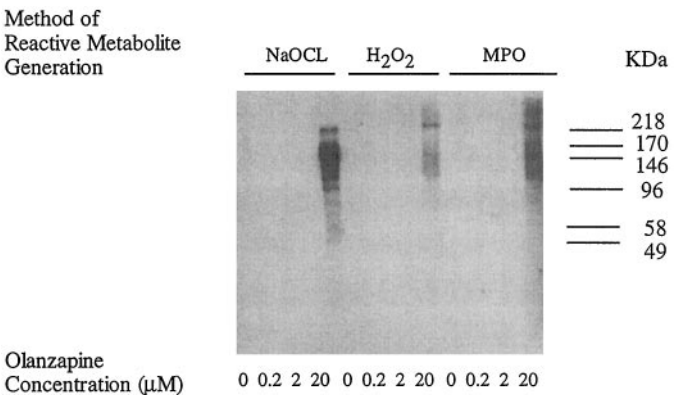
**Covalent binding of clozapine and olanzapine reactive metabolites to human MPO *in vitro*.** When human MPO (1 or 5 units) was incubated with clozapine or olanzapine in the presence of  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ), the drugs became covalently bound to the protein and were detected as a 58-kDa adduct by immunoblotting (data not shown). With clozapine, a concentration-dependent increase in covalent binding was observed at 2–200  $\mu\text{M}$ . In contrast, with olanzapine,

maximal detection of covalent binding was seen at 20  $\mu\text{M}$ , whereas at the higher concentration, a decrease in covalent binding was observed. No covalent binding was observed in the absence of drug or  $\text{H}_2\text{O}_2$ . When the clozapine-MPO adduct and neutrophils from patients taking clozapine were run on the same gel, the 58-kDa adducts comigrated (data not shown).

**Covalent binding of clozapine to rat bone marrow *in vivo*.** Using the anti-clozapine-NAC-KLH antiserum, it was possible to show that clozapine becomes covalently bound to the bone marrow of rats dosed with the drug (40 mg/kg/day) *in vivo*. The major clozapine polypeptide adduct had a molecular mass of 49 kDa (Fig. 11A). It was possible to inhibit binding of the antiserum to this band by preincubating it with clozapine (10  $\mu\text{M}$ ) before adding the antiserum to the nitrocellulose (Fig. 11B). In the rats treated with clozapine for 6 weeks, the plasma concentrations at the time of death were 0.23 and 0.29  $\mu\text{M}$ .



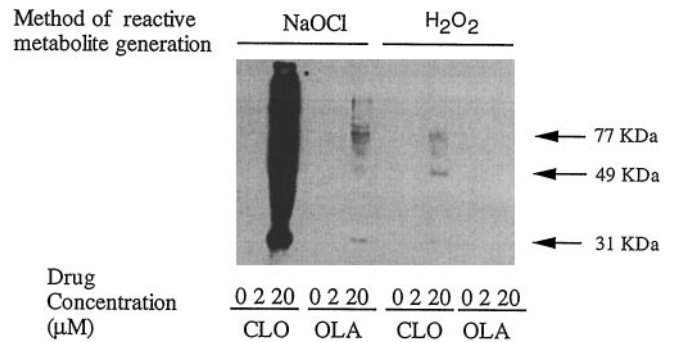
**Fig. 5.** Immunoblotting experiment showing the effect of glutathione (1 mM) on the covalent binding of clozapine reactive metabolite to human neutrophil proteins *in vitro*. Reactive metabolites were generated by directly reacting clozapine (20  $\mu\text{M}$ ) with NaOCl or by incubating neutrophils with clozapine and  $\text{H}_2\text{O}_2$  (10 mM) in the absence ( $\text{H}_2\text{O}_2$ ) or presence of 1 unit MPO. Experiments were performed in the presence (+) and absence (-) of glutathione (1 mM). Protein loading was 15  $\mu\text{g}/\text{lane}$ , the primary antiserum was used at a final dilution of 1:3000, and the film was exposed to the nitrocellulose sheet for 30 sec.



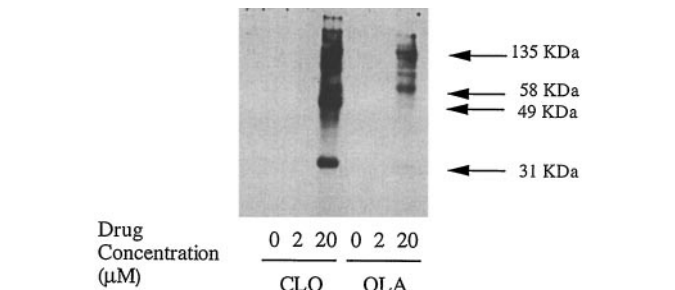
**Fig. 6.** Immunoblotting experiment showing covalent binding of olanzapine reactive metabolite to human neutrophil proteins *in vitro*. Reactive metabolites were generated by directly reacting olanzapine (0–20  $\mu\text{M}$ ) with NaOCl or by incubating neutrophils with olanzapine (0–20  $\mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  (10 mM) in the absence ( $\text{H}_2\text{O}_2$ ) or presence of 1 unit MPO. Protein loading was 8  $\mu\text{g}/\text{lane}$ , the primary antiserum was used at a final dilution of 1:3000, and the film was exposed to the nitrocellulose sheet for 5 min. This result is representative of results obtained with neutrophils from four different individuals.

Discussion

Bioactivation of clozapine to a reactive metabolite and the subsequent covalent binding of this reactive intermediate to



**Fig. 7.** Immunoblot experiment to compare directly the intensity of ECL signal obtained after covalent binding of clozapine and olanzapine to human neutrophil proteins *in vitro*. Human neutrophils were exposed to reactive metabolites of clozapine (CLO) or olanzapine (OLA) (0–20  $\mu\text{M}$ ) generated by direct reaction with NaOCl or by incubation of the drug with neutrophils in the presence of  $\text{H}_2\text{O}_2$  (10 mM). Protein loading was 3.5  $\mu\text{g}/\text{lane}$ , the primary antiserum was used at a final dilution of 1:3000, and the film was exposed to the nitrocellulose sheet for 5 min. This result is representative of results obtained with neutrophils from four different individuals.



**Fig. 8.** Immunoblot experiment to directly compare the intensity of ECL signal obtained after covalent binding of clozapine and olanzapine to human neutrophil proteins *in vitro* and exposed for a longer period of time to better visualize the olanzapine binding. Human neutrophils were incubated with clozapine (CLO) or olanzapine (OLA) (0–20  $\mu\text{M}$ ) in the presence of  $\text{H}_2\text{O}_2$  (10 mM). Protein loading was 5  $\mu\text{g}/\text{lane}$ , the primary antiserum was used at a final dilution of 1:3000, and the film was exposed to the nitrocellulose sheet for 5 min. This result is representative of results obtained with neutrophils from four different individuals.



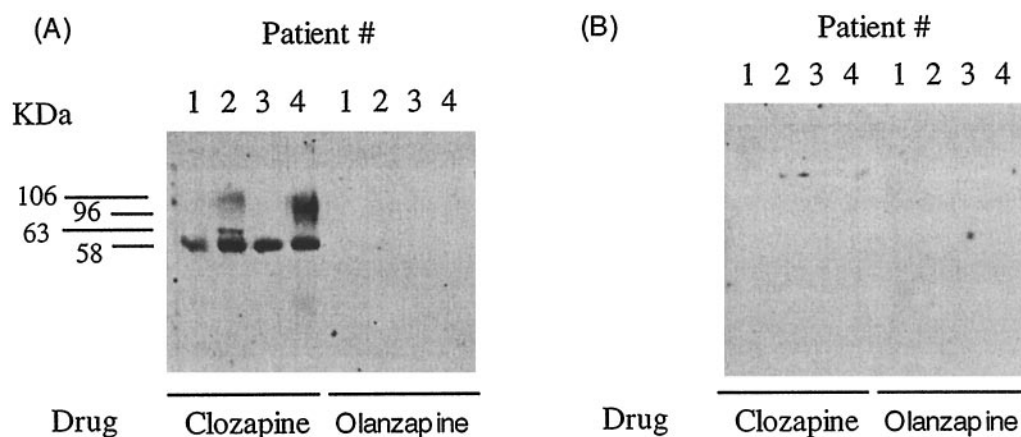
human neutrophils, or their precursors in the bone marrow, have been suggested to play a role in clozapine-induced agranulocytosis. In contrast, although olanzapine is structurally related to clozapine and can be bioactivated to an analogous reactive intermediate (Gardner *et al.*, 1998), it has not been associated with agranulocytosis (Fulton and Goa, 1997). In this study, we used immunochemical techniques to compare the covalent binding of clozapine and olanzapine to human neutrophils both *in vitro* and *in vivo*.

By trapping the reactive metabolite of clozapine with NAC and then coupling this conjugate to KLH, it was possible to produce an immunogen that subsequently was used to raise a polyclonal antiserum. This antiserum was shown to recognize clozapine and the structurally related compound olanzapine with high affinity but did not recognize vesnarinone, a structurally unrelated drug that also causes agranulocytosis (Furusawa *et al.*, 1996) (Figs. 1–3). In view of the similar orientation of the binding of both clozapine and olanzapine to sulfhydryl groups and the positive charge on the piperazine ring, which could offer a site of high affinity binding, the piperazine ring may be a major portion of the epitope for both haptens.

We demonstrated that exposure of neutrophils to clozapine (2 or 20  $\mu\text{M}$ ), but not olanzapine, in the presence of  $\text{H}_2\text{O}_2$  results in toxicity to the neutrophils (Gardner *et al.*, 1998). However, when HOCl was used to generate reactive metabolite, neither clozapine nor olanzapine reactive metabolites were toxic to neutrophils (Gardner *et al.*, 1998). In this study, we used the anti-clozapine-NAC-KLH antiserum to explore the relationship between covalent binding of clozapine and olanzapine to human neutrophils *in vitro* and resultant toxicity. As with toxicity, both the extent of covalent binding of clozapine to human neutrophils and the nature of the modified neutrophil proteins were highly dependent on the method of reactive metabolite generation (Fig. 4). At a clozapine concentration of 20  $\mu\text{M}$ , covalent binding was greatest in neutrophils exposed to HOCl-generated reactive metabolite and least in neutrophils incubated with clozapine and  $\text{H}_2\text{O}_2$  (Fig. 4), whereas at lower clozapine concentrations (0.2 and 2  $\mu\text{M}$ ), which span the range of clozapine concentrations seen clinically (Weigmann and Hiemke, 1992), covalent binding of clozapine to human neutrophils was observed only using the  $\text{H}_2\text{O}_2$ -based methods of reactive metabolite generation. At these lower clozapine concentrations, clozapine-polypeptide adducts of 49 and 58 kDa were particularly prominent (Fig. 4B).

Thus, there does not seem to be a simple relationship between the total covalent binding of clozapine to neutrophils *in vitro*, as estimated by immunoblotting, and subsequent toxicity. However, there are differences between the HOCl and  $\text{H}_2\text{O}_2$  methods, which could explain the apparent lack of relationship between total covalent binding and toxicity *in vitro*. Mixing HOCl and clozapine produces clozapine reactive metabolite extracellularly, whereas the  $\text{H}_2\text{O}_2$  presumably is used as a cofactor by intracellular enzymes to generate the clozapine reactive metabolite within the cell. This idea is supported by the observation that the addition of extracellular glutathione to incubations was able to inhibit covalent binding when HOCl was used to generate reactive metabolite, whereas neither covalent binding of the  $\text{H}_2\text{O}_2$ -generated metabolite to neutrophil proteins (Fig. 5) nor toxicity (Gardner I and Uetrecht JP, unpublished observations) were inhibited by addition of extracellular glutathione. In experiments with a combination of MPO and  $\text{H}_2\text{O}_2$ , glutathione was able to partly inhibit covalent binding (Fig. 5); under these conditions, clozapine presumably is bioactivated both intracellularly (by MPO in the neutrophils) and extracellularly (by the exogenously added MPO). Thus, when  $\text{H}_2\text{O}_2$  is used to catalyze the formation of clozapine reactive metabolite, in either the presence or absence of MPO, at least a portion of the reactive metabolite is formed in an intracellular compartment to which glutathione does not have access. When neutrophils ingest bacteria, the external cell membrane forms a phagosome around the ingested microorganisms that seems to show remarkable integrity in the presence of the powerful oxidants released into the phagosome to kill the bacteria (Klebanoff, 1990). Perhaps this helps to explain why the neutrophils seem to be relatively resistant to extracellularly produced reactive intermediates of clozapine. An alternative explanation is that the toxicity observed in this simple *in vitro* system is not dependent on the covalent binding of clozapine reactive metabolite to neutrophil polypeptides.

Because both olanzapine and clozapine can be bioactivated to a reactive nitrenium ion by HOCl, the major oxidant of human neutrophils, it is not clear why *in vitro* exposure of human neutrophils to the clozapine reactive metabolite produces toxicity, whereas *in vitro* exposure of human neutrophils to the olanzapine reactive metabolite does not produce toxicity (Gardner *et al.*, 1998). Using the anti-clozapine-NAC-KLH antiserum, it was possible to show that compared with clozapine, olanzapine became covalently bound to a different subset of neutrophil polypeptides (compare Figs. 4 and 6).



**Fig. 9.** Detection of drug-modified polypeptides in neutrophils of patients treated *in vivo* with clozapine but not olanzapine. A, Neutrophils were isolated from four subjects taking clozapine and four subjects taking olanzapine. Protein from  $1 \times 10^6$  cells was loaded onto each lane. Primary antiserum concentration was 1:2000, and nitrocellulose was exposed to film for 5 min. B, Identical experiment to that in A except the primary antiserum was incubated with clozapine (10  $\mu\text{M}$ ) for 30 min before the addition to the nitrocellulose.

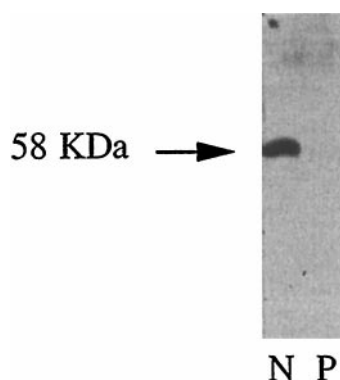
The polypeptides to which olanzapine became bound tended to exhibit a high molecular mass (>96 kDa). In particular, only relatively low levels of 49- and 58-kDa olanzapine-polypeptide adducts were observed, whereas 49- and 58-kDa adducts were major clozapine-polypeptide adducts, particularly at low substrate concentrations. This raises the possibility that binding of clozapine to the 49- or 58-kDa proteins, or both, is responsible for toxicity in the *in vitro* test system. Similar arguments have been used to explain the differences in hepatotoxicity of acetaminophen (which is hepatotoxic) and the acetaminophen regioisomer 3'-hydroxyacetanilide (which is not hepatotoxic) even though both compounds undergo extensive covalent binding to liver proteins (Myers *et al.*, 1995; Pumford and Halmes, 1997). It will be important in future studies to purify the clozapine-modified polypeptides and address these issues. Another factor that may explain the difference between the toxicity of clozapine and olanzapine is the difference in the total amount of reactive metabolite that becomes covalently bound to intracellular polypep-

tides of neutrophils. When the same method is used to generate reactive metabolite, olanzapine seems to covalently bind to human neutrophils to a markedly lower extent than clozapine (Figs. 6 and 7). Because the antiserum was raised against clozapine and has differing affinities for clozapine and olanzapine, it is not possible to quantify accurately the difference in covalent binding of the two drugs.

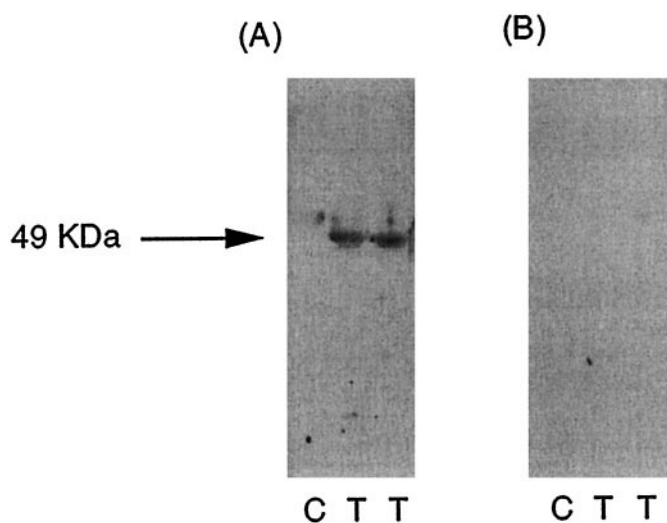
Previous studies with radiolabeled drug have demonstrated that clozapine can be metabolized to a reactive intermediate by MPO and by PMA-activated human neutrophils (Liu and Uetrecht, 1995) or bone marrow cells (Maggs *et al.*, 1995) *in vitro*. However, to date, metabolism of clozapine by this pathway has not been documented in humans *in vivo* under normal clinical conditions. Using the immunochemical techniques that we developed, it was possible to show that clozapine became covalently bound to the neutrophils of patients clinically treated with the drug (Fig. 9). It is not clear whether the clozapine becomes covalently bound to human neutrophils during their maturation in the bone marrow or after release of the neutrophils into the peripheral circulation. However, because clozapine becomes covalently bound to the bone marrow of rats treated with the drug *in vivo* (Fig. 11) and the plasma clozapine concentrations seen in these rats were similar to the plasma concentrations observed clinically (Weigmann and Hiemke, 1992), it seems likely that clozapine also becomes covalently bound to the bone marrow in humans during normal clinical use.

Although demonstration of clozapine-modified neutrophils in patients treated with the drug does not prove that MPO is catalyzing the *in vivo* bioactivation of clozapine, there is evidence to support this contention; for example, it is possible that the covalent binding of clozapine to neutrophils in patients treated with clozapine is not due to reactive metabolite generated *in situ* by the neutrophils but rather to clozapine reactive metabolite produced in the liver (or other metabolically active tissues) and then released into the blood. As an example, there is evidence that dapsone-induced methemoglobinemia is due to a reactive dapsone intermediate produced in the liver and then released into the bloodstream (Park *et al.*, 1995). Studies in rodents demonstrate that *in vivo*, clozapine undergoes hepatic bioactivation to a reactive intermediate that is excreted in the bile as glutathione conjugates (Maggs *et al.*, 1995). Thus, hepatic release of clozapine reactive metabolite is a possible mechanism by which neutrophils become covalently modified by the reactive metabolite. However, *in vitro* studies clearly demonstrate that human PBMCs also are covalently modified by extracellularly generated clozapine reactive metabolite (data not shown). Indeed, when a mixture of neutrophils and PBMCs, as would occur in the blood, are exposed to extracellular clozapine reactive metabolite and then separated by centrifugation over Ficoll-Paque, both types of cells are modified by the reactive metabolite to a similar extent (data not shown). Thus, it seems unlikely that only neutrophils, and not PBMCs, would be covalently modified if clozapine was bioactivated in the liver and released into the bloodstream. If the clozapine reactive metabolite is generated *in situ* in neutrophils, the MPO/H<sub>2</sub>O<sub>2</sub>/Cl<sup>-</sup> system seems the most likely to be responsible (Uetrecht, 1992).

*In vitro* studies have shown that clozapine is not bioactivated by human neutrophils unless the neutrophils are activated with agents such as PMA (Liu and Uetrecht, 1995;



**Fig. 10.** Immunoblot of neutrophils (N) and PBMCs (P) isolated from the peripheral blood of a patient (#3) taking clozapine *in vivo*. Protein from  $1 \times 10^6$  cells was loaded onto each lane. A primary antiserum dilution of 1:2000 was used. Nitrocellulose was exposed to film for 3 min.



**Fig. 11.** Immunochemical detection of covalent binding of clozapine to rat bone marrow *in vivo*. A, Bone marrow was isolated from rats fed a diet of powdered lab chow (C) or powdered lab chow containing clozapine (T) such that the intake of clozapine was 40 mg/kg/day. Protein loading was 30  $\mu$ g/lane, and the primary antiserum was used at a dilution of 1:2000. Nitrocellulose was exposed to the ECL film for 2 min. B, Samples were the same as used in A except that the primary antiserum was incubated with clozapine (10  $\mu$ M) for 30 min before the addition to the nitrocellulose.



Maggs *et al.*, 1995) or incubated with high concentrations of  $H_2O_2$  (Gardner *et al.*, 1998). It is not clear whether the covalent binding of clozapine observed *in vivo* represents a low level of neutrophil activation or whether neutrophils are able to catalyze the bioactivation of clozapine without activation *in vivo*. Recent work by Pollmacher *et al.* (1996) demonstrates that clozapine has immunomodulatory effects that result in changes in plasma cytokine levels and cytokine-induced fever in patients taking clozapine. As discussed by Pollmacher *et al.* (1996), some of the cytokines increased by clozapine (e.g., tumor necrosis factor- $\alpha$ ) have the potential to activate neutrophils (van der Poll *et al.*, 1992), and this may provide a mechanism by which clozapine bioactivation and subsequent covalent binding occur *in vivo*. Interestingly, fever was not reported as a side effect of olanzapine treatment (Fulton and Goa, 1997), and this may be one factor explaining why olanzapine was not detected covalently bound to neutrophils *in vivo*.

Although these studies show that clozapine becomes covalently bound to human neutrophils *in vivo*, the relationship between this binding and induction of agranulocytosis is less clear. The patients taking the clozapine in this study were not experiencing any adverse reactions to the medication. They had been taking clozapine for 12–66 months and so were past the stage at which clozapine-induced agranulocytosis usually develops (within 6 months of onset of treatment) (Alvir *et al.*, 1993). However, it is not difficult to imagine that there are additional risk factors in the development of clozapine agranulocytosis and that clozapine-modified polypeptides could trigger an immune reaction in certain susceptible individuals. There are certain parallels between the findings in this study and previous observations regarding halothane hepatitis, a probable immune-mediated idiosyncratic adverse drug reaction (Kenna and Neuberger, 1995). In all experimental animals and patients exposed to halothane, the drug can become covalently bound to the liver (Kenna and Neuberger, 1995; Kenna *et al.*, 1988), but only between 1:35,000 (on primary exposure) and 1:3,500 of these patients (on secondary exposure) go on to develop halothane hepatitis (National Halothane Study, 1966). This has led to the suggestion that differences in immune system response are a major susceptibility factor for halothane hepatitis (Gut *et al.*, 1993).

Although clozapine was detected bound to the neutrophils in all patients, none of the olanzapine patients exhibited covalent binding of olanzapine to the neutrophils. Although this may reflect an inability of the antiserum to detect low levels of olanzapine bound to the cells, it is surprising that use of low antiserum dilutions (1:1000) and prolonged exposure of the ECL-treated nitrocellulose to film (>60 min) failed to detect olanzapine covalently modified proteins in neutrophils of patients dosed with olanzapine *in vivo* (data not shown). Particularly, as in all the *in vitro* experiments, it was possible to detect covalent binding of both clozapine and olanzapine. Furthermore, in experiments with human MPO, equivalent amounts of binding of clozapine and olanzapine were observed (data not shown). One factor to bear in mind when comparing the binding of clozapine and olanzapine *in vivo* is the differing doses and resultant plasma concentrations of the two drugs. In this study, the mean dose of clozapine was 331 mg/day, whereas the mean dose of olanzapine was 7.5 mg/day. In a study of 16 psychiatric patients treated

with 75–400 mg of clozapine/day, the plasma concentrations ranged from 0.2 to 1.6  $\mu M$  (Weigmann and Hiemke, 1992). In contrast, olanzapine concentrations ranged from 0.03 to 0.1  $\mu M$  (Aravagiri *et al.*, 1997; Fulton and Goa, 1997). The lower plasma concentrations of olanzapine may, in part, explain why covalent binding of olanzapine to human neutrophils was not observed *in vivo*.

The clozapine-treated patients used in this study had been taking the drug for longer periods of time than the olanzapine-treated patients (43.5 versus 4.5 months); however, because it only takes ~12–14 days for mature neutrophils to develop from stem cells in the bone marrow (Hellewell and Williams, 1994), and given that once released into the circulation, human neutrophils have a relatively short half-life (8–20 hr; Edwards, 1994), the neutrophils in both sets of patients would have been exposed to the relevant drug for their full lifespan. Thus, differences in the duration of drug treatment in the two groups is unlikely to explain the lack of covalent binding of olanzapine to human neutrophils *in vivo*.

If recognition of drug-modified proteins by the immune system is important in clozapine-induced agranulocytosis, the observed difference in covalent binding of clozapine and olanzapine to neutrophils *in vivo* may help to explain the difference in toxicity of the two drugs when they are used clinically.

MPO exists as a covalently linked homodimer with a molecular weight of 140,000 (Andrews and Krinski, 1981). Each half of the dimer consists of a large (58-kDa) and a small (13-kDa) subunit. In human MPO, there are 14 cysteine residues. Twelve are involved in intrasubunit disulfide bonds (five disulfide bonds in the heavy chain and one in the light chain), and one is involved in an intermolecular disulfide bond between the two heavy chains of MPO; thus, there is one free cysteine residue on the heavy subunit of MPO (Zeng and Fenna, 1992). Previously, the reactive metabolites of clozapine and olanzapine have been shown to bind selectively to sulfur rather than nitrogen-containing nucleophiles. This specificity is supported by the finding that *in vitro*, human MPO oxidizes clozapine and olanzapine to reactive intermediates that were detected covalently bound to the heavy (58-kDa), but not the light (13-kDa), subunit of MPO (data not shown).

Although in these experiments we did not attempt to identify directly the enzymes that catalyze clozapine bioactivation, when human neutrophils are incubated with drug and  $H_2O_2$  *in vitro*, it is of interest that a 58-kDa clozapine-polypeptide adduct also is formed (Fig. 4). In addition, in each clozapine patient studied, the major covalently modified polypeptide had a molecular mass of 58 kDa (Fig. 9), and this polypeptide comigrated with clozapine-modified human MPO heavy subunit (58 kDa) when the two samples were run on the same gel (data not shown). Interestingly, anti-MPO antibodies have been demonstrated in the sera of some patients who developed clozapine-induced agranulocytosis (Jaunkalns *et al.*, 1992). Studies are under way to purify and characterize the 58-kDa protein.

In summary, we developed an anti-clozapine-NAC-KLH antiserum and used the antiserum to investigate the binding of clozapine and olanzapine to human neutrophils *in vitro*. Furthermore, we demonstrated for the first time that clozapine becomes covalently bound to human neutrophils *in vivo*.

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