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Chemical Research in Toxicology

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Effect of Clozapine and Olanzapine on Neutrophil Kinetics: Implications for Drug-Induced Agranulocytosis

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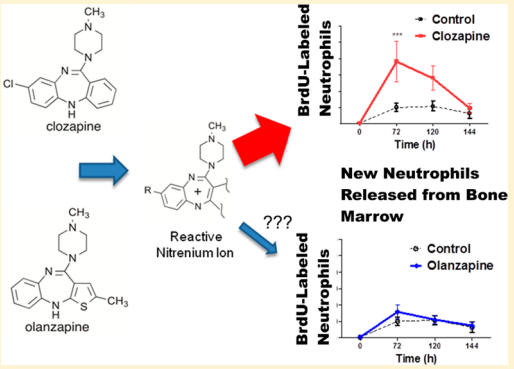
ABSTRACT: Clozapine is effective in the treatment of schizophrenia; however, its use is limited by a relatively high incidence of idiosyncratic agranulocytosis. The mechanism of clozapine-induced idiosyncratic agranulocytosis is unknown. Although most patients treated with clozapine do not develop agranulocytosis, most do have an immune response with an increase in inflammatory cytokines such as IL-6 and a release of immature neutrophils with neutrophilia rather than agranulocytosis. We have previously shown that treatment of rabbits with clozapine also causes an early release of neutrophils. Clozapine is oxidized to a reactive nitrenium ion that covalently binds to neutrophils, and this reactive metabolite may be responsible for the observed effects. Olanzapine and clozapine have very similar structures, and olanzapine is also oxidized to a reactive nitrenium ion; however, if it ever causes agranulocytosis, the incidence is much lower than that of clozapine. One possible basis for the difference in incidence of agranulocytosis between clozapine and olanzapine is that the therapeutic dose of olanzapine is much lower than that of clozapine. In this study, we compared the effects of clozapine and olanzapine in Sprague–Dawley rats at an equimolar dose and found that only clozapine had a significant effect on neutrophil kinetics. This suggests that the immune response and effects on neutrophil kinetics induced by clozapine are related to its ability to cause agranulocytosis.



INTRODUCTION

Clozapine is an atypical antipsychotic with unique efficacy for the treatment of refractory schizophrenia.[1] The use of clozapine has been limited because of its propensity to induce idiosyncratic drug reactions (IDRs) including agranulocytosis,[2] liver injury,[3] and myocarditis.[4] Approximately 0.8% of patients treated with clozapine develop agranulocytosis,[2] which is characterized by a neutrophil count of $<0.5 \times 10^9$/L and puts individuals at risk of severe infections, which are often fatal. However, mandatory monitoring of hematological parameters has decreased the incidence of agranulocytosis and increased patient safety.[5] Instead of agranulocytosis, most patients treated with clozapine have an increase in the number of immature neutrophils in the circulation[6] and an elevated total neutrophil count.[7] This neutrophilia is associated with an immune response and an increase in inflammatory cytokines such as IL-6.[8] We found that clozapine also causes an increase in the release of neutrophils from the bone marrow and total neutrophil count in rabbits as well as a shortened neutrophil half-life.[9] An increase in neutrophils has also been observed in rats treated with aminoglutethimide,[10] which is also associated with a relatively high risk of drug-induced agranulocytosis. This suggests that these early changes in neutrophil kinetics could be a biomarker to predict the risk that a drug would cause agranulocytosis.

Clozapine is oxidized to a reactive nitrenium ion,[11] which covalently binds to neutrophils,[12] and it has been proposed that this reactive metabolite is responsible for the effects of clozapine on neutrophils. Olanzapine and clozapine have very similar structures (Figure 1); however, olanzapine does not have the same unique efficacy as clozapine. Even though olanzapine also forms a reactive nitrenium ion metabolite,[13] it is

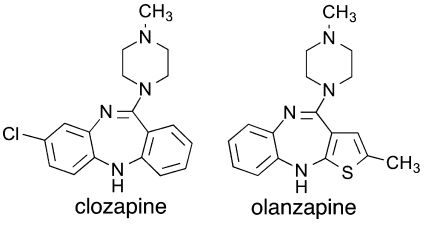


Figure 1. Structures of clozapine and olanzapine.

Received: May 11, 2014
Published: June 26, 2014

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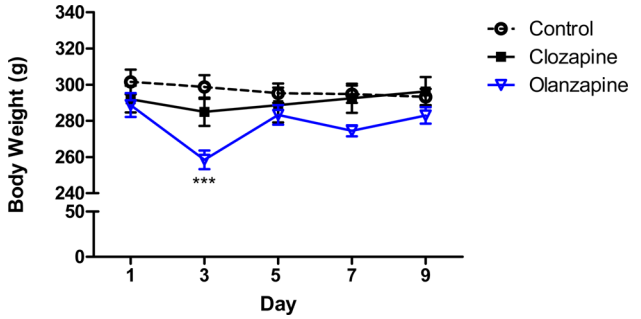

Figure 2. Body weight of rats during experimental treatment. Rats were administered clozapine (30 mg/kg/day by i.p. injection) or the molar equivalent of olanzapine (28.7 mg/kg/day), and body weights were monitored. No significant body weight changes were observed for clozapine during the 10 day treatment period as compared to that of control rats; however, a transient decrease in body weight was observed at day 3 in olanzapine-treated rats. Values expressed are the mean ± SEM for each group (n = 4). ***p < 0.001 compared to the control at the specific time point.

not associated with a significant incidence of agranulocytosis or other idiosyncratic reactions. One possible reason for the difference in the risk of agranulocytosis between clozapine and olanzapine is that the therapeutic dose of olanzapine is much lower than that of clozapine. In this study, we compared the effect of clozapine and olanzapine on neutrophil kinetics in Sprague–Dawley rats using the same dose of each.

MATERIALS AND METHODS

Chemicals and Reagents. Clozapine was provided by Novartis (Dorval, QC). Olanzapine was purchased from Toronto Research Chemicals (North York, ON). 5-Bromo-2′-deoxyuridine (BrdU), 10% neutral buffered formalin, and dextran-500 were purchased from Sigma (Oakville, ON). HCl and NaOH were acquired from Caledon Laboratory Chemicals (Georgetown, ON). Phosphate buffered saline (PBS), heat-inactivated fetal bovine serum (FBS), and trypan blue were obtained from Life Technologies (Burlington, ON, Canada).

Animals. Female Sprague–Dawley rats (250–300 g) were purchased from Charles River (Montreal QC) and housed under standard conditions (12:12 h light/dark cycle at 22 °C) at the Department of Comparative Medicine (Medical Sciences Building, University of Toronto). Rats were given standard rodent chow. Food and water were provided ad libitum. Experiments were started after 1 week of acclimatization. This animal protocol was approved and performed in concordance with the Faculties of Medicine and Pharmacy Animal Care Committee.

Treatments. Rats were treated by intraperitoneal injection with clozapine (30 mg/kg/day) or olanzapine (28.7 mg/kg/day); these are equimolar doses. Drugs were added to saline, and a small volume of HCl was added to facilitate dissolution. The pH of the drug solution was adjusted to pH 5.5 with NaOH prior to injection. The 30 mg/kg/day dosage of clozapine used in this experiment has been used in previous studies of neutrophil kinetics and was found to produce clozapine blood levels of 0.41–1.24 μg/mL in female Sprague–Dawley rats (unpublished observations) which is within the range of clozapine blood levels observed in patients treated with clozapine (0.35–0.60 μg/mL). Controls rats were given saline as a vehicle control. Rats were treated for a total of 10 days.

Leukocyte Counts. Whole blood from the tail vein of rats was diluted 40-fold in Turks solution (Ricca Chemical Company, Arlington, TX), and the total white blood cell count (WBC) was determined using a hemocytometer. The differential WBC count was obtained manually under a light microscope on blood smeared slides (from 5 μL whole blood) stained using CAMCO Stain Pak (Cambridge Diagnostic Products Inc., Fort Lauderdale, FL), as per the manufacturer's instructions. Differential counts were determined by characterizing 100 leukocytes per slide.

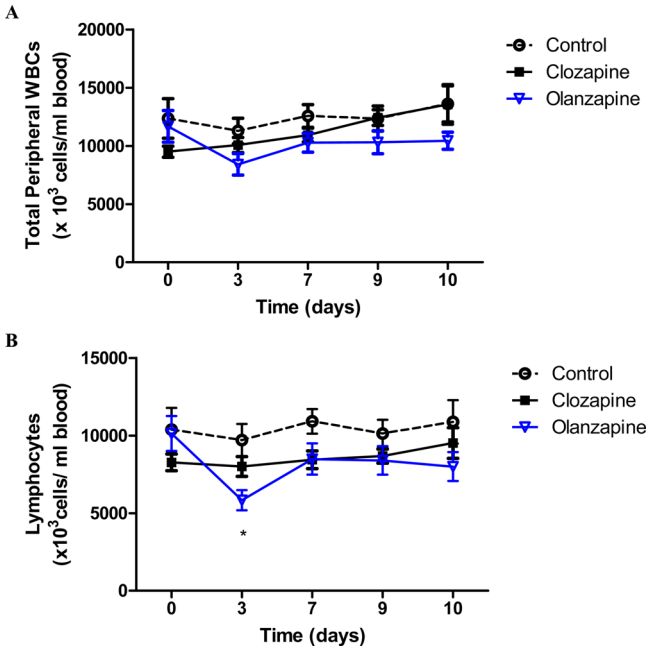

Figure 3. Effect of clozapine and olanzapine on white blood cells. (A) Total peripheral WBCs and (B) peripheral lymphocyte count. Values are expressed as the mean ± SEM (n = 7). *p < 0.05 compared to the control at the specific time point.

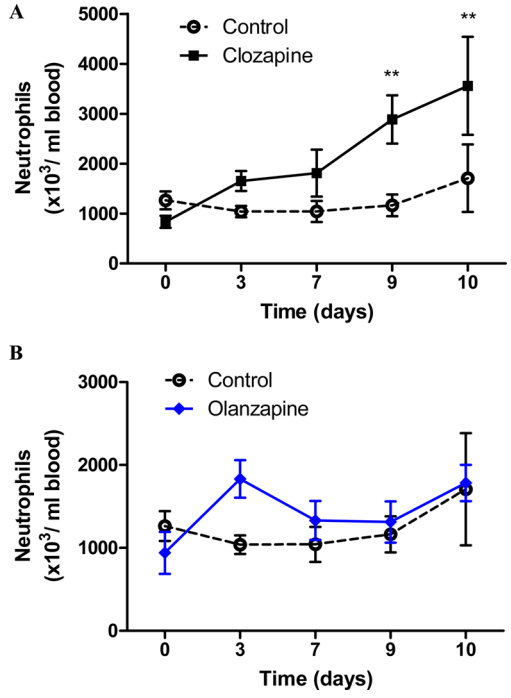

Figure 4. Changes in peripheral blood neutrophils induced by clozapine and olanzapine in rats treated for 10 days. (A) Clozapine-treated animals and (B) olanzapine-treated animals. Values are expressed as the mean ± SEM (n = 7). **p < 0.01 compared to the control at the specific time point.

Measurement of Neutrophil Release from the Bone Marrow. On the fourth day of the 10 day treatment, BrdU (100 mg/kg) was administered by a single intraperitoneal injection to label new neutrophils released from the bone marrow. Serial samples of blood

dx.doi.org/10.1021/tx500183x | Chem. Res. Toxicol. 2014, 27, 1104–1108

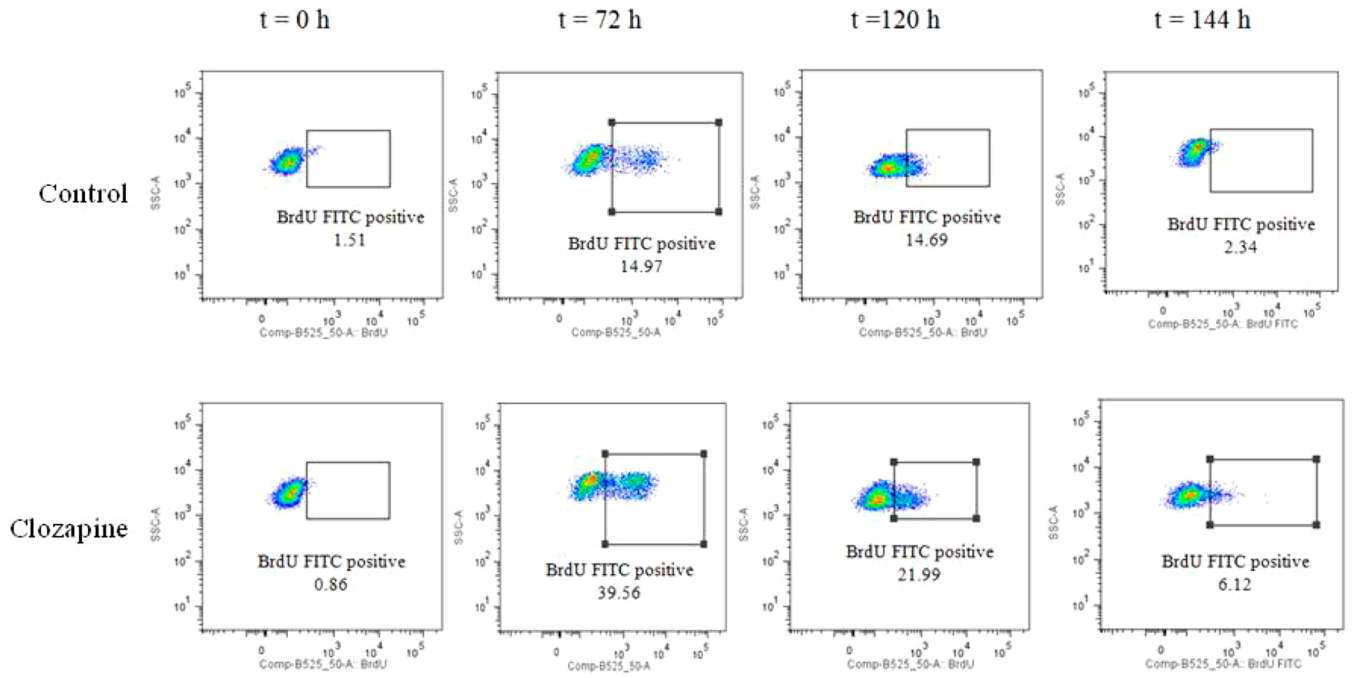

Figure 5. Representative flow plots for the measurement of BrdU-labeled neutrophils released from the bone marrow. Rats were treated with clozapine or olanzapine for 10 days. On the fourth day of treatment, 100 mg/kg BrdU was injected i.p., and blood was taken from the tail vein of rats at 0, 72, 120, and 144 h post-BrdU injection and analyzed by flow cytometry. Neutrophils were gated based on forward and side scatter characteristics, and newly released bone marrow neutrophils were identified as BrdU positive on the B525/50 (FITC) filter. A representative time course of expression is illustrated with a control and clozapine-treated rat.

were taken at 0, 72, 120, and 144 h post-BrdU injection, and leukocytes were isolated. Briefly, blood collected from the tail vein of rats was incubated with equal volumes of 3% dextran (prepared in saline) for 18 min at room temperature. The leukocyte-rich upper layer was extracted and centrifuged at 350*g* for 5 min. The cell pellet was then incubated with red cell lysis buffer for 10 min to remove the red blood cells. Cells were washed and resuspended in florescence-activated cell-sorting (FACS) buffer (5% FBS in PBS), and a Countess Automated Cell Counter (Invitrogen, Life Technologies) was used for cell counting with trypan blue. BrdU-labeled leukocytes were determined using the FITC BrdU Flow Kit (BD Biosciences) as per the manufacturer's protocol and analyzed by flow cytometry using the BD FACSCalibur (BD Biosciences) instrument and FACSDiva software (BD Biosciences) at a flow rate of no more than 400 events/s. Detailed analyses of the flow cytometry data were performed using FlowJo software (Tree Star, Inc., Ashland, OR). BrdU is carcinogenic, and procedures for carcinogenic substances were followed throughout the study as outlined by the University of Toronto Faculties of Medicine and Pharmacy Animal Care Committee.

Bone Marrow Histology. Rats were sacrificed at day 11. The femur and tibia were removed using standard procedures and immediately immersed in 10% neutral buffered formalin (Sigma) for 2−5 days. Paraffin-embedded hematoxylin and eosin (H&E) stained slides were prepared by the Toronto Centre for Phenogenomics Histology Laboratory (Toronto, ON). Imaging of slides was performed at the Microscopy Imaging Lab (Faculty of Medicine, University of Toronto) using a Zeiss fluorescence microscope with deconvolution.

Data Analysis. GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) was used for statistical analysis. ANOVA with Bonferroni post-tests were performed to determine statistical significance between treated and control groups.

RESULTS

Effect of Clozapine and Olanzapine on Rats. Treatment of rats with clozapine did not lead to overt adverse effects. Clozapine treatment has been previously observed to induce central sedation in Sprague−Dawley rats (unpublished observations); however, this effect was mild and lasted only a few hours. This sedative effect was also observed in rats treated with olanzapine; olanzapine was generally less well-tolerated than clozapine. Olanzapine-treated rats appeared unwell and developed a significant decrease in body weight by day 3; however, upon careful monitoring and administration of mash (a mixture of powdered rodent chow with water) to replace dry pelleted rodent food, the body weight of the rats returned to levels found in the control and clozapine treatment groups (Figure 2).

Effect of Clozapine and Olanzapine on Leukocyte Counts. Treatment of clozapine or olanzapine did not change the total WBC count in rats, although a subtle but non-significant decrease in WBCs was observed for olanzapine (Figure 3A). In addition, no changes were observed in the lymphocyte population with clozapine treatment; however, olanzapine significantly decreased the lymphocyte count at day 3 (Figure 3B), which was associated with the decrease in body weight. As expected, clozapine induced a significant increase in neutrophils compared to that of the control; however, this effect was not observed in olanzapine-treated animals (Figure 4).

Bone Marrow Changes Induced by Clozapine and Olanzapine. To investigate new neutrophil release from the bone marrow, rats were treated with the thymidine analogue BrdU. BrdU is incorporated into the DNA of newly formed cells, and flow cytometry was used to track neutrophils labeled with BrdU as a representation of the new neutrophils released into the blood (Figure 5). As observed previously, clozapine induced an increase in new neutrophil release from the bone marrow; however, no difference in neutrophil release was observed with olanzapine (Figure 6). Upon histological examination, this corresponded to an increase in myeloid cells in the

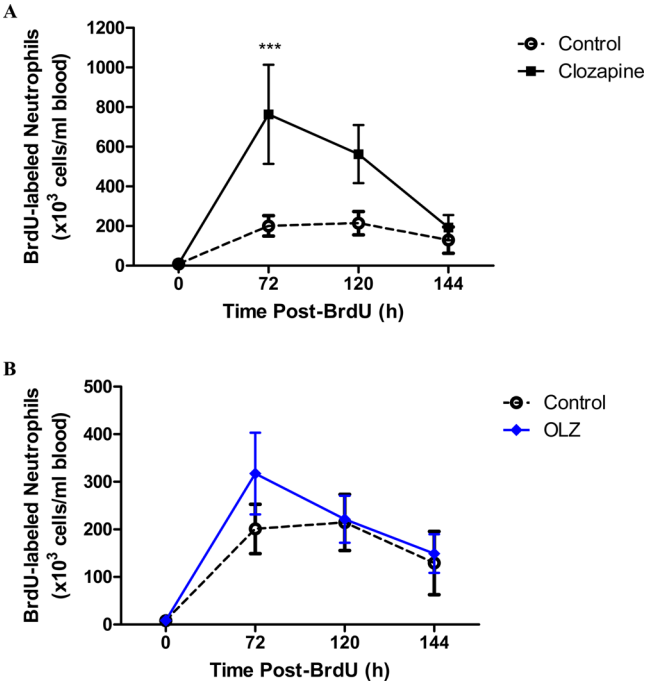

Figure 6. Kinetics of BrdU-labeled neutrophils released after treatment with (A) clozapine or (B) olanzapine (OLZ).

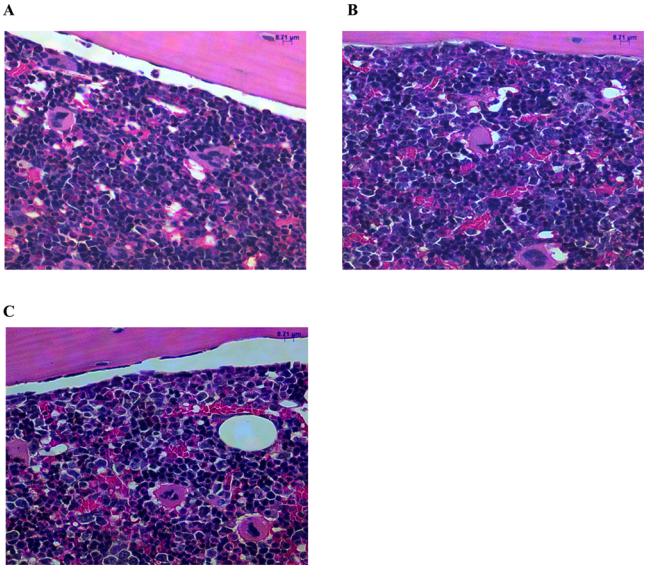

Figure 7. Changes in the bone marrow induced by clozapine or olanzapine after treatment for 10 days. Representative H&E-stained biopsies of femurs from (A) the control, (B) clozapine-, or (C) olanzapine-treated rats.

bone marrow of clozapine-treated but not in the olanzapine-treated animals (Figure 7).

DISCUSSION

As discussed in the Introduction, clozapine causes a variety of IDRs, of which agranulocytosis is the principle concern. The fact that clozapine-induced agranulocytosis is associated with specific HLA genotypes suggests that it is immune-mediated.[14] Although clozapine does not cause a serious IDR in most patients, there is an immune response in most patients that is accompanied by neutrophilia. Olanzapine also forms a reactive nitrenium ion metabolite, but it is not associated with the same type of IDRs, possibly because the therapeutic dose is much lower. However, in this study we found that clozapine also causes neutrophilia in Sprague–Dawley rats, while at the same dose, olanzapine does not, even though olanzapine caused more central nervous system effects than clozapine. This suggests that despite their structural similarity there is something fundamentally different about clozapine and olanzapine. This discrepancy could involve differences in the covalent binding of the reactive metabolite, or it could be totally independent of reactive metabolite formation. Together with the finding that other drugs that cause agranulocytosis also cause neutrophilia in rats, specifically aminoglutethimide[10] and amodiaquine (unpublished observation), this suggests that the ability of a drug to induce neutrophilia may represent a biomarker of agranulocytosis risk. The accompanying article investigates the effects of clozapine on bone marrow and inflammatory markers in more detail.

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Funding

This research was funded by the Canadian Institutes of Health Research and support from National Pharmaceutical Technologies.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We acknowledge Alexandra Lobach for her technical insight and Imir Metushi for assistance during bone marrow extraction. J.U. is the Canada Research Chair in Adverse Drug Reactions.

ABBREVIATIONS

IDRs, idiosyncratic drug reactions

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