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# Clozapine bioactivation induces dose-dependent, drug-specific toxicity of human bone marrow stromal cells: A potential in vitro system for the study of agranulocytosis

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*Abbreviations:*
ANOVA, analysis of variance
FCS, fetal calf serum
GSH, glutathione (reduced)
GSSG, glutathione (disulfide)
HBSS, Hanks balanced salt solution
$H_2O_2$, hydrogen peroxide
HOCl, hypochlorous acid
HRP, horseradish peroxidase
$IC_{50}$, 50% inhibitory concentration
IMDM, Iscove's modified Dulbecco's medium

ABSTRACT

Clozapine, an atypical antipsychotic drug effective in treatment of refractory schizophrenia causes potentially life-threatening agranulocytosis. The drug undergoes bioactivation to a toxic, chemically reactive intermediate with capacity to target stromal cells, central components of the bone marrow microenvironment implicated in neutrophil development. To identify possible mechanisms underpinning disruption of stroma as a site of drug bioactivation, toxicity was induced in vitro. Therefore metabolite generation procedures utilizing HOCl or HRP–$H_2O_2$ as primary components involved in clozapine metabolism were adapted for stromal culture and coupled with viability determinations. Drug oxidation by HOCl was less toxic to stromal cells than HRP–$H_2O_2$ based methods. More specifically, clozapine bioactivation by HRP–$H_2O_2$ caused dose-dependent inhibition of stromal viability at therapeutically relevant concentrations. Differences in susceptibility of HAS303 and LP101 cells to the clozapine nitrenium ion were also evident. Stromal cell death was attributed to clozapine in the presence of a complete metabolising system comprising HRP and $H_2O_2$. In the absence of a complete metabolising system clozapine was not cytotoxic. For LP101 cells, drug plus HRP (minus $H_2O_2$) also induced toxicity. Importantly, other antipsychotic drugs including risperidone, olanzapine and haloperidol when bioactivated, were not cytotoxic, indicating system specificity for clozapine. Exogenous GSH, *N*-acetylcysteine, L-ascorbic acid, catalase, and sodium azide afforded protection to cells whereas *S*-methylGSH, GSSG, ketoprofen and proadifen did not. Thus functional data derived from the in vitro stromal system defined in these studies may enable further investigation of the mechanisms subserving stromal impairment in clozapine-induced agranulocytosis and direct attention to improved methods for its prevention.



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MPO, myeloperoxidase
NaHOCl, sodium hypochlorite
PBS, phosphate-buffered saline
SEM, standard error of the mean

## 1. Introduction

Clozapine, an antipsychotic drug indicated for treatment-resistant schizophrenia can induce agranulocytosis, a potentially fatal decrease in circulating neutrophils [1]. In defining the chemical entity responsible for clozapine-induced agranulocytosis, evidence centers on drug bioactivation to a chemically reactive intermediate, thought to be a nitrenium ion [2–4]. It has been postulated that covalent modification of neutrophil/bone marrow proteins by this reactive species could be directly toxic to cellular processes causing cell death, or alternatively, hapten formation and immune-mediated toxicity or hypersensitivity [5,6]. Whichever is the relevant mechanism, the cellular targets of this toxic process are unknown.

Clozapine-induced agranulocytosis has been suggested to involve disruption of the bone marrow microenvironment in a way that may interfere with the normal regulation of granulopoiesis [7]. A pivotal component of this microenvironment is the bone marrow stromal cell. Stromal cells and the extracellular matrix which they secrete harbour myeloid precursor cells in established niches which provide signals for differentiation and enable proximity between adhesion molecules, cytokines and growth factors [8–10]. It seems plausible therefore, that clozapine bioactivation may impair stromal cell function and in turn arrest neutrophil production.

To determine the mechanism that may underlie drug-induced agranulocytosis, clozapine oxidation has been undertaken both in vitro and in vivo to identify potential candidate reactive metabolites [2,3,11]. Interestingly, comparative studies with olanzapine, an antipsychotic drug designed as a pharmacological equivalent to clozapine but not associated with agranulocytosis or as effective in treatment resistance have indicated differing abilities to cause cytotoxicity [12]. These studies utilized either hypochlorous acid (HOCl) or a combination of myeloperoxidase (MPO) or horseradish peroxidase (HRP) and hydrogen peroxide ($H_2O_2$) as the primary components involved in drug metabolism. HOCl, whether derived endogenously from MPO released by activated neutrophils or generated by sodium hypochlorite (NaHOCl) added directly to cell suspensions oxidizes clozapine to an initial nitrenium ion. The same metabolites were also produced by the MPO or HRP–$H_2O_2$ system [2,13,14]. Importantly with clozapine, the metabolites produced by HRP in conjunction with $H_2O_2$ were identical to those produced by neutrophils and their precursors when activated [3].

More recent investigations have focused on assessment of the functional toxicity of the nitrenium ion on the neutrophil. Accordingly, in vitro assays were developed in which the in situ generation of reactive metabolite was coupled to determination of peripheral leukocyte viability and chemical characterization of clozapine metabolism [4,6,13]. The current experiments stem from these findings, albeit examining impairment of bone marrow stromal cell function by the reactive intermediates of clozapine as a target for toxicity. An in vitro bone marrow stromal cell system was therefore established to permit mechanistic evaluation of the pathways involved. Hence, the effects of clozapine derivatives on HAS303 and LP101 stromal cell viability were studied. These two cell lines are of endothelial and macrophage origin, respectively, [15] and are reported to possess in vitro hematopoietic supportive functions [16,17]. Exposure of stromal cultures to chemical reaction of clozapine with HOCl or the oxidation system of HRP–$H_2O_2$ was thus undertaken and viability parameters measured. The defining of metabolite generation procedures for use in cell culture enabled functional characterization of the reaction profile and importantly highlighted system specificity for clozapine.

## 2. Materials and methods

### 2.1. *HAS303 and LP101 stromal cultures*

Normal human bone marrow cloned stromal cell lines HAS303 and LP101 were kindly donated by Dr. Shin Aizawa (Department of Anatomy, Nihon University School of Medicine, Tokyo, Japan). Cells were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal calf serum (FCS) (Gibco BRL) at 37 °C, 5% $CO_2$ until semi-confluent, then washed with Versene (Gibco BRL) followed by phosphate-buffered saline (PBS), detached with 0.25% trypsin (Gibco BRL), resuspended in IMDM and counted. Cell suspensions were then centrifuged at 170 × *g* for 5 min at 20 °C, the resulting pellets resuspended and diluted as stipulated.

### 2.2. *Oxidation of clozapine by HOCl and determination of HAS303 cell viability*

The production of clozapine reactive metabolites by chemical reaction of drug with HOCl was adapted from the method of Gardner et al. [4], to accommodate the cell systems under study. Hence HAS303 cells were cultured and transferred at $5.0 \times 10^5$ cells in 1 ml of Hanks balanced salt solution (HBSS) (Gibco BRL) to 2 ml microfuge tubes. Clozapine (Sigma–Aldrich, Saint Louis, MO, USA) was dissolved in methanol and diluted to concentrations of 500, 1000 and 2000 μM using PBS pH 7.4. NaHOCl was diluted in PBS pH 6.0 to 450, 900 and 1800 μM or 0.9 molar equivalent concentrations. Hundred microlitres of drug was then added to the 1 ml cell suspension followed by immediate addition of 100 μl of NaHOCl at the corresponding concentration. Cells were exposed to each

component of the oxidation system either alone or in combination as follows: (A) cells + [clozapine] + [NaOCl], (B) cells + [clozapine], (C) cells + [NaOCl] and (D) cells only in total 1.2 ml volumes with final clozapine concentrations being 42, 83 and 166 μM. Reaction samples were then mixed before $4.2 \times 10^4$ cells in 100 μl aliquots were plated in 96-well microtitre plates (Nunc, Roskilde, Denmark). Plates were incubated for 2 h at 37 °C, 5% $CO_2$ before removal of solution and addition of 100 μl IMDM for cell recovery. Cells were then incubated overnight for 18 h and viability measured following addition of 10 μl of Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals, Mannheim, Germany) to each well. Cells were incubated for a further 2 h and absorbance at 450 nm quantified using a spectrophotometric microtitre plate reader (Molecular Devices, Sunnyvale, CA, USA). Results were expressed as a percentage of the number of viable cells present in untreated cell samples (control) and then subtracted from 100 to calculate the percentage inhibition of cell viability (cell cytotoxicity). Thus, control or cell only samples represented zero percent inhibition of viability or cell toxicity.

### 2.3. Bioactivation of clozapine by HRP–$H_2O_2$ and determination of HAS303 cell viability

Preliminary method development was undertaken to determine suitable concentrations of HRP and $H_2O_2$ for use in subsequent activation assays (data not shown).

Clozapine dissolved in methanol and diluted in HBSS to final concentrations of 42, 83 and 166 μM was added in 500 μl volumes to $5.0 \times 10^5$ HAS303 cells in 500 μl HBSS. Following addition of 1 U of HRP (Type I, Sigma–Aldrich) in a 5 μl volume, the reaction was started by the further addition of 195 μl of $H_2O_2$ (3%, w/w solution, Sigma–Aldrich) in PBS pH 7.4 at a final concentration of 25 μM. Cells were exposed to components of the oxidation system as follows: (A) cells + [clozapine] + HRP + $H_2O_2$, (B) cells + HRP + $H_2O_2$, (C) cells + HRP, (D) cells + $H_2O_2$, (E) cells + [clozapine] and (F) cells only. Each sample was immediately mixed, plated, incubated and viability assessed as previously outlined.

### 2.4. Bioactivation of clozapine by HRP–$H_2O_2$ and effects on HAS303 and LP101 viability

Experiments were extended to examine the dose-dependent effects of clozapine bioactivation on stromal viability. Clozapine concentrations chosen were based on those used in preliminary spectrophotometric time course experiments (in the absence of cells) in which drug metabolism was assessed (data not shown). Hence clozapine at final concentrations of 0.83, 1.67, 2.5, 4.2, 5.2, 8.3 and 16.6 μM was bioactivated as detailed above with HAS303 cells exposed to components of the oxidation system as follows: (A) cells + [clozapine] + HRP + $H_2O_2$ and (B) cells only. In similar experiments $2.4 \times 10^5$ LP101 cells were exposed to the bioactivation system at final clozapine concentrations of 0.83, 2.5, 3.3, 4.2, 8.3, 16.6 and 166 μM. $4.2 \times 10^4$ HAS303 and $2.0 \times 10^4$ LP101 cells were then incubated for 2 h at 37 °C, 5% $CO_2$ before removal of solution, addition of 100 μl IMDM and subsequent cell recovery for 18 and 16 h, respectively. Viability was then determined as previously described.

### 2.5. Stromal cell viability in the presence of a complete and incomplete metabolite-generating system

To establish whether toxicity towards HAS303 and LP101 cells could occur in the absence of the complete activating system, cells were exposed to 8.3 μM of clozapine (a concentration toxic to HAS303 and LP101 cells as indicated in 3.3) in the following experimental setups: (A) cells + [clozapine] + HRP + $H_2O_2$, (B) cells + HRP + $H_2O_2$, (C) cells + [clozapine] + HRP, (D) cells + [clozapine] + $H_2O_2$, (E) cells + HRP, (F) cells + $H_2O_2$, (G) cells + [clozapine] and (H) cells only. $4.2 \times 10^4$ HAS303 and $2.0 \times 10^4$ LP101 cells were then incubated as detailed previously, followed by assay of viability.

### 2.6. Bioactivation of antipsychotic drugs by HRP–$H_2O_2$ and determination of HAS303 cell viability

To assess whether the toxicity generated by the HRP–$H_2O_2$ oxidation system was specific for clozapine, HAS303 cells were exposed to other antipsychotic drugs under the same experimental paradigm. Thus, risperidone (Jansen Cilag Pharmaceutica, Beerse, Belgium), olanzapine (Eli Lilly, Indianapolis, IN, USA) and haloperidol (Sigma–Aldrich) were each dissolved in methanol and diluted in HBSS to a final concentration of 8.3 μM thereby enabling viability data to be compared with clozapine. All drugs were bioactivated in the presence of HAS303 cells as described for clozapine with the following systems used: (A) cells + [drug] + HRP + $H_2O_2$, (B) cells + [drug] and (C) cells only. Cells were then incubated and viability assessed as detailed previously.

### 2.7. Determination of HRP–$H_2O_2$ mediated clozapine cytotoxicity in the presence of biological modifiers

To determine whether clozapine mediated cytotoxicity could be attenuated, incubations with stromal cells, the activating system and exogenous agents were undertaken. 8.3 μM clozapine was bioactivated in the presence of HAS303 cells by modification of the method outlined with final cell, HRP and $H_2O_2$ concentrations remaining the same. Hence, 100 μl $H_2O_2$ was added to the reaction system followed by final 100 μl addition of the agents (Sigma–Aldrich) listed in Table 1. All agents were dissolved in PBS pH 7.4 except ketoprofen, and diluted to final concentrations of 10, 100 and 1000 μM. Ketoprofen was dissolved in methanol and similarly diluted. Catalase was diluted to final concentrations of 10, 50 and 100 U/ml. Components of the oxidation system included were as follows: (A) cells + [clozapine] + HRP + $H_2O_2$, (B) cells + [clozapine] + HRP + $H_2O_2$ + [agent], (C) cells + [agent] and (D) cells only. $4.2 \times 10^4$ HAS303 cells were then incubated and viability assayed.

### 2.8. Data analysis

Data are presented as the mean ± standard error of the mean (S.E.M.) from three separate experiments, each measured in quadruplicate unless indicated otherwise. Statistical analyses were undertaken using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Treatment groups were compared to the control group plus pairwise comparisons

**Table 1 – Biological modifiers added to clozapine bioactivation reactions to determine antioxidation potential**

| Agent antioxidant | Mechanism of action | Reference |
|---|---|---|
| Glutathione (reduced) (GSH) | Reducing agent | [18] |
| S-Methylglutathione (S-methylGSH) | Non-reactive glutathione | [18] |
| Glutathione disulfide (GSSG) | Oxidizing agent | [18] |
| N-Acetylcysteine | Sulphydryl donor, hydroxyl radical scavenger | [19] |
| L-Ascorbic acid | Reducing agent, free radical scavenger | [20] |
| Catalase | Catalyzes breakdown of $H_2O_2$ | [21] |
| Proadifen | Cytochrome P450 inhibitor | [22] |
| Ketoprofen | Anti-inflammatory agent, inhibitor of cyclooxygenase | [23] |
| Dimethylthiourea | Hydroxyl radical scavenger, inhibitor of laminin and entactin degradation | [24] |
| Sodium azide | Inhibitor of myeloperoxidase | [25] |

between treatment groups made using one-way ANOVA, with Bonferroni corrected multiple comparison tests applied post hoc to determine the significance levels reached. Unpaired Student's (two-tailed) *t*-tests for comparison between pairs of variables were also used.

## 3. Results

### 3.1. *Effect of clozapine oxidation by HOCl on HAS303 cell viability*

Forty-two and 83 μM clozapine in the presence of corresponding 0.9 molar equivalent concentrations of NaHOCl, caused no significant inhibition of HAS303 viability relative to control (cells only) (Fig. 1A and B). However, 166 μM of clozapine when activated was significantly toxic to HAS303 cells ($F = 97.42$, d.f. = 3, 6, $p < 0.0001$) (Fig. 1C). When cells were incubated with an incomplete activating system (that is, in the absence of either NaHOCl or drug), no appreciable cell death above control was noted ($p > 0.05$, in all cases) (Fig. 1A–C).

### 3.2. *Effect of clozapine bioactivation by HRP–$H_2O_2$ on HAS303 cell viability*

In the presence of both HRP and $H_2O_2$, clozapine was bioactivated to a metabolite that was significantly toxic to HAS303 cells at each drug concentration utilized (42 μM: $F = 30.45$, d.f. = 5, 6, $p = 0.0003$ (Fig. 2A); 83 μM: $F = 196.8$, d.f. = 5, 12, $p < 0.0001$ (Fig. 2B); 166 μM: $F = 39.27$, d.f. = 5, 12, $p < 0.0001$ (Fig. 2C)). Significant toxicity by incubation of cells with 166 μM clozapine alone, was also detected post hoc ($p < 0.05$) (Fig. 2C). By contrast, in the absence of drug but in the presence of components of the system, there was no significant increase in cell death above background control values ($p > 0.05$, in all cases) (Fig. 2A–C).

### 3.3. *Dose-dependent effect of clozapine bioactivation by HRP–$H_2O_2$ on stromal cell viability*

Assessment of HAS303 cells following bioactivation and overnight cell recovery indicated toxicity proportional to clozapine concentration ($IC_{50}$ 4.4 μM), reaching maximum levels at 8.3 μM drug ($F = 155.6$, d.f. = 7, 16, $p < 0.0001$) (Fig. 3A). At drug concentrations of 2.5 μM and above, cell viability was significantly inhibited relative to control ($p < 0.01$, in all cases) (Fig. 3A).

With LP101 cells, concentration-dependent cytotoxicity ($IC_{50}$ 3.9 μM), was also demonstrated ($F = 102.7$, d.f. = 7, 16, $p < 0.0001$) (Fig. 3B). Increased cell sensitivity relative to control at clozapine concentrations of 3.3 μM and above when activated, was shown post hoc ($p < 0.01$, in all cases) (Fig. 3B).

### 3.4. *Effect of components of the HRP–$H_2O_2$ activating system on stromal cell viability*

An overall significant effect on HAS303 cell viability was noted when components of the activating system were analyzed

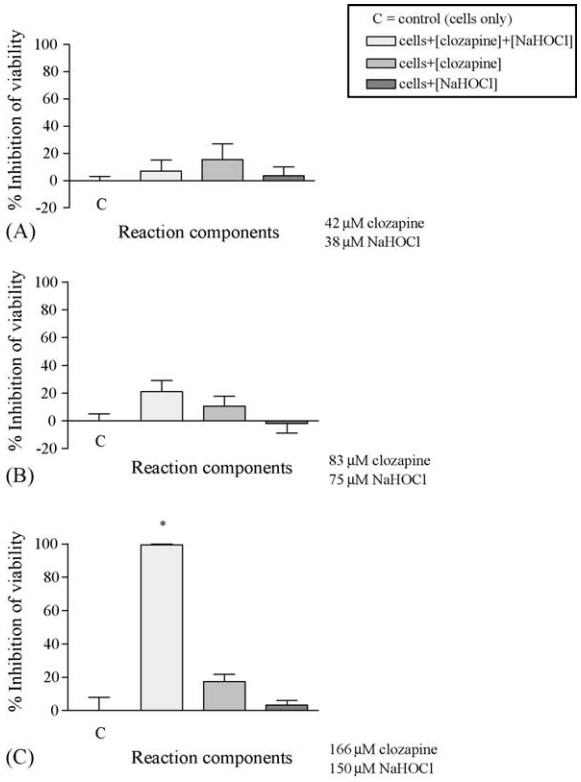


**Fig. 1 – The effect of clozapine oxidation by HOCl and components of the system on inhibition of HAS303 viability at various drug concentrations: (A) 42 μM clozapine, (B) 83 μM clozapine and (C) 166 μM clozapine. Data are expressed relative to untreated control cells (C = 0%) and represent the mean ± S.E.M. of quadruplicate incubations from three separate experiments. *$p < 0.01$. Statistical difference compared with control values.**

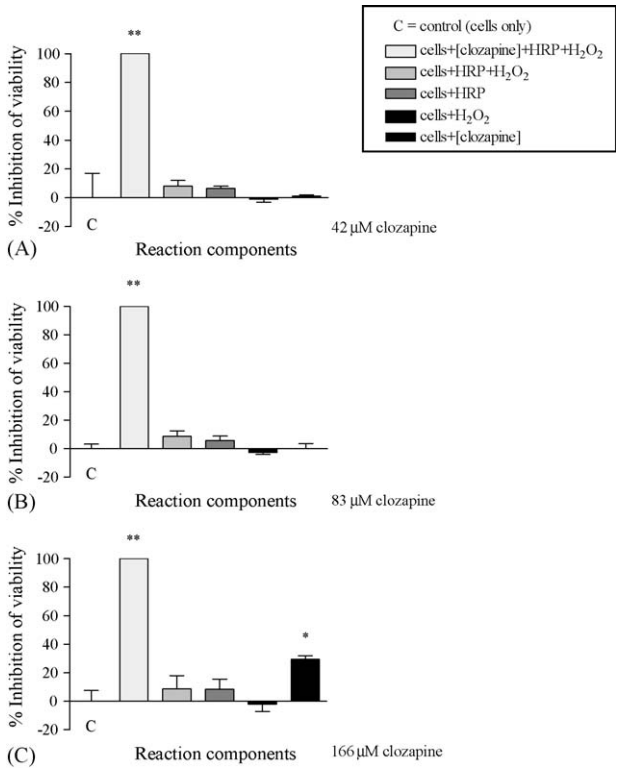


**Fig. 2 – The effect of clozapine bioactivation by HRP–$H_2O_2$ and components of the system on inhibition of HAS303 viability at various drug concentrations: (A) 42 μM clozapine, (B) 83 μM clozapine and (C) 166 μM clozapine. Data are expressed relative to untreated control cells (C = 0%) and represent the mean ± S.E.M. of quadruplicate incubations from three separate experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. Statistical differences compared with control values.**

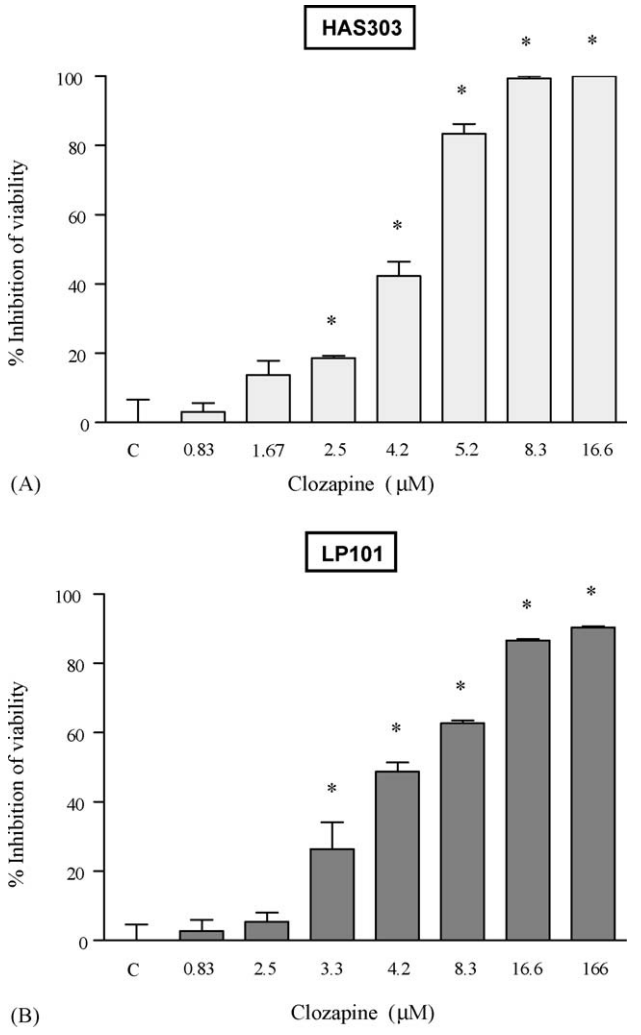


**Fig. 3 – The effect of clozapine bioactivation by HRP–$H_2O_2$ over a range of drug concentrations on inhibition of stromal cell viability: (A) HAS303 cells [$IC_{50}$ 4.4 μM] and (B) LP101 cells [$IC_{50}$ 3.9 μM]. Data are expressed relative to untreated control cells (C = 0%) and represent the mean ± S.E.M. of quadruplicate incubations from three separate experiments. $^{*}p < 0.01$. Statistical differences compared with control values.**

either in combination or alone ($F = 89.99$, d.f. = 7, 16, $p < 0.0001$) (Fig. 4A). This was attributed to complete cell death in the presence of the full system [cells + clozapine + HRP + $H_2O_2$] at 8.3 μM clozapine ($p < 0.01$), in contrast to the absence of toxicity above control for cells incubated with an incomplete system ($p > 0.05$, in all cases) (Fig. 4A).

For LP101 cells, significant overall effects on viability were also found when components of the system were analyzed ($F = 40.06$, d.f. = 7, 16, $p < 0.0001$) (Fig. 4B). However for these cells, significantly increased inhibition of viability was observed with two systems as follows: [cells + clozapine + HRP + $H_2O_2$] and [cells + clozapine + HRP] at 8.3 μM clozapine, relative to control ($p < 0.01$, in both cases) with no significant difference between the two noted ($t = 1.294$, d.f. = 4, $p = 0.2652$) (Fig. 4B). All other systems were not significantly toxic ($p > 0.05$, in all cases) (Fig. 4B).

### 3.5. *Effect of antipsychotic drug bioactivation by HRP–$H_2O_2$ on HAS303 cell viability*

When risperidone, olanzapine and haloperidol were tested for cytotoxicity following bioactivation at 8.3 μM drug, in the same manner as clozapine, neither agent caused significant HAS303 cell death ($F = 1.984$, d.f. = 6, 14, $p = 0.1365$) (Fig. 5). As well, each drug incubated with cells alone did not inhibit viability relative to control ($p > 0.05$, in all cases) (Fig. 5).

### 3.6. *Effect of biological modifiers on HRP–$H_2O_2$ mediated clozapine cytotoxicity*

Significant overall effects on clozapine-induced toxicity were recorded with the exclusion and inclusion of agents listed in Table 1 in activation incubations with HAS303 cells ($p < 0.0001$, in all cases) (Fig. 6a and b). Post hoc comparisons with control expectedly showed significant toxicity with the complete activating system at 8.3 μM clozapine in all experiments ($p < 0.001$) (Fig. 6a(A–F) and 6b(G–J)). No significant reduction in this toxicity was then achieved by any agent

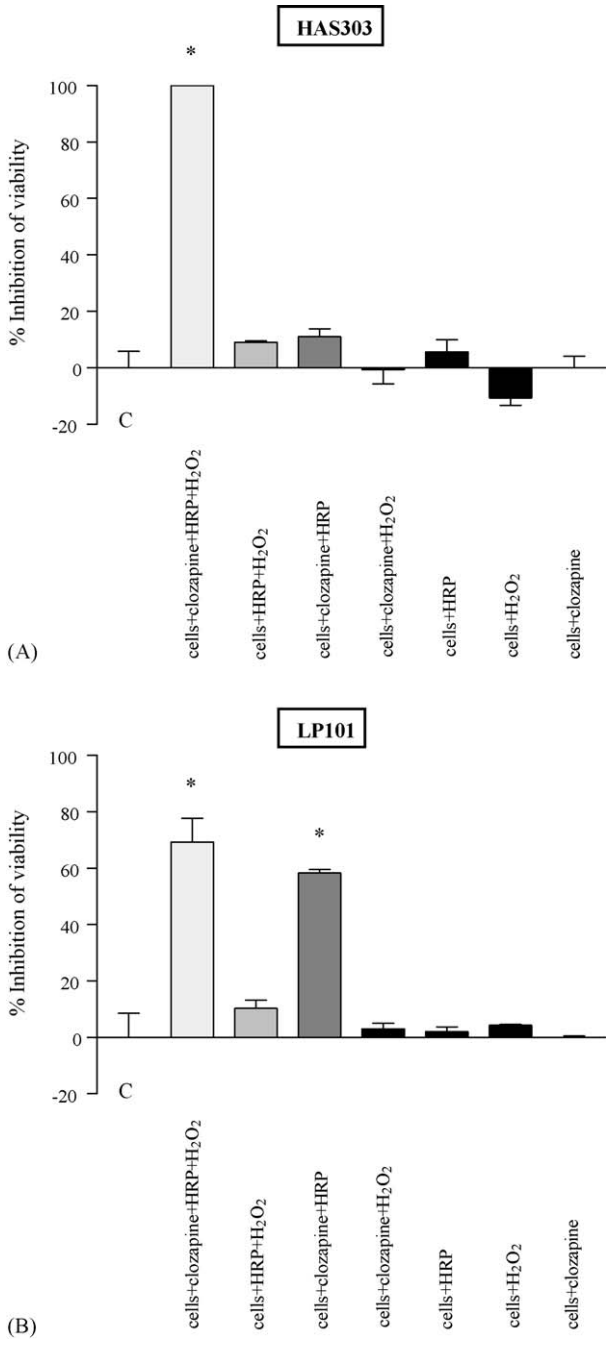


**Fig. 4 – The effect of a complete and incomplete HRP–$H_2O_2$ activating system at 8.3 μM clozapine on inhibition of stromal cell viability: (A) HAS303 cells and (B) LP101 cells. Data are expressed relative to untreated control cells (C = 0%) and represent the mean ± S.E.M. of quadruplicate incubations from three separate experiments. $^{*}p < 0.01$. Statistical differences compared with control values.**

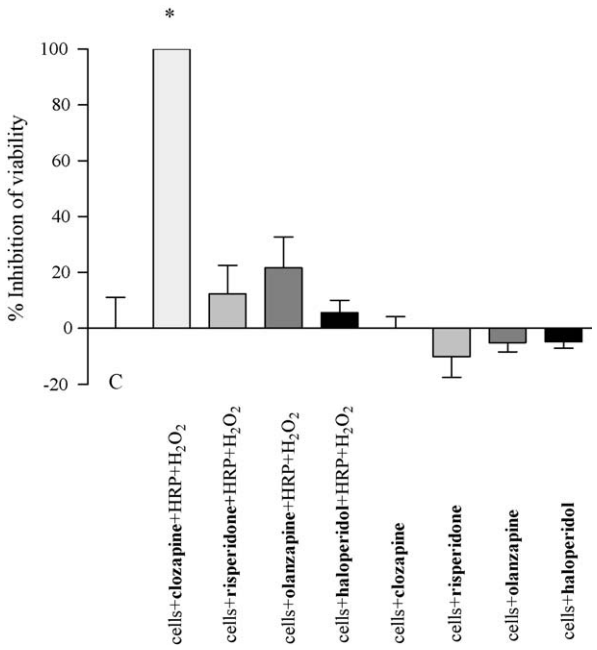


**Fig. 5 – The effect of antipsychotic bioactivation by HRP–$H_2O_2$ at 8.3 μM drug, on inhibition of HAS303 viability. Data are expressed relative to untreated control cells (C = 0%) and represent the mean ± S.E.M. of quadruplicate incubations from three separate experiments. Clozapine data were excluded from analysis but included graphically, as a point of comparison. $^{*}p < 0.01$. Statistical difference compared with control values.**

added to the complete system at a concentration of 10 μM (Fig. 6a(A–F) and 6b(G–J)). However, toxicity was significantly attenuated when 100 and 1000 μM GSH, *N*-acetylcysteine, L-ascorbic acid, catalase (50, 100 U/ml), dimethylthiourea (at 1000 μM only) and sodium azide were incubated with the complete system (Fig. 6a(A, D–F) and 6b(I and J), respectively).

All other agents including *S*-methylGSH, GSSG, proadifen and ketoprofen at 100 and 1000 μM in the presence of the activated system caused no change in inhibition of viability relative to the complete system (Fig. 6a(B and C) and 6b(G and H), respectively).

In addition, HAS303 cells in the presence of proadifen (1000 μM) showed significant increases in inhibition of viability, whilst *S*-methylGSH (100 μM) and sodium azide (100, 1000 μM) at the concentrations noted, caused significant decreases in inhibition or stimulation of viability compared with control (Fig. 6b(G), 6a(B) and 6b(J), respectively). GSH, GSSG, *N*-acetylcysteine, L-ascorbic acid, catalase, ketoprofen and dimethylthiourea when incubated with cells alone, had no significant effect on cell death above control values (Fig. 6a(A, C–F) and 6b(H and I), respectively).

## 4. Discussion

### 4.1. *Reactive metabolite formation by HOCl and the HRP–$H_2O_2$ system*

The oxidation of clozapine to reactive intermediates by HOCl and the bioactivation system of HRP–$H_2O_2$ was established in vitro with induction of stromal cell death ascribed to the products formed. At comparable clozapine concentrations and incubation conditions, drug oxidation by HOCl was less toxic to stromal cells than HRP–$H_2O_2$ methods.

Although clozapine reactive metabolite was produced extracellularly, with the HOCl method critical sites inside cells may not have been reached whereas with the HRP–$H_2O_2$ method, $H_2O_2$ may in part act as a cofactor by intracellular enzymes to generate metabolites within cells. Support for this supposition comes from studies with neutrophils in which extracellular GSH inhibited covalent binding of HOCl generated reactive intermediate to cells but only partially inhibited binding of MPO–$H_2O_2$ generated metabolite. Thus in the latter case, at least some reactive metabolite was formed in an intracellular compartment, inaccessible by GSH [4,12]. In the absence of detectable peroxidase in HAS303 cells [15], other hydrolytic enzymes or glutathione transferases could be responsible for clozapine oxidation coupled with binding of products to stromal protein, initiating death [26]. Furthermore, Gardner et al. [4] suggest that with the HOCl method, cell exposure to reactive intermediate occurs for a brief period whilst with the alternative, exposure is longer and may coincide with toxicity.

### 4.2. *Characterization of bone marrow stromal cell toxicity by clozapine reactive metabolites*

Clozapine bioactivation by HRP–$H_2O_2$ over an extended concentration range caused dose-dependent inhibition of stromal viability. Damage to cells was sustained at higher drug concentrations but not at the lower end of the range. Differences in sensitivity of HAS303 and LP101 cells to the nitrenium ion of clozapine were also evident.

Complete HAS303 cell death was the outcome of exposure to 8.3–16.6 μM clozapine (2712–5429 ng/ml), concentrations deemed supratherapeutic based on plasma levels which range from 20 to 1479 ng/ml, with 350 to 420 ng/ml clozapine considered the threshold for clinical response [27]. At the

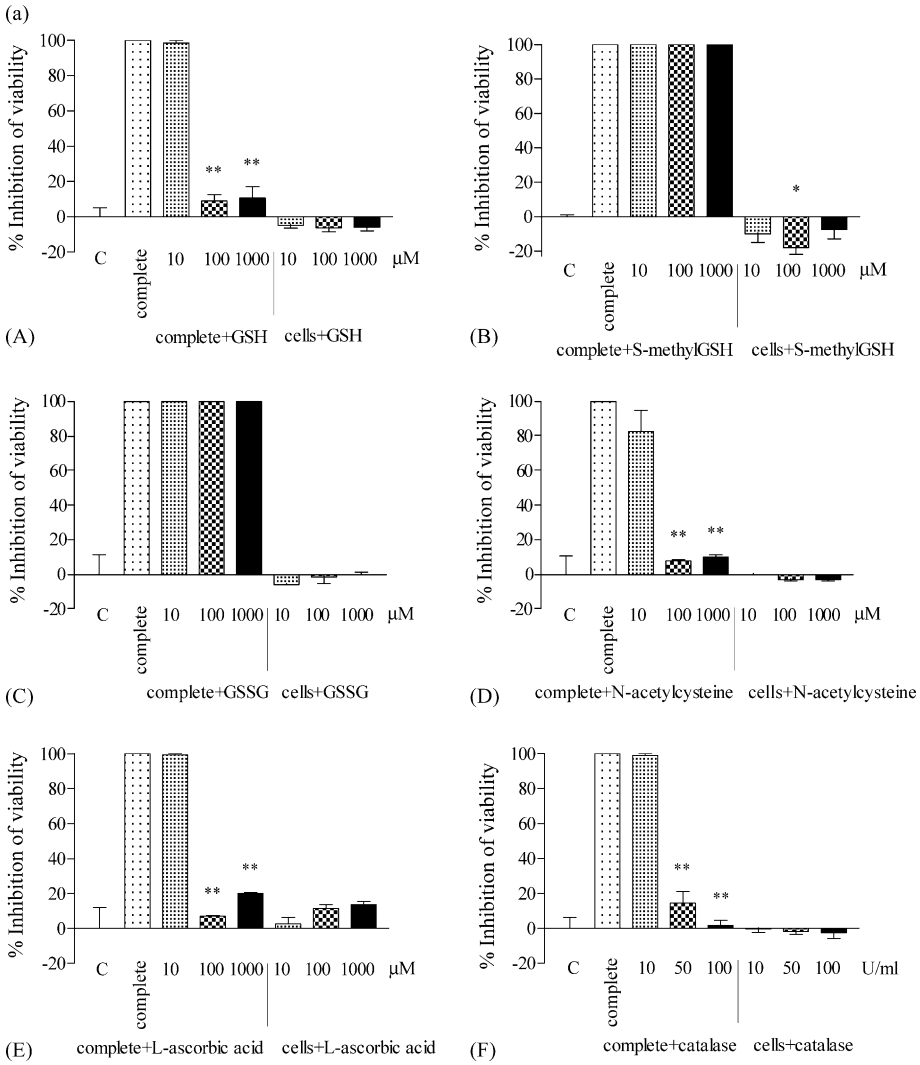


**Fig. 6 – The effect of biological modifiers at various concentrations on HRP–$H_2O_2$ mediated clozapine cytotoxicity toward HAS303 cells. Part (a): (A) GSH, (B) S-methylGSH, (C) GSSG, (D) N-acetylcysteine, (E) L-ascorbic acid and (F) catalase. Part (b): (G) proadifen, (H) ketoprofen, (I) dimethylthiourea and (J) sodium azide. Data are expressed relative to untreated control cells (C = 0%) and represent the mean ± S.E.M. of quadruplicate incubations from three separate experiments. $^{*}p < 0.05$; $^{**}p < 0.001$. Statistical differences between the complete system [cells + 8.3 μM clozapine + HRP + $H_2O_2$] in the absence and presence of agent and cells in the absence (C) and presence of agent are indicated.**

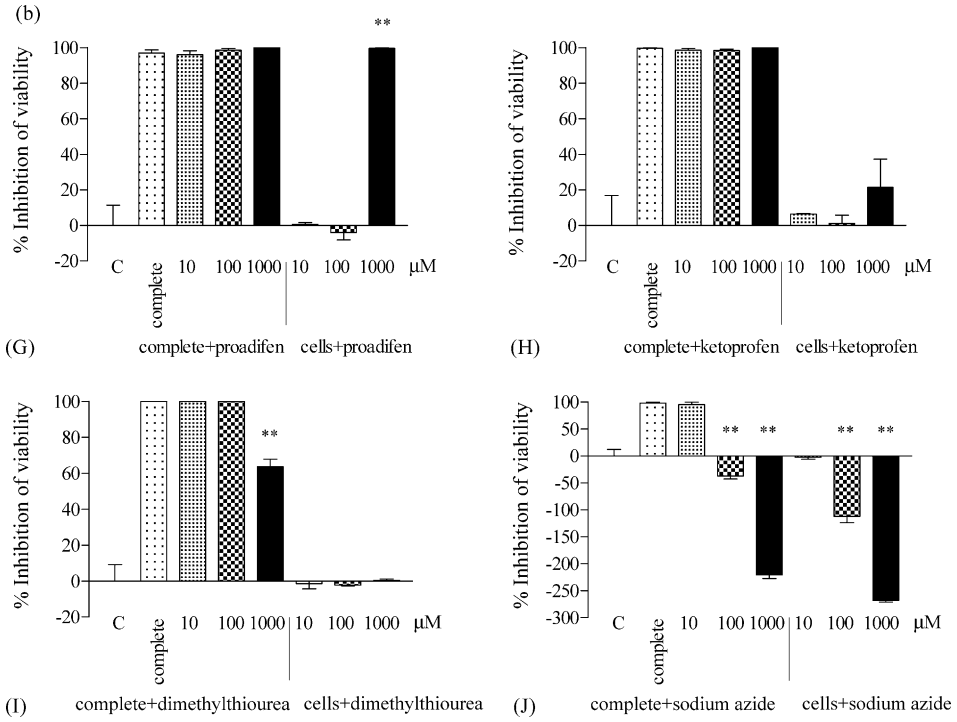


**Fig. 6. (Continued)**.

more therapeutic-equivalent concentrations of 2.5–5.2 μM (817–1699 ng/ml) significant toxicity was still detectable. By comparison, for LP101 cells toxicity of approximately 63–90% was achieved at clozapine concentrations of 8.3–16.6 μM and above, with marked increases of 26–49% across the therapeutic range (3.3–4.2 μM = 1078–1373 ng/ml). Thus LP101 cells seemed less susceptible to the effects of drug bioactivation than HAS303 cells particularly at higher drug concentrations. Whilst there is evidence for a lower limit to its therapeutic range, the upper limit for clozapine efficacy has yet to be defined with concentrations as high as 2000 ng/ml observed clinically [28]. Hence, wide drug concentration ranges covering therapeutic and supratherapeutic plasma levels were used in this study.

Cell death was attributed to drug in the presence of both HRP and $H_2O_2$, but not with an incomplete activating system, with one exception. Marked toxicity was also apparent with LP101 cells plus clozapine in conjunction with HRP. LP101 cells being of macrophage origin [15] may contain peroxide-forming enzymes (D-amino acid oxidase) leading to intracellular generation of $H_2O_2$ not adequately eliminated by enzymic defence mechanisms utilising catalase [21]. Bioactivation of clozapine in the absence of exogenous additions of $H_2O_2$ may therefore have been accomplished. This presupposes that drug and enzyme permeate LP101 cell membranes, the mechanism by which remains unidentified.

Variation in stromal cell susceptibility to clozapine probably resides in differences in cellular composition. Cell-specific stromal impairment has been documented with benzene and ceftazidine in which macrophage and endothelial cells/adipocytes were targeted, respectively [29,30]. Since stromal cells are vital in the control of neutrophil production, damage to a subset could plausibly be instrumental in agranulocytosis. Individual susceptibility to idiosyncratic toxicity may then depend on balance between clozapine bioactivation and detoxification by cellular defence mechanisms [5]. Thus greater sensitivity of cells of some patients due to age, diet, enzyme induction or genetic factors, may allow toxic metabolites to escape inactivation [26].

In light of recent data, clozapine-induced impairment of stromal function may also involve interaction of drug with selective neurotransmitter receptors identified on these cells, sites at which toxicity may be mediated [31]. In this regard, neurotransmitters such as dopamine, serotonin and histamine have been implicated in regulation of hematopoiesis and as mediators of committed precursor cell proliferation and differentiation via receptor mechanisms [32]. In the case of clozapine bioactivation to reactive species, a link between drug-cell binding and functional toxicity has been established, with haptenation greater in those cells undergoing apoptosis [6]. Notwithstanding this, whether the mechanisms underpinning these toxic interactions involve neurotransmitter receptors and are related to known pharmacological effects of the drug (or its metabolites) remains undefined. However, of particular relevance to agranulocytosis is the finding of histamine H4 receptors primarily in bone marrow and eosinophils to which clozapine has moderate affinity, given speculation that these sites may be a peripheral immune target with respect to asthma and allergy [33]. The detection of h1 mRNA in mononuclear and stromal cells [31] is also in line with reports of immunoregulatory control of lymphocytes by histamine binding and histamine release from mast cells [34]. Nevertheless, whether bioactivated clozapine can affect bone marrow function through such receptor activity still awaits clarification.

### 4.3. *Reaction specificity*

Peroxidase-catalyzed oxidation of the atypical antipsychotic drugs risperidone and olanzapine, pharmacologically related to clozapine and the conventional antipsychotic haloperidol, indicated limited impairment of HAS303 viability, in contrast to the significant inhibition recorded with clozapine. These findings remain preliminary but suggest that system specificity may be of benefit in functional screening of oxidation or hematotoxic potential of candidate drugs.

Risperidone has been classified as safe from a hematological standpoint [35] in seeming accordance with current minimal reactivity. Similarly, findings with haloperidol coincide with those recorded in mononuclear cells [36]. With olanzapine, data suggest that the drug does not cause agranulocytosis although induction has been documented in more recent case reports [37]. Reduced stromal toxicity associated with olanzapine compared to clozapine in this study was in line with previous data indicating that although both drugs produced reactive nitrenium ions, toxicity varied between agents [4]. Thus, whilst olanzapine did not induce neutrophil toxicity at concentrations up to 20 μM, clozapine did so at 2 μM when bioactivated [4]. Moreover, no drug-modified polypeptides were detected in neutrophils from patients administered olanzapine, in contrast to clozapine. These in vivo differences in binding may in part be explained by difference in dosage (olanzapine 5–20 mg/day; clozapine 200–900 mg/day) and resultant lower plasma concentrations of olanzapine compared to clozapine [28]. Furthermore, olanzapine may be less prone to hematotoxic reactions than clozapine due to formation of a 10-*N*-glucuronide derivative [38].

### 4.4. *Reaction with exogenous biological modifiers*

Upon bioactivation, clozapine-mediated toxicity toward HAS303 cells was attenuated by GSH, *N*-acetylcysteine, L-ascorbic acid, catalase, and sodium azide which afforded protection to cells. Furthermore, inclusion of dimethylthiourea to activation incubations partially reduced stromal toxicity. By contrast, proadifen and ketoprofen caused no reversal of clozapine-induced stromal cytotoxicity. Existing experimental data can explain these results, for example, GSH and *N*-acetylcysteine form stable adducts by covalent binding with clozapine [13]. Such conjugations are thought to neutralize the toxic intermediate, consistent with present data. Incubation with each compound also eliminated reaction of the nitrenium ion with water to form phenolic products [2]. Both thiols also caused a concentration-dependent decrease in irreversible binding of clozapine to microsomal protein and partial inhibition of binding to neutrophils [12]. Moreover, since GSH can scavenge $H_2O_2$, decreased levels could prevent transformation of peroxidase to its respective intermediate in the oxidation pathway [14]. To counter this possibility, agents were added last to incubations to prevent reduction of $H_2O_2$ before reaction with HRP. It would seem unlikely therefore, that GSH was acting as a simple reducing agent when exerting its effects. In vivo, GSH removes peroxides and free radicals and undergoes xenobiotic and protein conjugation that aids detoxification [21]. As well, the compound may be a candidate neuropeptide with an active uptake mechanism [39], and if transported by stromal cells may exert an intracellular role in minimizing the harmful effects of reactive species. By comparison, the non-reactive analogue of GSH, *S*-methylGSH, did not inhibit clozapine oxidation and hence cytotoxicity. This effect could conceivably be due to lack of adduct formation or the non-reducible state of the molecule with viability data similar to that seen with glutathione disulfide (GSSG). These bioactivation data also suggest that the tripeptide structure of GSH and *S*-methylGSH and hexapeptide structure of GSSG per se, had no apparent effect on viability given outcomes noted between agents.

Ascorbic acid decreased stromal cell death in accordance with its function as an inhibitor of clozapine oxidation, ability to reduce reactive intermediates to relatively stable molecules and lack of adduct formation [14]. Of interest are reports of low ascorbate levels in schizophrenic patients [40] and the suggestion that co-administration with clozapine may reduce risk of agranulocytosis due to its cytoprotective properties [11]. Likewise, in the presence of catalase HAS303 cytotoxicity was reduced to control levels. Catalase does not cross the plasma membrane of cultured cells when added exogenously to medium [41]. Therefore, lowered extracellular turnover to reactive metabolite due to decomposition of $H_2O_2$ by catalase may account for the HAS303 finding.

Since proadifen was without antioxidation effect, it would appear that metabolism of clozapine by cytochrome P450 isoforms if expressed in HAS303 cells, did not significantly contribute to toxicity. Similarly as ketoprofen was unable to prevent clozapine-induced toxicity it would seem the process is not mediated by cyclooxygenase, in agreement with findings in human neutrophils [4]. By contrast, dimethylthiourea significantly lowered HAS303 cell death upon clozapine bioactivation. As a hydroxyl radical scavenger, dimethylthiourea inhibits degradation of laminin and entactin, primarily caused by reactive oxygen species [24]. Reduced damage of these plasma membrane associated glycoproteins of the extracellular matrix may thereby assist stromal cell survival. Of interest also were the data that sodium azide attenuated the toxic effects of clozapine bioactivation having a stimulatory effect on HAS303 metabolism. Inhibition of HRP activity by azide [25], resulting in reduced clozapine metabolism would partly account for present observations, although the physiological basis of the stimulation noted remains unknown. Azide stimulated WST-1 reduction has also been reported [42]. However, the current experimental design in which agent was removed prior to viability measurements, and the cell-specific nature of the finding (data not shown) would argue against attribution to such factors.

In summary, an in vitro stromal system was established in which clozapine when oxidized to reactive metabolites by HOCl or HRP–$H_2O_2$, caused dose-dependent inhibition of cell viability. Differences in HAS303 and LP101 metabolism of clozapine were observed. System specificity was highlighted by lack of comparable cell death when antipsychotics including risperidone, olanzapine and haloperidol were bioactivated. Furthermore, inhibition of the metabolism-dependent pathway at different points by biological modifiers may suggest a role for oxidative stress in the toxicity described. Hence, the methods developed allowed functional examination of the chemical and cellular consequences of

toxicity induced by clozapine bioactivation directed at the stroma. These systems may permit the mechanisms subserving such effects, their causal relationship to agranulocytosis and interaction with susceptibility factors in patients to be deciphered.

## Conflict of interest

No conflict of interest arises from this work.

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