

Clozapine and its Major Stable Metabolites, N-desmethylclozapine and Clozapine N-oxide do not Affect Human Bone Marrow Stromal Cells *in vitro*

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Abstract: Treatment of refractory schizophrenia with the atypical antipsychotic drug clozapine is associated with life-threatening agranulocytosis, characterised by a drop in neutrophil count. Theoretically, toxicity may be accounted for by direct action of parent drug or one of its stable metabolites on bone marrow stroma given importance of these cells to neutrophil maturation. Effects of clozapine, N-desmethylclozapine and clozapine N-oxide on stromal cell viability were therefore assessed using human primary long-term bone marrow culture and stromal cell lines, HAS303 and LP101, to define cell-specificity of response. Clozapine, N-desmethylclozapine and clozapine N-oxide had no significant effect on bone marrow stromal, HAS303 and LP101 viability over a wide drug concentration range (10-20000 ng mL⁻¹) compared with cells in absence of drug. Hence it is unlikely that parent drug or its stable metabolites are directly toxic to stroma under clinical conditions. Reduced capability of stroma to support myelopoiesis, however, cannot be excluded.

Key words: Clozapine, metabolites, stroma, agranulocytosis, schizophrenia

INTRODUCTION

Clinical studies have defined the effectiveness of the antipsychotic drug clozapine in the treatment of refractory schizophrenia^[1]. Its demonstrated efficacy and expanding indications for use, however, are mitigated by its propensity to cause agranulocytosis, a potentially fatal reduction in neutrophil cell numbers^[2]. The mechanism by which clozapine-induced agranulocytosis occurs has been posited to involve direct drug toxicity to hematopoietic progenitor cells of the bone marrow^[3]. In most instances, higher concentrations of clozapine and its derivatives than those found in serum were required for suppressive effects on myeloid progenitor growth to be observed^[3]. Furthermore, the myeloid progenitor cell IC₅₀ of both clozapine and N-desmethylclozapine a principal metabolite, was higher in CD34⁺ purified cells than in low density mononuclear cells suggesting a predominant effect on more differentiated rather than primitive hematopoietic cells^[4]. These findings lent support to the postulate that clozapine-induced toxicity could also be due to effects on accessory cells (macrophages, lymphoid cells), which in turn produce inhibitory factors. An extension of this postulate would include toxicity directed at stromal cells of the marrow, not considered in previous studies.

The stromal cell mesenchymal population establishes the milieu in which hematopoietic cells develop *in-vivo* by deposition of extracellular matrix components which, as well as providing physical support, regulate hematopoiesis through interaction with cytokines and adhesion molecules^[5]. Given that a balanced interplay between hematopoietic stem cells and the marrow stroma characterizes normal hematopoiesis, alterations in this microenvironment may result in abnormal blood cell production. In accordance with this view, drugs such as vesnarinone, etoposide, gold sodium thiomalate and diclofenac, associated clinically with agranulocytosis have documented effects on the stroma that may impair function^[6]. Importantly, in the case of clozapine, demonstration of targeted neurotransmitter receptor expression in these cells raises the possibility of their modulation by drug interaction^[7].

The present studies have thus used long-term bone marrow culture as an *in-vitro* model to examine the suppressive or stimulatory potential of clozapine and its major stable metabolites, N-desmethylclozapine and clozapine N-oxide on stromal cell function. Interest in N-desmethylclozapine stems from its identification as a potent muscarinic M1 receptor agonist^[8] and partial dopamine D2/D3 receptor agonist^[9], properties which may confer antipsychotic efficacy. The procedures

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outlined whilst direct in their approach nevertheless require bone marrow samples that are often difficult to obtain. For this reason, alternate methodology was developed using the cloned human stromal cell lines, HAS303 and LP101 of endothelial and macrophage origin, respectively, and reported to possess *in-vitro* hematopoietic supportive functions^[10,11]. Therefore, whilst the primary adherent stromal layer of long-term marrow cultures is a heterogeneous population of cells, the stromal cell lines represent a subset, originating from specific cell types with varying biological functions. Hence, these studies were conducted to determine whether cells of the marrow stroma are selectively sensitive to the effects of clozapine and its metabolites. Stromal cell viability was measured by microtitre assay optimised for the culture systems under study.

MATERIALS AND METHODS

Long-term bone marrow stromal culture: Bone marrow specimens were obtained by needle aspiration from normal donors and collected with informed consent in accordance with The Royal Melbourne Hospital and The Alfred Hospital approved guidelines. Bone marrow mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation and were used to initiate long-term bone marrow cultures using modification of a previous method^[12]. Samples were diluted with an equal volume of Iscove's modified Dulbecco's medium (IMDM) (Gibco BRL) before 5 ml of cell suspension was layered over 5 ml of density gradient and centrifuged at 700 *g* for 20 minutes at 20°C. After centrifugation, the supernatant was aspirated to the white cell (low density) layer. The white cell layer was then collected, diluted with IMDM and centrifuged at 700 *g* for 5 minutes at 20°C. The cell pellet was resuspended with 10 ml of IMDM and the cells counted. The cell suspension was then re-centrifuged at 700 *g* for 10 minutes at 20°C and the resulting pellet resuspended and diluted to 7.5 x 10⁴ cells per 96-well tissue culture microtitre plates (Nalge Nunc) in a final 100 µl volume. Cells cultured in IMDM supplemented with 10% fetal calf serum (FCS) (Gibco BRL), penicillin-streptomycin-amphotericin antibiotic/antimycotic mixture (Gibco BRL), 1 µM hydrocortisone and 50 µM mercaptoethanol (Sigma-Aldrich) termed complete IMDM were then maintained in a 37°C, 5% CO₂ humidified incubator, for up to 17 days. At weekly intervals, cultures were fed by removal of a 30 µl aliquot of non-adherent cell suspension from wells and replacement with complete IMDM.

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

IMDM supplemented with 10% FCS at 37°C, 5% CO₂ until semi-confluent, then washed with Versene (Gibco BRL) followed by phosphate-buffered saline, detached with 0.25% trypsin, resuspended in IMDM and counted. Cell suspensions were then centrifuged at 170 *g* for 5 minutes at 20°C, the resulting pellets resuspended and diluted as stipulated.

Primary and cloned stromal cell viability: Bone marrow mononuclear cells were isolated and plated to generate a primary bone marrow stromal layer as outlined. Clozapine (Novartis) and N-desmethylozapine (Sigma-Aldrich) were dissolved in methanol and clozapine N-oxide (Sigma-Aldrich) in DMSO and diluted in complete IMDM to final concentrations of 10, 50, 100, 500, 1000, 5000 and 20000 ng mL⁻¹. Camptothecin (Sigma-Aldrich) was similarly diluted to final concentrations of 500 and 1000 ng mL⁻¹ and included in all experiments as a positive metabolic inhibitor or control. At incubation's end the non-adherent cell layer was removed, 100 µl of diluted drug added and plates incubated overnight at 37°C, 5% CO₂. Untreated cells in the absence of drug were plated in 100 µl of IMDM and included in all experiments. Stromal cell viability was then measured by WST-1 assay following addition of 10 µl of Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals) to each well. Cells were incubated for a further 2 hours and absorbance at 450 nm then quantified and corrected for against background absorbance (blank), using a spectrophotometric microtitre plate reader (Molecular Devices). Results were expressed as a percentage of the number of viable cells present in untreated cell samples (control). In similar experiments, 5.0 x 10⁴ HAS303 and 2.0 x 10⁴ LP101 cells were plated in 90 µl of IMDM and 10 µl of diluted drug added. Cells were then incubated overnight at 37°C, 5% CO₂ for 18 and 16 hours, respectively, and viability measured as described.

Data analysis: Data are presented as the mean ± standard error of the mean (SEM) from three separate experiments, each measured in quadruplicate. Statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc.) by comparing incubations in the absence and presence of clozapine, its stable metabolites and camptothecin at different drug concentrations. Results of assessment of variables were analysed using one-way analysis of variance with post hoc Dunnett's multiple comparison tests to establish significant differences between control and treatment groups.

RESULTS AND DISCUSSION

Effect of clozapine, N-desmethylozapine and clozapine N-oxide on stromal cell viability: Clozapine, N-desmethylozapine and clozapine N-

oxide had no significant effect on bone marrow primary stromal, HAS303 and LP101 cell viability over the drug concentration range 10 to 20000 ng mL⁻¹ compared with cells in the absence of drug ($p > 0.05$, in all cases) (Fig. 1 A-C, Fig. 2 A-C and Fig. 3 A-C, respectively).

Camptothecin at 500 and 1000 ng mL⁻¹ caused a significant decrease in viability of primary bone marrow stroma relative to control in each experiment (clozapine: $F = 374.8$, $df = 2,6$, $p < 0.0001$ (Fig. 1 A); N-desmethylozapine: $F = 25.23$, $df = 2,6$, $p = 0.0012$ (Fig. 1 B); clozapine N-oxide: $F = 59.56$, $df = 2,6$, $p = 0.0001$ (Fig. 1 C)). Similarly, camptothecin also inhibited LP101 cell viability (clozapine: $F = 12.94$, $df = 2,6$, $p = 0.0067$ (Fig. 3 A); N-desmethylozapine: $F = 16.43$, $df = 2,6$, $p = 0.0037$ (Fig. 3B); clozapine N-oxide: $F = 16.02$, $df = 2,6$, $p = 0.0039$ (Fig. 3 C). By contrast, whilst camptothecin decreased HAS303 viability, these differences were not significant compared with cells in the absence of drug ($p > 0.05$, in all cases) (Fig. 2 A-C).

Exposure of human bone marrow stromal cells, both primary and cloned to clozapine and its major stable metabolites, N-desmethylozapine and clozapine N-oxide at a range of concentrations did not alter cell viability as indicated by assay of metabolic activity. Direct toxicity resulting in stromal cell death was therefore not a consequence of treatment with clozapine at therapeutic concentrations.

Whilst previous investigations have assessed the cytotoxic potential of clozapine or a derivative on hematopoietic stem cells, direct effects on the bone marrow stroma have not been documented. Clozapine inhibition of fibroblast and HeLa cell survival at concentrations that exceeded 120 μM has been reported^[13], data in relative agreement with the present study (20000 ng mL⁻¹ clozapine equates to 61.2 μM , about half the concentration required to achieve inhibitory effects). In light of the current stimulatory potential of clozapine evident, residual effects on bone marrow stromal cells could increase levels of inhibitory factors which impact on granulopoiesis.

Plasma levels of clozapine and its metabolites can vary widely between individuals with results dependent on pharmacokinetics, metabolism, compliance and study design^[14,15]. Whilst 350 to 420 ng mL⁻¹ drug is considered the threshold for clinical response^[15], the upper limit for clozapine efficacy has yet to be defined with concentrations as high as 2000 ng mL⁻¹ observed^[16,17]. Hence a wide drug concentration range covering therapeutic and supratherapeutic plasma levels was used in this study. Incubations were conducted in culture medium with reportedly greater than 80% of clozapine free and not protein bound^[3].

In response to N-desmethylozapine stromal cell viability was not altered. These findings are of particular note since N-desmethylozapine has muscarinic M1 receptor agonist properties with potential as a cognitive enhancer in schizophrenia^[8].

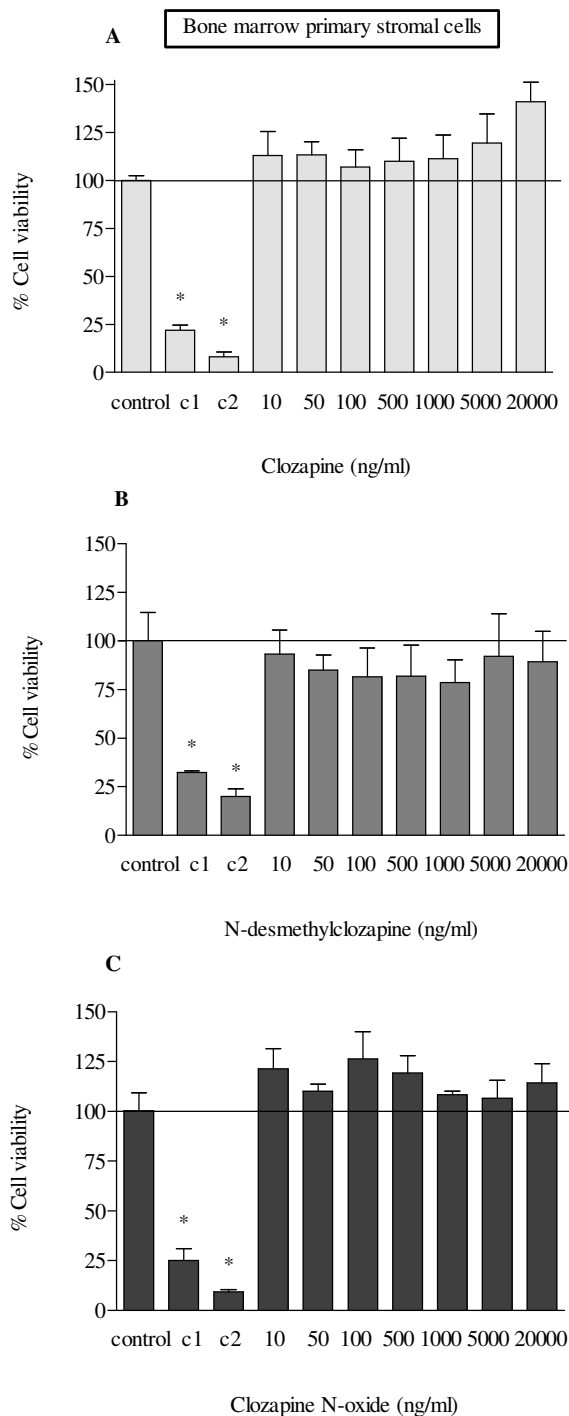


Fig. 1: The effect of clozapine and its metabolites over a range of concentrations on the viability of bone marrow primary stromal cells A) Clozapine B) N-desmethylozapine C) Clozapine N-oxide. Data are expressed as a percentage of untreated control cells (solid line) and represent the mean \pm SEM of quadruplicate incubations from three separate experiments. c1 = 500 ng mL⁻¹ camptothecin c2 = 1000 ng mL⁻¹ camptothecin * $p < 0.01$, Statistical differences compared with control values

Present data indicates that the metabolite would seem unlikely to cause agranulocytosis. This contrasts with reports of complete abolition of myeloid and erythroid

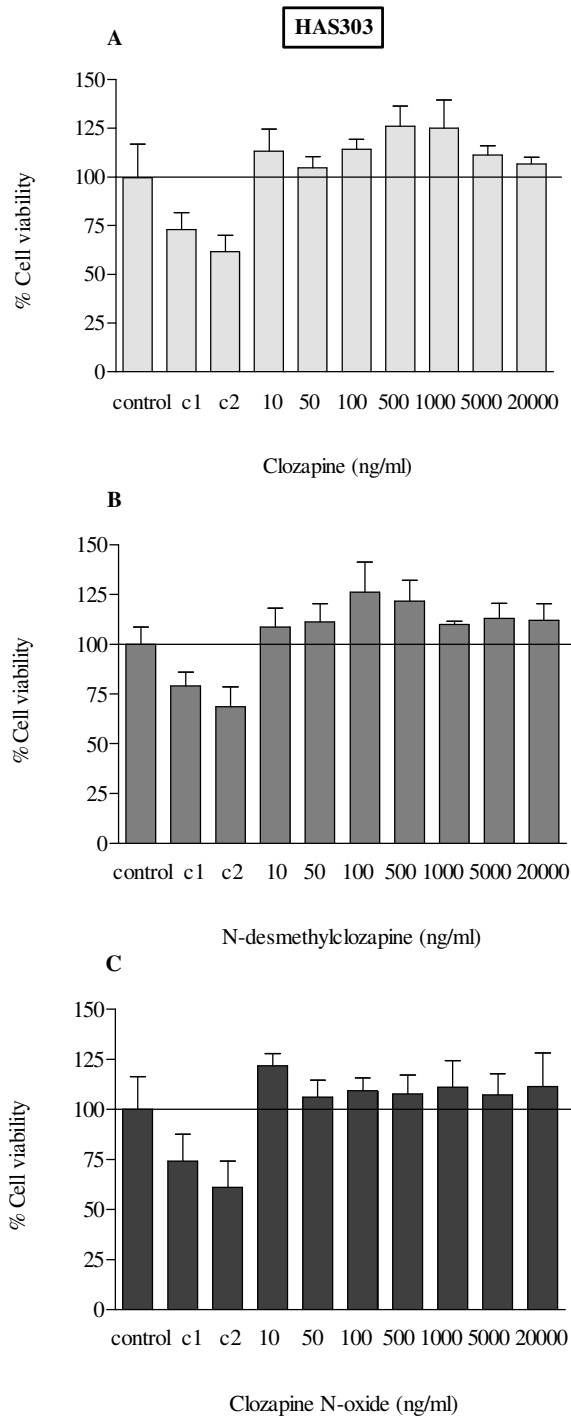


Fig. 2: The effect of clozapine and its metabolites over a range of concentrations on the viability of HAS303 cells A) Clozapine B) N-desmethylclozapine C) Clozapine N-oxide. Data are expressed as a percentage of untreated control cells (solid line) and represent the mean \pm SEM of quadruplicate incubations from three separate experiments. c1 = 500 ng mL⁻¹ camptothecin c2 = 1000 ng mL⁻¹ camptothecin

progenitor cell growth at more than 20 μ g mL⁻¹ N-desmethylclozapine^[4]. Based on such outcomes the possibility that patients with agranulocytosis are hypersensitive to N-desmethylclozapine or have a

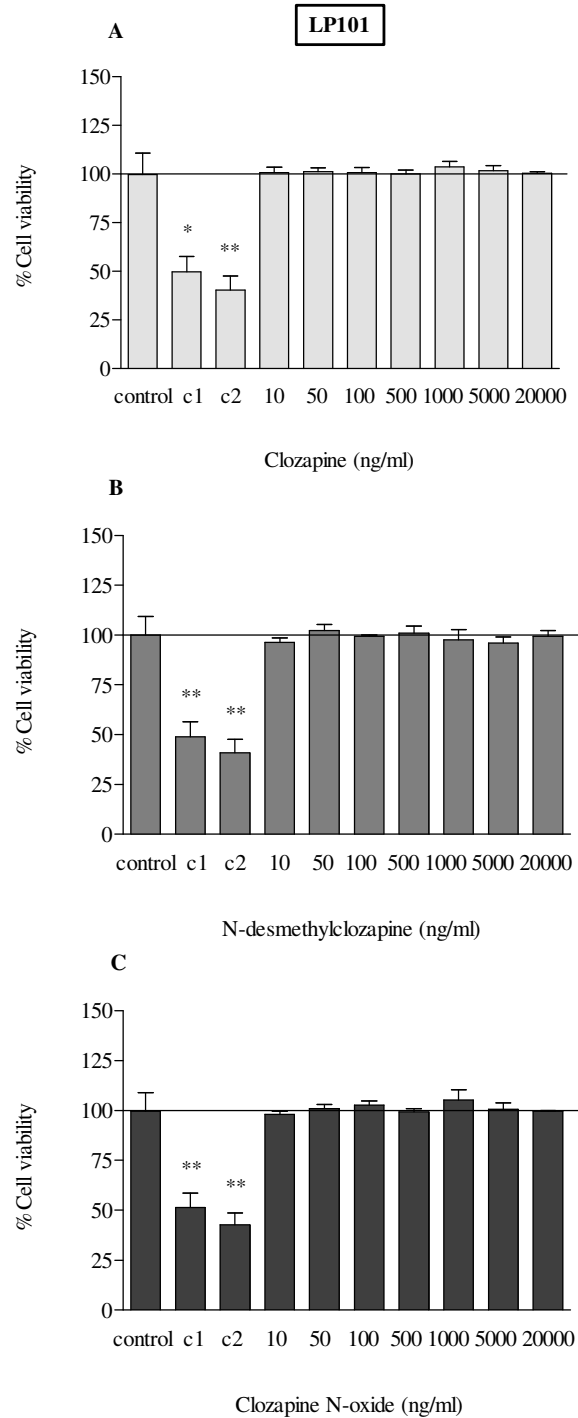


Fig. 3: The effect of clozapine and its metabolites over a range of concentrations on the viability of LP101 cells A) Clozapine B) N-desmethylclozapine C) Clozapine N-oxide. Data are expressed as a percentage of untreated control cells (solid line) and represent the mean \pm SEM of quadruplicate incubations from three separate experiments. c1=500ng mL⁻¹ camptothecin c2 = 1000 ng mL⁻¹ camptothecin * p < 0.05 ** p < 0.01, Statistical differences compared with control values

defect in clozapine metabolism resulting in increased levels of the metabolite has been raised^[3]. However bone marrow mononuclear cells from two patients who

developed agranulocytosis and were studied at onset, were not more sensitive to N-desmethylclozapine than those from normal donors. As well, elevated plasma levels of N-desmethylclozapine were not found^[3].

In this study, N-desmethylclozapine-induced reduction in viability of a specific cell type other than macrophage or endothelial cells represented by LP101 and HAS303 cells respectively, within the heterogeneous stromal cell population cannot be excluded. The dose-independent nature of the response may then be explained by an uneven distribution of the target cell within the primary cultured samples. However in the absence of differential cell counts this remains speculative. Cell specific stromal dysfunction has been indicated with benzene^[18] and ceftazidime^[19] with the macrophage targeted by the former agent and significantly lower numbers of endothelial cells and adipocytes compared to untreated cultures with the latter, in the absence of marked reduction in total stromal cell number.

Clozapine N-oxide did not affect the viability of stromal cells to any significant extent. Given that the circulatory concentration of clozapine N-oxide is typically only 10 to 35% that of clozapine^[20], the concentrations used were in excess of that of parent drug. Furthermore, the metabolite can potentially be metabolized back to clozapine^[21] which may in part explain the variability in profiles of primary stromal and HAS303 cell viability across clozapine N-oxide concentrations. Studies with the neutrophil have also shown no concentration dependent induction of cell death in the presence of up to 300 μ M clozapine N-oxide^[22].

It is noteworthy that LP101 cells in the presence of clozapine and its metabolites showed limited fluctuations in viability across drug concentrations, compared with that seen with primary stromal and HAS303 cells. The data may tentatively support the conjecture that LP101 cells are less sensitive to these compounds.

Stromal cells showed differing sensitivity to camptothecin which targets human DNA topoisomerase I^[23,24]. The viability or metabolic activity of primary stromal cells was significantly reduced upon exposure to the drug in the absence of cell lysis (data not shown). Thus it would appear that these cells although having intact plasma membranes were in a state of cell cycle arrest or delay, likely irreversibly damaged and committed to die. The possibility of partial or full recovery of cell subsets within the heterogeneous sample remains. By contrast, HAS303 cells in the presence of camptothecin showed no significant reduction in viability, whilst LP101 metabolism was impaired. Such differences in cellular response suggested varied determinants of camptothecin cytotoxicity. For example, levels of expression of topoisomerase I protein, the site of lesion formation

based on local DNA sequence and chromatin structure, the ability of cells to process or repair sites of damage, or complex functions of the cell cycle and apoptosis control systems^[23], could confer diverse cytotoxic potential as observed.

In conclusion, current evidence suggests that clozapine and its stable metabolites are not directly toxic to bone marrow stromal cells. Whilst the viability of stromal cells was unaffected, reduced capability of the stroma to support myelopoiesis via a network of different regulatory systems cannot be discounted. Similarly, it remains plausible that patients who develop clozapine-induced agranulocytosis may have stroma that are inherently susceptible to the effects of the drug due to genetic predisposition. Notwithstanding this, these data indicate that it is unlikely that the parent drug exerts direct cytotoxicity under clinical conditions. Therefore, given that clozapine can be bioactivated to a reactive nitrenium ion by liver microsomes, activated neutrophils and myeloid precursor cells with potential to be covalently modified by this metabolite^[25], consideration should be given to this chemical species as a factor that might mediate stromal cell toxicity.

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