



## In vitro effect of clozapine on hemopoietic progenitor cells

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### Abstract

**Background and Objective.** Clozapine is a diabenzo-diazepine derivative characterized by a high therapeutic index in schizophrenic patients resistant to traditional neuroleptic drugs, because of the rarity of any extrapyramidal side effects, and its particular hematologic toxicity. According to the international literature, clozapine-induced neutropenia occurs mainly during the first 4-6 months of treatment, and its incidence decreases considerably over time. This neutropenic effect is not dose-dependent and normally clears up after drug discontinuation, although it may evolve into agranulocytosis. The aim of this study is to evaluate the *in vitro* toxic effect of clozapine and N-desmethylclozapine on both committed and immature human hematopoietic progenitor cells.

**Design and Methods.** Cytotoxic assays were performed *in vitro* on normal human bone marrow samples treated with clozapine or with its metabolite N-desmethylclozapine. The clonogenic potential after treatment with both compounds was assessed on low density mononuclear cells (LD-MNC), purified CD34<sup>+</sup> cells, cytokine driven liquid cultures and long term culture initiating cell (LTC-IC).

**Results.** Clozapine and N-desmethylclozapine had a dose-dependent inhibitory effect on *in vitro* growth of CFU-GM and BFU-E from normal bone marrow. The two drugs had toxic effects on purified CD34<sup>+</sup> progenitor cells but no significant effect on LTC-IC.

**Interpretations and Conclusions.** Our data indicate a cytotoxic effect, which is more pronounced with N-desmethylclozapine and at high doses, on the committed progenitor cell compartment but not on primitive hematopoietic cells. Furthermore, our data show that clozapine and N-desmethylclozapine have a direct effect on treated cells and do not induce apoptotic death.

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Key words: clozapine, agranulocytosis, bone marrow cultures, CD34<sup>+</sup> cells, LTC-IC

**N**eutropenia and agranulocytosis in patients treated with clozapine for refractory schizophrenia are well-known adverse events that

were initially reported in the late 1960s,<sup>1,2</sup> and have been more recently documented in larger clinical trials and various national registries.<sup>3-8</sup> Although all these studies make it possible to obtain an accurate estimate of the cumulative incidence (0.8% over 15 months), the period of occurrence (first 6-18 weeks of treatment) and the predisposing risk factors (older age, female gender, ethnic groups), the mechanisms underlying the development of clozapine-associated blood dyscrasia have not yet been identified. It has been suggested that bone marrow damage may be caused by immunologic<sup>9</sup> or toxic processes that are mediated by metabolites rather than by clozapine itself.<sup>5,8,10,11</sup> Furthermore, on the basis of results obtained from human bone marrow cultures incubated with increasing concentrations of clozapine or its metabolites, Gerson and Meltzer<sup>10</sup> have postulated that the drug target is likely to be the early hemopoietic stem cell compartment. A similar analysis of cytotoxicity was made in the present study but, in addition, the effects of clozapine and its major metabolite, N-desmethylclozapine, on clonogenic potential were also assessed on purified bone marrow CD34<sup>+</sup> progenitor cells, cytokine-driven liquid long term cultures and early hematopoietic progenitor long term culture initiating cells (LTC-IC). Our results show that the drug and its metabolite have a direct effect on committed but not on primitive hematopoietic cells.

### Materials and Methods

#### Sample preparation and cell lines

Five bone marrow samples from healthy donors were studied. The toxicity induced by clozapine and its metabolite N-desmethylclozapine (kindly provided by Sandoz, Basel, Switzerland) was evaluated both in low-density mononuclear cells (LD-MNC) and in selected CD34<sup>+</sup> progenitor cells.

We also studied the toxicity and the potential apoptotic effect mediated by clozapine or its metabolite on the following human cell lines: KG1a (ECACC #91030101),<sup>12</sup> which is a CD34<sup>+</sup> myeloid leukemia cell line, K562 (ICLC HTL 94001),<sup>13</sup> which is a chronic myeloid leukemia cell line, HL60 (ICLC HTL 95010),<sup>14</sup> which is a promyelocytic leukemia cell line and RW Leu4 (kindly provided by Dr. M.A. San-

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tucci, Bologna, Italy),<sup>15,16</sup> which is a chronic myeloid leukemia cell line. All the cell lines were cultured with RPMI 1640 medium supplemented with 10% of FCS at 37°C and 5% CO<sub>2</sub> in fully humidified atmosphere.

The bone marrow specimens were layered on a Ficoll-Paque gradient (specific gravity 1.077 g/mL; Nycomed Pharma AS, Oslo, Norway) in order to separate the LD-MNC; the samples were then washed twice in Hanks' balanced salt solution (HBSS). The adherent cells were removed by means of two 30-minute pre-adherence cycles at 37°C in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum (FBS) in 25 cm<sup>2</sup> culture flasks. The non-adherent fraction was then resuspended in phosphate buffer saline (PBS) containing 0.5% bovine serum albumin (BSA) and 5 mM EDTA.

### **CD34<sup>+</sup> cell purification**

To isolate the CD34<sup>+</sup> cells, the LD-MNC were incubated for 15 min at 4°C with the QBEND10 monoclonal antibody directed against the CD34 antigen.<sup>17</sup> The cells were then washed and incubated for 15 min at 4°C with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) against QBEND10. For the flow cytometry analysis, CD34-phycoerythrin (CD34-PE) conjugated antibody (HPCA-2, Becton Dickinson, Mountain View, CA, USA) was added to the cells for 15 min at 4°C. The samples were then filtered through a 50 mm nylon mesh in order to remove clumps and placed on a column in a miniMACS cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Separated by a high gradient magnetic field, the labeled cells were eluted from the column after being removed from the magnet. The positive fraction was then placed on a new column and the magnetic separation step repeated. At the end of the separation, the cells were counted and assessed for viability by means of trypan blue dye exclusion; their purity was determined by means of flow cytometry analysis. All of these steps were performed under aseptic conditions.

The mean percentage of purity of the CD34<sup>+</sup> cells after immunomagnetic separation was 90.6%, the mean yield was 52% and the mean enrichment was 45.8-fold.<sup>18</sup>

### **Clonogenic assays**

The effect of clozapine and N-desmethylclozapine on the clonogenic potential of human bone marrow LD-MNC and CD34<sup>+</sup> cells was evaluated using two different treatment schedules:

a) the cells were cultured in the presence of clozapine or N-desmethylclozapine at concentrations ranging from 0 to 40 µg/mL; in these experiments the substances remained in contact with the cells throughout the culture period;

b) the cells were pre-treated with clozapine or N-desmethylclozapine at concentrations ranging from 0 to 40 µg/mL for 24 hours, and subsequently washed twice with the medium and seeded in a semisolid

assay. Both clozapine and N-desmethylclozapine were dissolved in ethanol and the same quantity of this solvent was also used in the control samples.

In both models, the clonogenic assays (CFU-GM and BFU-E) were carried out by plating 1×10<sup>5</sup> LD-MNC or 5×10<sup>3</sup> CD34<sup>+</sup> cells in a methylcellulose culture medium (Iscove's Methylcellulose, StemCell Technologies Inc., Vancouver, Canada) containing four parts of 2.2% methylcellulose, three parts of pre-tested FCS, one part of 10% pretested BSA, 0.1 part of 10<sup>-2</sup> M mercaptoethanol, one part of 3 U/mL human urinary erythropoietin and 15% of the supernatant of the 5637 bladder carcinoma cell line. Triplicate dishes were incubated at 37°C in a 5% CO<sub>2</sub> fully humidified atmosphere. Aggregates of > 40 and < 40 cells were respectively scored as colonies and clusters after 14 days of culture.

### **Cytokine-driven long-term liquid cultures**

The effects of clozapine and N-desmethylclozapine on cytokine-driven LD-MNC liquid cultures<sup>19</sup> were also evaluated. The cells were seeded at a concentration of 6.5×10<sup>5</sup>/mL in a 25 cm<sup>2</sup> culture flask, and cultivated for one week at 37°C in 100% humidified 5% CO<sub>2</sub> in air in an IMDM stroma-free liquid culture with 10% FBS; every 48 hours, r-hu SCF (10 ng/mL), kindly provided by Amgen (Thousand Oaks, CA, USA), r-hu IL-3 (100 ng/mL) (Sandoz, Basel, Switzerland), r-hu IL-6 (10 ng/mL) (Pharmacia-Farmitalia, Milan, Italy), r-hu GM-CSF (100 ng/mL) (Sandoz, Basel, Switzerland) were added to the cultures, as well as clozapine or N-desmethylclozapine. After seven days of culture, the cells were counted, assessed for viability and seeded in methylcellulose for clonogenic evaluation.

### **Long term culture initiating cells (LTC-IC)**

The effect of clozapine and N-desmethylclozapine on more primitive stem cells was studied in five bone marrow samples by means of a long-term culture-initiating cell assay (LTC-IC). In particular, the bone marrow LD-MNC from five donors were preincubated for 24 hours with different concentrations of clozapine (ranging from 0 to 100 µg/mL) or N-desmethylclozapine (ranging from 0 to 20 µg/mL). The untreated bone marrow LD-MNC from five normal subjects served as normal controls. The frequency of LTC-IC was evaluated using a limiting dilution assay (LDA).<sup>20,21</sup> The pre-treated LD-MNC were seeded on the M2-10B4 murine cell line in 96-well microtiter plates (1×10<sup>4</sup> M2-10B4, 80 Gy irradiated cells per well). The dilution steps (16 replicates for each dilution) were 3000, 6000, 9000, 12000 and 15000 cells/well in 200 µL/well of LTBM medium (MyeloCult™ H5100, StemCells, Vancouver, Canada). After five weeks of culture at 33°C with 5% CO<sub>2</sub>, the adherent and non-adherent cells were harvested by removing the medium and adding 50 µL of trypsin per well for 10 minutes at 37°C. The trypsinized cells

were washed from the wells using 50  $\mu\text{L}$  of FBS, and pooled with the non-adherent cells. In order to evaluate colony formation, the cells from each well were plated in 24-well plates in 250  $\mu\text{L}$  of colony assay semisolid medium (MethoCult™ H4230, StemCells, Vancouver, Canada) as described for clonogenic assays. The incidence of negative wells was then determined, and the frequency of LTC-IC evaluated by means of Poisson statistics.

### Cytotoxicity assay

The WST-1 cell proliferation assay (Boehringer Mannheim GmbH, Germany) was used to evaluate the toxic effect of clozapine and N-desmethylclozapine on cell lines and bone marrow samples. The WST-1 assay was performed as recommended by the kit protocol. The results were evaluated by means of the 1420 VICTOR multilabel counter (EG&G Wallac, Finland).

### Cell cycle analysis and apoptotic test

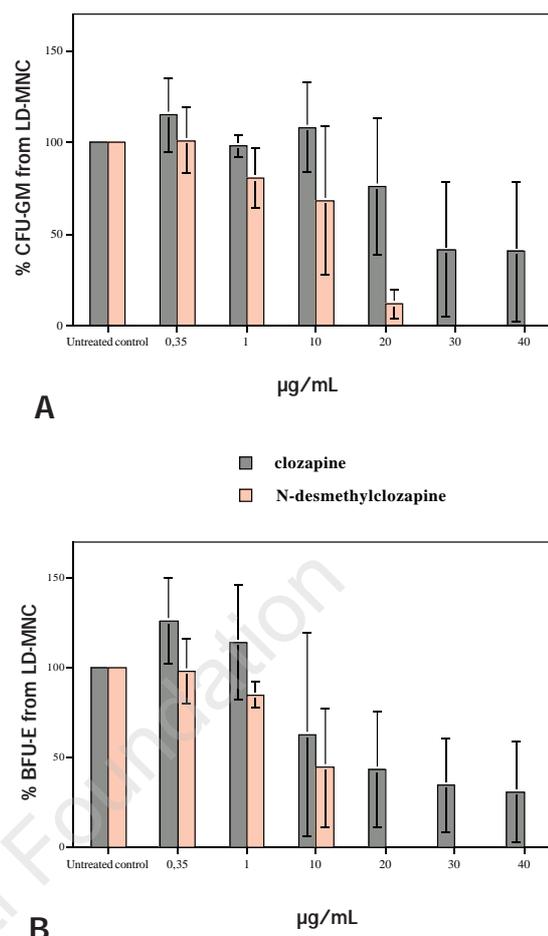
In order to evaluate the possible role of clozapine or N-desmethylclozapine in bone marrow cell apoptosis, the DNA content of  $4 \times 10^5$  cells of each of the above mentioned cell lines or normal bone marrow LD-MNC were analyzed. The cells were incubated for 24 hours at 37°C in the presence of clozapine (10  $\mu\text{g}/\text{mL}$  or 40  $\mu\text{g}/\text{mL}$ ) or N-desmethylclozapine (1  $\mu\text{g}/\text{mL}$  or 10  $\mu\text{g}/\text{mL}$ ), and then fixed in 70% ethanol, permeabilized with 0.1% saponin, treated with 0.5 mg/mL RNase (Sigma, St. Louis, MO, USA), and incubated at 37°C for 30 minutes before staining with 20  $\mu\text{g}/\text{mL}$  propidium iodide (Sigma) for 30 minutes at 4°C.<sup>18</sup> The cell cycle analysis was carried out by means of flow cytometry using a FACSVantage (Becton Dickinson, San José, CA, USA).

The apoptotic cells were identified by the method of TdT-mediated dUTP-biotin nick end labeling (TUNEL) (Boehringer Mannheim GmbH, Germany). The cells were incubated with clozapine and N-desmethylclozapine at different concentrations for 24 hours. The cells were then fixed with 70% ethanol and labeled and analyzed by means of FACSVantage (Becton Dickinson).

## Results

### Low density mononuclear cells (LD-MNC)

It was found that the toxicity of both clozapine and N-desmethylclozapine on human normal bone marrow LD-MNC was dose-dependent. In particular, there was a significant reduction in CFU-GM output in the samples treated with doses of clozapine of between 10 and 20  $\mu\text{g}/\text{mL}$  and doses of N-desmethylclozapine ranging from 0.35 to 1  $\mu\text{g}/\text{mL}$  (Figure 1). Although the addition of N-desmethylclozapine at concentrations of more than 20  $\mu\text{g}/\text{mL}$  completely abolished the growth of CFU-GM, some CFU-GM colonies still persisted in the presence of very high



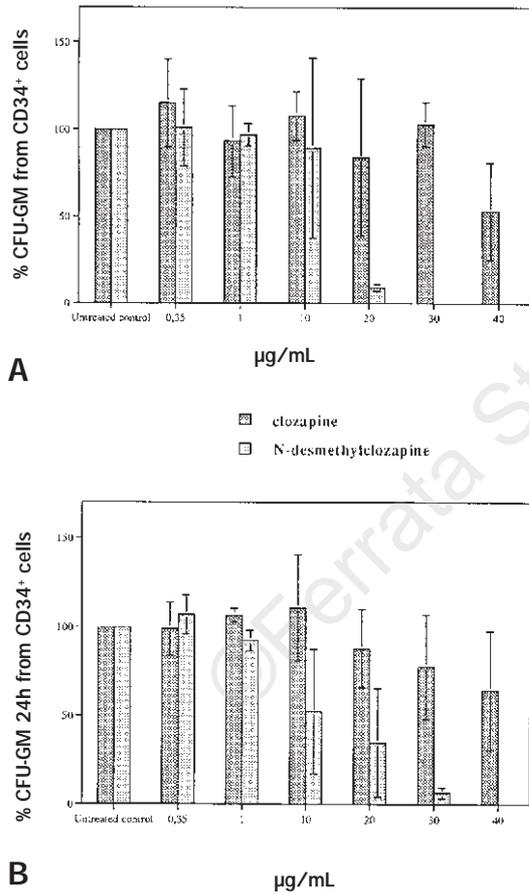
**Figure 1.** Effect of different concentrations of clozapine and N-desmethylclozapine on CFU-GM (a) and BFU-E (b) growth of bone marrow LD-MNC from five normal subjects. The data are expressed as mean percentages ( $\pm$ SD) of colony output, normalized to the untreated control.

doses of clozapine. When we calculated the drug dose capable of inhibiting the growth of half of the colonies in the clonogenic assays ( $\text{IC}_{50}$ ), we found that the mean value was 32  $\mu\text{g}/\text{mL}$  for clozapine and 14  $\mu\text{g}/\text{mL}$  for N-desmethylclozapine when the drugs were added directly to the methylcellulose; similar data were obtained when the cells were pretreated with the drugs for 24 hours, and then washed before culture (Table 1).

The effect on BFU-E growth was even more pronounced (Figure 1), with clozapine and its metabolite reducing BFU-E output at doses of between 1 and 10  $\mu\text{g}/\text{mL}$ , even though some erythroid colonies were still detectable at very high drug concentrations: no BFU-E growth was observed at concentrations of more than 20  $\mu\text{g}/\text{mL}$ . The mean  $\text{IC}_{50}$  was 24  $\mu\text{g}/\text{mL}$  for clozapine and 12  $\mu\text{g}/\text{mL}$  for N-desmethylclozapine when added directly to methylcellulose, and

**Table 1.** Expression of cell toxicity in terms of the clozapine or N-desmethylclozapine concentrations producing 50% inhibition of colony growth ( $IC_{50}$ ): A) CFU-GM  $IC_{50}$  values of the LD-MNC, the LD-MNC pretreated for 24 hours and then washed and plated, the CD34<sup>+</sup> cells, and finally the CD34<sup>+</sup> cells pretreated for 24 hours and then washed and plated. B) BFU-E  $IC_{50}$  values of the LD-MNC, the LD-MNC pretreated for 24 hours and then washed and plated, the CD34<sup>+</sup> cells and finally the CD34<sup>+</sup> cells, pretreated for 24 hours and then washed and plated.

A - CFU-GM $IC_{50}$				
	LD-MNC	LD-MNC24h	CD34	CD34 24 h
Clozapine ( $\mu\text{g/mL}$ )	32	36	59	57
N-desmethylclozapine ( $\mu\text{g/mL}$ )	14	13	14	16
B - BFU-E $IC_{50}$				
	LD-MNC	LD-MNC24h	CD34	CD34 24 h
Clozapine ( $\mu\text{g/mL}$ )	24	50	24	38
N-desmethylclozapine ( $\mu\text{g/mL}$ )	12	15	11	16

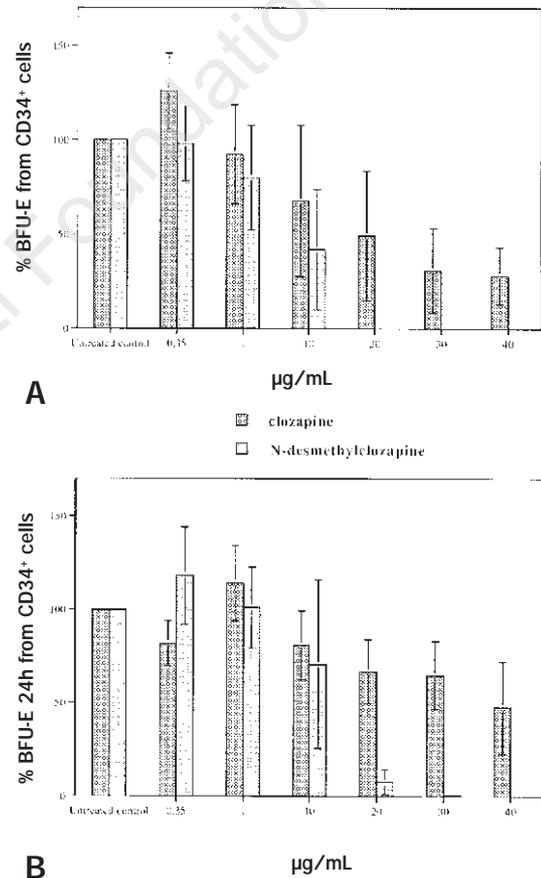


**Figure 2.** Effect of different concentrations of clozapine and N-desmethylclozapine on CFU-GM growth of CD34<sup>+</sup> separated cells from five normal subjects. a): the drugs were added directly to the methylcellulose; b): the cells were first incubated with the two drugs for 24 hours in liquid culture, and subsequently washed and plated in methylcellulose. Data are expressed as mean percentages ( $\pm$ SD) of colony output, normalized to the untreated control.

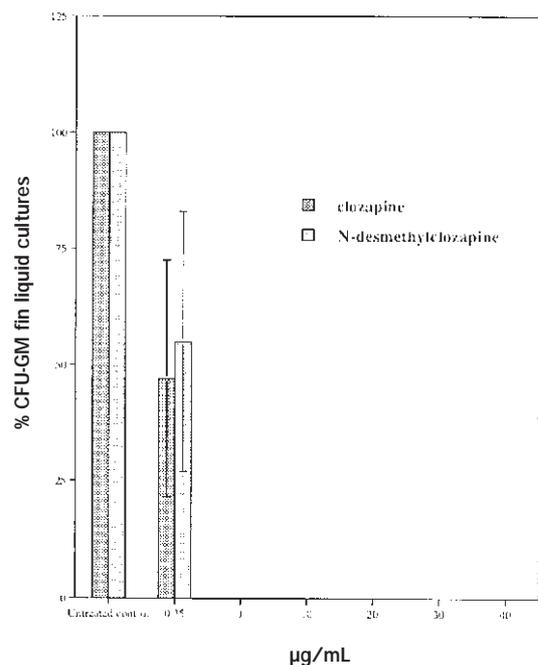
respectively 50  $\mu\text{g/mL}$  and 15  $\mu\text{g/mL}$  when the cells were pretreated with the drugs for 24 hours, and then washed before culture.

**CD34<sup>+</sup> purified cells**

CD34<sup>+</sup> cells showed decreased CFU-GM output after incubation with only very high concentrations of clozapine (>30  $\mu\text{g/mL}$ ) (mean  $IC_{50}$  59  $\mu\text{g/mL}$ ) (Figure 2). A decrease in CFU-GM was also observed with the higher doses of N-desmethylclozapine (>10  $\mu\text{g/mL}$ ) (mean  $IC_{50}$  14  $\mu\text{g/mL}$ ). The effect of both drugs on the BFU-E output of CD34<sup>+</sup> cells was similar to that observed in LD-MNC (mean  $IC_{50}$  24  $\mu\text{g/mL}$  for clozapine and 11  $\mu\text{g/mL}$  for N-desmethylclozapine) (Figure 3).



**Figure 3.** Effect of different concentrations of clozapine and N-desmethylclozapine on BFU-E growth of CD34<sup>+</sup> separated cells from five normal subjects. a): the drugs were added directly to the methylcellulose. b): the cells were first incubated with the two drugs for 24 hours in liquid culture, and subsequently washed and plated in methylcellulose. Data are expressed as mean percentages ( $\pm$ SD) of colony output, normalized to the untreated control.



**Figure 4.** Effect of different concentrations of clozapine and N-desmethylclozapine on CFU-GM growth after one week of liquid culture with SCF, IL-3, IL-6 and GM-CSF. Data are expressed as mean percentages ( $\pm$ SD) of colony output, normalized to the untreated control.

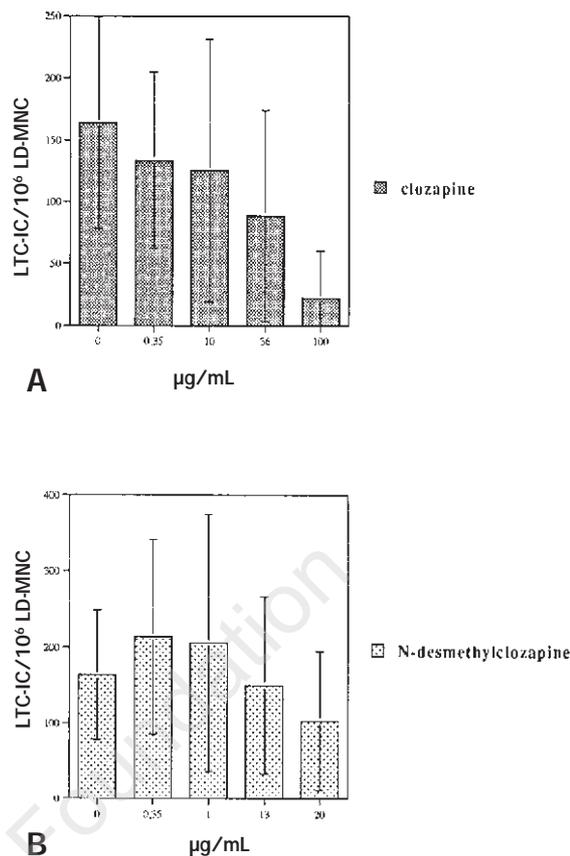
No significant differences in CFU-GM output were found in the samples that were pretreated with the drugs for 24 hours. However, the treatment of CD34<sup>+</sup> cells seemed to be less toxic than for the LD-MNC (see mean IC<sub>50</sub> Table 1).

#### Cytokine-driven liquid cultures

Bone marrow LD-MNC grown in liquid cultures with the addition of cytokines also showed a decrease in clonogenic output with the addition of escalating doses of the two drugs (Figure 4). In particular, there was a 50% reduction in CFU-GM growth at a dose of 0.35 µg/mL, and a complete absence of growth after seven days of culture at concentrations of more than 1 µg/mL.

#### Long term culture initiating cells (LTC-IC)

LTC-IC assays were performed in five cases, with the bone marrow LD-MNC being preincubated for 24 hours with different doses of clozapine or N-desmethylclozapine and then seeded in limiting dilutions on pre-irradiated stromas. The results suggested that neither clozapine nor N-desmethylclozapine induce a significant decrease in the early stem cell compartment even at high doses; only when the cells were treated with a dose of 100 µg/mL of clozapine could there be a significant reduction of the fre-



**Figure 5.** Effect of different concentrations of clozapine (a) and N-desmethylclozapine (b) on LTC-IC growth of bone marrow LD-MNC from five normal subjects. Data are expressed as mean values ( $\pm$ SD).

quency of the LTC-IC (Figure 5). These data seemed to indicate that neither clozapine nor its metabolite affected more immature stem cells.

#### Cytotoxic assay and DNA content analysis

We demonstrated that with the WST-1 cytotoxicity assay on the cell lines and normal bone marrow cells showed IC<sub>50</sub> similar to those obtained with the CFU-GM assays. In particular when the samples were treated with clozapine we obtained the following IC<sub>50</sub> values: HL60 48 µg/mL; RW Leu4 38 µg/mL; K562 30 µg/mL; KG1a 45 µg/mL and normal bone marrow 35 µg/mL; when the cells were treated with N-desmethylclozapine we obtained the following IC<sub>50</sub> values: HL60 23 µg/mL; RW Leu4 19 µg/mL; K562 13 µg/mL; KG1a 20 µg/mL and normal bone marrow 15 µg/mL.

In order to investigate whether clozapine and its metabolite N-desmethylclozapine can induce the

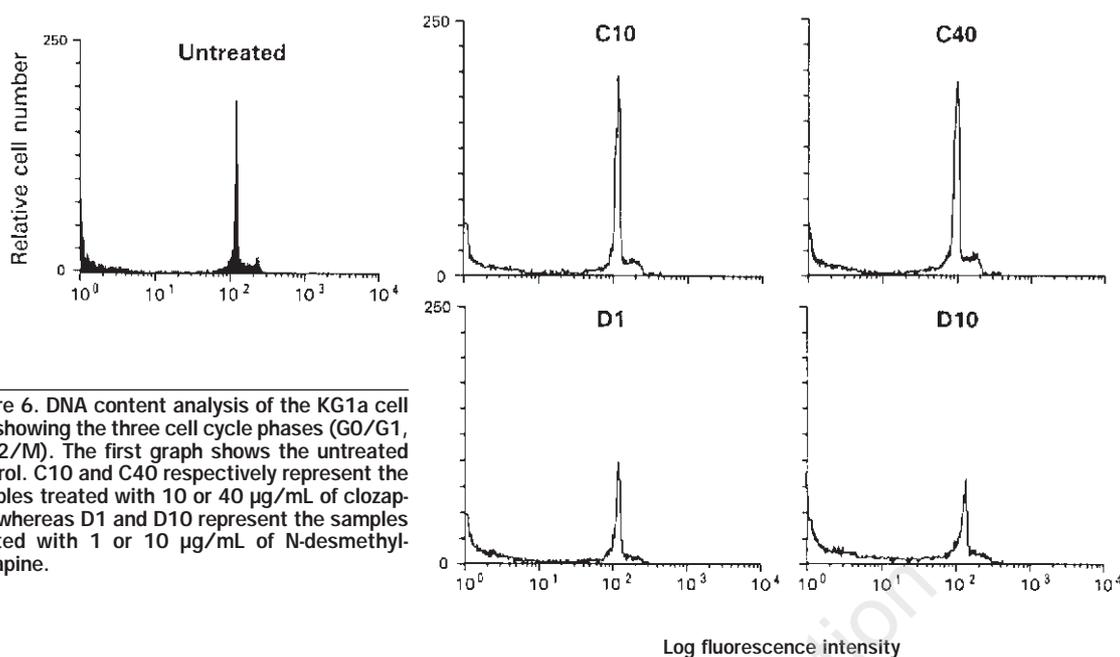


Figure 6. DNA content analysis of the KG1a cell line showing the three cell cycle phases (G0/G1, S, G2/M). The first graph shows the untreated control. C10 and C40 respectively represent the samples treated with 10 or 40  $\mu\text{g}/\text{mL}$  of clozapine, whereas D1 and D10 represent the samples treated with 1 or 10  $\mu\text{g}/\text{mL}$  of N-desmethylclozapine.

apoptotic cell death of hemopoietic cells, KG1a (Figure 6), HL60, RW Leu4, K562 and normal bone marrow cells (data not shown) were incubated with the two drugs for 24 hours, stained with propidium iodide (as described above) and analyzed by means of flow cytometry. As shown in Figure 6, no difference was found between the cell cycle histograms of the control cells and the specimens treated with clozapine 10  $\mu\text{g}/\text{mL}$  or 40  $\mu\text{g}/\text{mL}$ . The cells treated with N-desmethylclozapine 1  $\mu\text{g}/\text{mL}$  or 10  $\mu\text{g}/\text{mL}$  showed a reduction in all the compartments of the cell cycle (G0/G1, S and G2), but no apoptotic peaks.

Furthermore these data were confirmed by means of the more refined technique for detection of apoptosis (TUNEL). In fact we obtained respectively the following percentage of apoptotic cells in samples treated with 20  $\mu\text{g}/\text{mL}$  clozapine: HL60 4.6%; RW Leu4 1.8%; K562 4.2%; KG1a 5% and normal bone marrow 1.6%; the samples treated with 10  $\mu\text{g}/\text{mL}$  N-desmethylclozapine showed the following percentage of apoptosis: HL60 3.5%; RW Leu4 5%; K562 6%; KG1a 5.5% and normal bone marrow 5.8%.

These results seem to indicate that, at least after 24 hours of treatment, clozapine and N-desmethylclozapine induce cytotoxic rather than apoptotic cell death.

## Discussion

Over the last decade, the introduction of specific hematologic monitoring<sup>3-7</sup> and the availability of growth factors in clinical practice<sup>23,24</sup> have significantly reduced mortality due to clozapine-induced agranulocytosis. However, the propensity of this highly effective<sup>25,26</sup> antipsychotic drug to affect granulocyte

production<sup>5-8</sup> still limits its indications for use. Elucidation of the mechanism underlying its hemopoietic toxicity may eliminate these restrictions, thus making it possible to administer the drug to patients with schizophrenia at first diagnosis or possibly to patients with other neurologic disorders.

A number of clinical observations<sup>8,10,27</sup> support the hypothesis that clozapine and/or its metabolites act centrally on bone marrow hemopoietic precursors: first, the agranulocytosis has a delayed onset, is not dose-dependent, and has a rapid course even if the drug is discontinued; secondly, bone marrow examinations at the time of diagnosis reveal an absence of myeloid precursors. Furthermore, *in vitro* culture experiments<sup>9,28</sup> indicate that clozapine has toxic effects on bone marrow progenitor cells, and that its metabolite N-desmethylclozapine is much more toxic than clozapine itself.<sup>10,29</sup>

The results of the present study confirm the fact that both compounds impair the clonogenic growth of both myeloid and erythroid bone marrow progenitor cells.

A sharp reduction in colony growth was also evident at N-desmethylclozapine concentrations of between 0.35 and 1  $\mu\text{g}/\text{mL}$ , which may correspond to the blood levels generally observed in treated patients:<sup>26</sup> although the range of plasma or serum concentrations of clozapine metabolites is quite variable, the level of N-desmethylclozapine is 90-93% that of clozapine.<sup>31</sup>

Although a common hemopoietic precursor seemed to be involved, toxicity to the erythroid-committed progenitor cells was slightly greater despite the

fact that in clozapine-treated patients neutropenia is a more clinically frequent complication than severe anemia:<sup>5</sup> this may be easily explained by the short half-life of terminally differentiated myeloid cells, and the rapid withdrawal of the drug as soon as the drop in the number of leukocytes is observed. A similar level of toxicity was found regardless of whether the bone marrow cells were exposed to the drug during the culture time or pre-incubated with the drug for 24 hours before being cultured: this seems to indicate that the toxic effects operate immediately and do not depend on prolonged exposure.

It was also found that clozapine had toxic effects on purified CD34<sup>+</sup> progenitor cells: these data clearly indicate that the drug has a direct effect on progenitor hematopoietic cells and exclude the possibility of indirect damage to accessory cells (macrophages, fibroblasts or lymphoid cells). However, the myeloid progenitor cell IC<sub>50</sub> of both clozapine and N-desmethylclozapine were much higher in CD34<sup>+</sup> purified cells than in LD-MNC: these data suggest a predominant effect on more differentiated rather than primitive hemopoietic cells. In fact, as the LD-MNC consist of a small percentage of primitive cells, a majority of differentiated hematopoietic cells and some accessory cells, the drugs could be toxic either to committed hematopoietic cells or to accessory cells. This hypothesis was further confirmed by our data on bone marrow liquid cultures and LTC-IC.

Recent studies<sup>19,32</sup> have attempted to expand hematopoietic cells in liquid cultures by replacing the normal hematopoietic microenvironment with an appropriate combination of growth factors. Using this system, a large number of cells and colony forming unit cells (CFU-C) have been generated after up to eight weeks of culture. However, the numbers of both the CD34<sup>+</sup> cells and the very early progenitor cells (LTC-IC) rapidly decline during culture, thus suggesting the preferential expansion of committed progenitor cells. When LD-MNC were grown in the presence of the two drugs under cytokine-driven stroma-free liquid culture conditions, a rapid decrease in clonogenic output was observed even at relatively low drug concentrations; these data seem to suggest that committed progenitor cells (particularly those forced to proliferate and expand by means of hemopoietic growth factor) are especially sensitive to clozapine and its metabolite. Furthermore, the present data seem to indicate that these drugs have no significant effect on the early stem cell compartment, as is demonstrated by the results of the LTC-IC assay on LD-MNC pretreated with clozapine and N-desmethylclozapine.

A preferential effect on committed rather than progenitor cells may explain the clinical presentation of the disease: agranulocytosis with milder involvement of the erythroid lineage and rapid reversibility of the picture (14-24 days) after drug discontinuation with no reported cases of severe aplastic anemia.<sup>5,10,27</sup>

Nevertheless, some questions still need to be answered, including the possibility of the existence of a genetic defect in one of the enzymes involved in clozapine metabolism,<sup>8</sup> and the controversial hypothesis that an immune mechanism may underlie clozapine-induced agranulocytosis.<sup>9</sup>

In an attempt to provide further insights into the mechanism behind the toxic effect of clozapine on hematopoietic cells, we explored the possibility that the drug may induce apoptotic rather than cytotoxic cell death. However, flow cytometry failed to reveal the presence of apoptotic peaks or the selective inhibition of the different phases of the cell cycle in the cell lines or normal bone marrow cells. These data were confirmed by the TUNEL technique.

In conclusion, the mechanism of clozapine-induced agranulocytosis remains to be clarified. The results reported in this study support the concept that a stable or reactive metabolite might affect bone marrow hematopoietic committed precursors and probably not early stem cells: if this hypothesis is clearly proved, the ultimate aim of pharmacological research should be the synthesis of clozapine analog that have a different metabolism, able to avoid interfering with the hematopoietic system.

#### **Contributions and Acknowledgments**

*GLD formulated the design of the study, provided the drugs and contributed to the writing of the paper. FS performed all the in vitro assays; she also contributed to the formulation of the design, analysis and writing of the paper. GL performed the flow cytometric (FACS) analysis and the cytotoxic assays. NQ did the long term culture assays and helped in writing and correcting the paper. DS contributed to the formulation of the study, the analysis of the results and the writing of the paper.*

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#### **Disclosures**

*Conflict of interest: none.*

*Redundant publications: no substantial overlapping with previous papers.*

#### **Manuscript processing**

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