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Involvement of the histamine H₄ receptor in clozapine-induced hematopoietic toxicity: vulnerability under granulocytic differentiation of HL-60 cells

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1 **Abstract**

2 Clozapine is an effective antipsychotic for treatment-resistant schizophrenia, but can cause fatal
3 hematopoietic toxicity as agranulocytosis. To elucidate the mechanism of hematopoietic toxicity induced
4 by clozapine, we developed an *in vitro* assay system using HL-60 cells, and investigated the effect on
5 hematopoiesis. HL-60 cells were differentiated by *all-trans* retinoic acid (ATRA) into three states
6 according to the following hematopoietic process: undifferentiated HL-60 cells, those undergoing
7 granulocytic ATRA-differentiation, and ATRA-differentiated granulocytic cells. Hematopoietic toxicity
8 was evaluated by analyzing cell survival, cell proliferation, granulocytic differentiation, apoptosis, and
9 necrosis. In undifferentiated HL-60 cells and ATRA-differentiated granulocytic cells, both clozapine (50
10 and 100 μ M) and doxorubicin decreased the cell survival rate, but olanzapine did not. Under granulocytic
11 differentiation for 5 days, clozapine, even at a concentration of 25 μ M, decreased survival without affecting
12 granulocytic differentiation, increased caspase activity, and caused apoptosis rather than necrosis.
13 Histamine H₄ receptor mRNA was expressed in HL-60 cells, whereas the expression decreased under
14 granulocytic ATRA-differentiation little by little. Both thioperamide, a histamine H₄ receptor antagonist,
15 and DEVD-FMK, a caspase-3 inhibitor, exerted protection against clozapine-induced survival rate
16 reduction, but not of live cell counts. 4-Methylhistamine, a histamine H₄ receptor agonist, decreased the
17 survival rate and live cell counts, as did clozapine. HL-60 cells under granulocytic differentiation are
18 vulnerable under *in vitro* assay conditions to hematopoietic toxicity induced by clozapine. Histamine H₄
19 receptor is involved in the development of clozapine-induced hematopoietic toxicity through apoptosis, and
20 may be a potential target for preventing its occurrence through granulocytic differentiation.

21

22 **Key words:** clozapine; agranulocytosis; HL-60 cells; histamine H₄ receptor; apoptosis

1 **Introduction**

2 Clozapine is an atypical antipsychotic used in patients with treatment-resistant schizophrenia because it is
3 more effective than any other antipsychotic drug (Fehsel *et al.*, 2005; Saha *et al.*, 2005). It has the benefit
4 of a lack of extrapyramidal side effects, such as Parkinsonism, tardive dyskinesia, and dystonias (Kane *et*
5 *al.*, 1988; Meltzer, 2002). However, despite such advantages, the use of clozapine is heavily restricted
6 because agranulocytosis is observed in 0.8 % of patients treated with clozapine, which in turn can induce
7 life-threatening infections (Alvir *et al.*, 1993; Atkin *et al.*, 1996). Therefore, regular monitoring of
8 hematological parameters is needed for patients treated with clozapine (Honigfeld *et al.*, 1998).

9 The mechanisms involved in agranulocytosis have yet to be fully elucidated. Several studies have
10 suggested that hematopoietic toxicity as agranulocytosis is an immune-mediated response, or the result of a
11 direct toxicity of clozapine and/or its metabolites (Meltzer, 2002; Flanagan and Dunk, 2008; Lahdelma *et*
12 *al.*, 2010). Clozapine does not exhibit direct toxicity toward granulocytes (Gerson *et al.*, 1994; Williams
13 *et al.*, 1997) and affects myeloid precursor cells rather than neutrophils (Pirmohamed and Park, 1997). It
14 has recently been reported that clozapine is toxic towards myeloid precursor cells in the bone marrow,
15 which leads to hematopoietic toxicity (Deliliers *et al.*, 1998). However, bone marrow aspiration is an
16 invasive procedure and therefore is not performed routinely in schizophrenia patients to evaluate myeloid
17 precursor cells. Thus, *in vivo* assay using individual patient samples to monitor for hematopoietic toxicity
18 is difficult.

19 Clozapine binds to multiple receptors: dopamine D₁₋₅, serotonin 5-HT_{1A}, 2A, 2C, 3, 6, 7, adrenaline α_1 ,
20 muscarine M₁₋₅ and histamine H₁₋₃ receptors (Meltzer, 1995; Ashby and Wang, 1996; Kinon and Lieberman,
21 1996). Some studies also suggest that clozapine acts as a partial agonist for the histamine H₄ receptor
22 (Smits *et al.*, 2006; Sugata *et al.*, 2007). This receptor is highly expressed in bone marrow and leukocytes,
23 and plays a role in both the immune and inflammatory responses (Ciz and Lojek, 2013). Histamine H₄
24 receptor antagonist has been reported to prevent a decrease of neutrophils by histamine (Takeshita *et al.*,
25 2004). The pharmacological role of histamine H₄ receptor in hematopoietic toxicity is yet to be
26 elucidated.

1 To determine the mechanism of hematopoietic toxicity, we developed an *in vitro* assay system using
2 HL-60 cells (human promyelocytic leukemia cells) as myeloid precursor cells, and investigated the effect
3 on hematopoiesis. The objectives of this analysis were 1) to investigate which state of hematopoietic
4 process is vulnerable to toxicity by comparing olanzapine (atypical antipsychotic with a similar structure to
5 clozapine (Ng *et al.*, 2014)) and doxorubicin (chemotherapeutic agent known to cause agranulocytosis
6 (Ying *et al.*, 2013)), and 2) to investigate the pharmacological mechanisms of hematopoietic toxicity
7 focusing on the histamine H₄ receptor.
8

1 **Materials & methods**

2 *Cell line and culture*

3 HL-60 cells were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank
4 (Osaka, Japan). The cells were maintained in RPMI 1640 medium (Sigma Aldrich, MO, USA)
5 supplemented with 10 % heat-inactive fetal bovine serum (FBS: Thermo scientific, MA, USA) and 1 %
6 penicillin/streptomycin. The cells were routinely grown in 100 × 20 mm dishes (BD Bioscience, CA,
7 USA), and incubated at 37°C in a humidified atmosphere of 5 % CO₂ in air. Every 2 days, half of the
8 medium was replaced with fresh medium. Experiments were carried out with HL-60 cells between
9 passage levels of 10 and 60.

10

11 *Materials*

12 Clozapine, olanzapine (both atypical antipsychotics, Sigma Aldrich), and DEVD-FMK (caspase 3 inhibitor:
13 BD Bioscience) were diluted to their required concentrations with dimethyl sulfoxide (DMSO), providing a
14 final concentration of 0.1 % DMSO. Thioperamide (histamine H₄ receptor antagonist: Sigma Aldrich),
15 doxorubicin (anthracycline antineoplastic: Sigma Aldrich) and 4-methyl histamine (histamine H₄ receptor
16 agonist: Wako, Tokyo, Japan) were diluted with distilled water.

17

18 *Experimental schedule*

19 The effects of clozapine on HL-60 cells were evaluated in three different states of the hematopoietic
20 process: 1) undifferentiated HL-60 cells as myeloid precursor cells were exposed to clozapine (1, 10, 25, 50
21 or 100 µM) and cultured at a concentration of 4× 10⁵/mL for 2 days or 1× 10⁵/mL for 5 days (Breitman *et*
22 *al.*, 1980; Romero-Steiner *et al.*, 1997). 2) the myeloid precursor cells under granulocytic differentiation
23 were exposed to *all-trans* retinoic acid (ATRA) (1 µM) and clozapine (1, 10, 25, 50 or 100 µM) and
24 cultured at a concentration of 4× 10⁵/mL for 2 days or 1× 10⁵/mL for 5 days. 3) HL-60 cells were
25 suspended at a concentration of 1× 10⁵/mL and exposed to ATRA (1 µM) for 5 days to differentiate towards
26 granulocytic cells. After that, granulocytic cells were exposed to clozapine (1, 10, 25, 50 or 100 µM), and

1 cultured at a concentration of $4 \times 10^5/\text{mL}$ for 2 or 5 days. The effects of clozapine in three different
2 hematopoietic states were compared to those of olanzapine (1, 10, 25, 50 or 100 μM), or doxorubicin (0.2
3 μM).

4 5 *Trypan blue staining*

6 Cell survival and proliferation were evaluated using trypan blue staining. Only cells with intact
7 membranes remain impermeable to the dye, so that dead cells with compromised membranes become
8 stained. Cell suspension was added to an equal amount of trypan blue solution (Wako), and mixed
9 thoroughly. The total number of cells and the number of trypan blue positive cells were counted using an
10 automatic cell counter by TC-20 (Bio-Rad, CA, USA). Cell survival rate (%) was calculated as the
11 number of trypan blue negative cells in the total number of cells. The live cell counts (/ml) were
12 determined by counting the number of trypan blue negative cells.

13 14 *May-Giemsa staining*

15 Granulocytic differentiation was evaluated using May-Giemsa staining. May-Grünwald's eosin methylene
16 blue and Giemsa's azur eosin methylene blue are used for the histological staining of blood cells. HL-60
17 cells under granulocytic differentiation were centrifuged at 2,000 rpm for 5 min. The pellet was loaded
18 onto slides and dried. Cells were stained with May-Grünwald stain solution (Wako) for 1 min,
19 May-Grünwald diluted with 1 % phosphate buffer saline (PBS) for 3 min, and Giemsa staining solution
20 (Wako) diluted with 1 % PBS, for 15 min. The slides were rinsed in deionized water and air dried. In all,
21 100 cells per slide were evaluated to determine the morphological characteristics of the nuclei (%).

22 23 ***CD11b staining***

24 CD11b is surface marker that increases rapidly with granulocytic differentiation (Drayson *et al.*, 2001).
25 To measure expression levels of CD11b on the cell surface, HL-60 cells under granulocytic differentiation
26 were centrifuged at 2,000 rpm for 5 min. The pellet was resuspended in PBS containing 1 % FBS twice,

1 and cells were stained with a Human anti-CD11b antibody conjugated to Phycoerythrin (PE) or a
2 PE-conjugated isotype control IgG Ab (both from BD Bioscience) for 30 min in the dark on ice, then
3 washed with PBS containing 1 % FBS twice. The data were analyzed with FACS Canto II flow cytometer
4 (BD Biosciences) using FlowJo™ software (FLOWJO, OR, USA). A minimum of 10,000 events were
5 collected per sample.

7 ***Annexin V and 7-AAD staining***

8 To distinguish between early apoptotic and necrotic populations, we used Annexin V and 7-actinomycin D
9 (7-AAD). Annexin V and 7-AAD are used to detect the translocation of phosphatidylserine (PS) to the
10 cell surface of apoptotic cells (Zamai *et al.*, 2001), and alterations of cell membrane integrity (Lecoeur *et*
11 *al.*, 1997), respectively. The pellets were resuspended in PBS containing 1 % FBS twice, and
12 subsequently exposed to **Annexin** V conjugated to **PE** or APC (BD Bioscience), which binds exposed PS
13 residues on apoptotic cells, 7-AAD (BD Bioscience), **which intercalates into double-stranded nucleic**
14 **acids on dead cells, and Hoechst 33258 (Dojindo, Kumamoto, Japan), which stains the nuclei of both**
15 **live and dead cells,** for 15 **min** in the dark at room temperature. The data were analyzed with **Keyence**
16 **BZ 9000 fluorescence microscope using BZ-9000 analysis software (Keyence, Osaka, Japan) or** FACS
17 Canto II flow cytometer (BD Biosciences). **In immunocytochemistry, the rate of Annexin V- or**
18 **7-AAD-positive cells were quantified by scoring the number of them** over the total number of viable
19 **cells in 3 microscopic fields.**

21 *Caspase activity*

22 Caspase activity was quantified using Muse™ MultiCaspase Kit (Millipore, MA, USA) following the
23 manufacturer's instructions. HL-60 cells under granulocytic differentiation were centrifuged at 2,000 rpm
24 for 5 min. The pellet was incubated with caspase buffer and working solution. The quantity of
25 caspase-active cells was calculated and analyzed using the Muse™ Cell Analyzer (Millipore).

Real-time PCR

Total RNA was extracted from HL-60 cells using a NucleoSpin RNA kit (Takara Bio, Otsu, Japan) and converted into cDNA using a PrimeScript™ RT reagent Kit (Takara Bio). Real-time PCR analysis was undertaken using SYBR Premix Ex Taq (Takara Bio). Data were collected using a StepOnePlus System (Applied Biosystems, CA, USA). Primers used for PCR were as follows: H4R: forward 5'-CATCCCTCACACGCTGTTTCG-3', reverse 5'-GCAGGAACCTTCTGTCGATGC-3', β -actin: forward 5'-ACCTCATGAAGATCCTGAC-3', reverse 5'-ACTCCTGCTTGCTGATCC-3' (Ikawa *et al.*, 2005). The reaction profile consisted of a first round at 95°C for 15 min and then 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min, with a final extension reaction carried out at 72°C for 10 min. To standardize the quantification, β -actin was amplified simultaneously (Medina *et al.*, 2006). Expression levels were calculated using the delta-delta Ct method.

Statistics

All the experiments were triplicate cultures used for each drug concentration. The results of all the experiments were combined and expressed as the mean and standard error. Statistical analysis of the data was carried out by one-way or two-way analysis of variance (ANOVA) with application of Turkey's post-hoc test for multi-group comparisons, Student's *t*-test for two-group. Values of $p < 0.05$ were considered as statistically significant.

Results

HL-60 cells were differentiated by ATRA into three states according to their hematopoietic process: undifferentiated HL-60 cells, those undergoing granulocytic ATRA-differentiation, and ATRA-differentiated granulocytic cells. CD11b, a marker of granulocytic differentiation of HL-60 cells (Drayson *et al.*, 2001), was expressed in less than 5 % of undifferentiated HL-60 cells, was increased under ATRA-induced granulocytic differentiation, and was expressed in over 90 % of the cells 5 days after ATRA treatment. Hematopoietic toxicity was evaluated by analyzing cell survival, cell proliferation, granulocytic differentiation, apoptosis, and necrosis.

The effect of clozapine on survival and proliferation of HL-60 cells

Firstly, we evaluated the effects of clozapine on survival and proliferation of undifferentiated HL-60 cells as myeloid precursor cells. Exposure to clozapine (50 and 100 μ M) and doxorubicin for 2 days significantly decreased the survival rate (Fig 1A: one-way ANOVA, $F_{(11, 60)} = 372.40$, $p < 0.01$) and live cell counts (Fig 1B: one-way ANOVA, $F_{(11, 60)} = 74.57$, $p < 0.01$) of undifferentiated HL-60 cells, but these were not observed in the case with olanzapine (1-100 μ M). When undifferentiated HL-60 cells were exposed to the drugs for 5 days, clozapine (50 and 100 μ M) and doxorubicin continuously decreased the survival rate (Fig 2A: one-way ANOVA, $F_{(11, 60)} = 905.65$, $p < 0.01$) and live cell counts (Fig 2B: one-way ANOVA, $F_{(11, 60)} = 75.94$, $p < 0.01$). Olanzapine (1-100 μ M) caused a decrease in live cell counts, but not the survival rate.

Next, we evaluated the effect of clozapine on survival and proliferation of HL-60 cells under ATRA-induced granulocytic differentiation. Exposure of clozapine (survival rate: 50 and 100 μ M; live cell counts; 1, 25-100 μ M) and doxorubicin, but not olanzapine (1-100 μ M) with ATRA for 2 days significantly decreased the survival rate (Fig 1C: one-way ANOVA, $F_{(11, 60)} = 167.24$, $p < 0.01$) and live cell counts (Fig 1D: one-way ANOVA, $F_{(11, 60)} = 63.62$, $p < 0.01$) of HL-60 cells. When HL-60 cells were exposed to the drugs with ATRA for 5 days, clozapine (25 μ M) with ATRA even significantly decreased the survival rate (Fig 2C: one-way ANOVA, $F_{(11, 60)} = 135.04$, $p < 0.01$) of HL-60 cells. Clozapine (50-100

1 μM) and doxorubicin, but not olanzapine (1-100 μM) with ATRA significantly decreased live cell counts
2 (Fig 2D: one-way ANOVA, $F_{(11, 60)} = 34.10$, $p < 0.01$).

3 Finally, we evaluated the effect of clozapine on the survival and proliferation of granulocytic
4 differentiated HL-60 cells. Exposure to clozapine (50-100 μM) and doxorubicin, but not olanzapine
5 (1-100 μM), for 2 days significantly decreased the survival rate (Fig 1E: one-way ANOVA, $F_{(11, 60)} = 85.70$,
6 $p < 0.01$) and live cell counts (Fig 1F: one-way ANOVA, $F_{(11, 60)} = 21.28$, $p < 0.01$) of granulocytic
7 differentiated HL-60 cells. When differentiated granulocytic HL-60 cells were exposed to vehicle for 5
8 days, the survival rate and live cell counts of granulocytic differentiated HL-60 cells was decreased
9 (survival rate of null cells and live cell counts was about 30 % and $2 \times 10^5/\text{mL}$, respectively, on 5 days).
10 These results indicated that HL-60 cells under ATRA-induced granulocytic differentiation were vulnerable
11 for the toxicity of clozapine.

13 *The effect of clozapine on the ATRA-induced granulocytic differentiation of HL-60 cells*

14 In May-Giemsa staining, clozapine (1-25 μM) did not affect ATRA-induced nuclear segmentation (Fig 3A
15 and B: one-way ANOVA, $F_{(4,10)} = 107.37$, $p < 0.01$) and ATRA-increased CD11b-positive cells (Fig 3C and
16 D: one-way ANOVA, $F_{(2,6)} = 202.72$, $p < 0.01$).

18 *Involvement of histamine H_4 receptor in the decreased survival and proliferation of HL-60 cells by* 19 *clozapine under ATRA-induced granulocytic differentiation*

20 Histamine H_4 receptor is mainly expressed in the bone marrow and leukocytes (Zampeli and Tiligada,
21 2009), in which they are expressed in eosinophils and mast cells, but less so in neutrophils or monocytes
22 (de Esch *et al.*, 2005). Histamine H_4 receptor mRNA was also expressed in undifferentiated HL-60 cells,
23 decreased under ATRA-induced granulocytic differentiation little by little, and eventually disappeared 5
24 days after treatment of ATRA (Fig 4A: one-way ANOVA, $F_{(5,12)} = 271.91$, $p < 0.01$). We thus investigated
25 the involvement of histamine H_4 receptors in the decreased cell survival and proliferation induced by
26 clozapine under ATRA-induced granulocytic differentiation in HL-60 cells. Thioperamide, a histamine H_4

receptor antagonist significantly reversed the decrease in the survival rate induced by clozapine (25 μ M, but not 50-100 μ M) (Fig 4B: two-way ANOVA: $F_{\text{clozapine (1, 20)}} = 57.57$, $p < 0.01$, $F_{\text{Thioperamide (1, 20)}} = 19.96$, $p < 0.01$) without affecting live cell counts (Fig 4C: two-way ANOVA: $F_{\text{clozapine (1, 20)}} = 13.81$, $p < 0.01$, $F_{\text{Thioperamide (1, 20)}} = 0.51$, $p = 0.48$). Conversely, 4-methyl histamine (4-MH), a histamine H_4 receptor agonist, significantly decreased the survival rate (Fig 4D: one-way ANOVA: $F_{\text{4-MH (3, 20)}} = 5.51$, $p < 0.01$) and live cell counts (Fig 4E: one-way ANOVA: $F_{\text{4-MH (3, 20)}} = 74.36$, $p < 0.01$) of HL-60 cells under ATRA-induced granulocytic differentiation. Thioperamide significantly prevented a 4-MH-induced decrease in the survival rate (Fig 4F: two-way ANOVA: $F_{\text{4-MH (1, 20)}} = 11.91$, $p < 0.01$, $F_{\text{Thioperamide (1, 20)}} = 1.69$, $p < 0.05$), but not of live cell counts (Fig 4G: two-way ANOVA: $F_{\text{4-MH (1, 20)}} = 157.29$, $p < 0.01$, $F_{\text{Thioperamide (1, 20)}} = 1.69$, $p = 0.21$).

11

Involvement of apoptosis and necrosis in the decreased survival and proliferation of HL-60 cells induced by clozapine via histamine H_4 receptor under ATRA-induced granulocytic differentiation

We investigated whether apoptosis and/or necrosis are involved in the decreased survival of HL-60 cells by a vulnerability to toxicity in HL-60 cells, and whether this occurs via histamine H_4 receptor under ATRA-induced granulocytic differentiation. **Exposure to clozapine (25 μ M) with ATRA for 5 days**

significantly increased the rate of Annexin V and 7-AAD-positive cells in immunocytochemistry (Annexin V: 33.4 %, $p < 0.01$ and 7-AAD: 9.1 %, $p < 0.01$; Fig 5A and B) and flow cytometry (Annexin V: 40.1 %, $p < 0.01$ and 7-AAD: 3.6 %, $p < 0.01$; Fig 5C and D). They also significantly

increased the rate of caspase-active cells, compared to that in DMSO with ATRA (Fig 5E and F: Student's t -test, $t_{10} = -5.82$, $p < 0.01$). DEVD-FMK, a caspase-3 inhibitor, prevented clozapine- and 4-MH-induced decrease the survival rate (clozapine: Fig 5G two-way ANOVA: $F_{\text{clozapine (1, 19)}} = 9.40$, $p < 0.01$, $F_{\text{DEVD-FMK (1, 19)}} = 6.13$, $p < 0.01$; 4-MH: Fig 5H two-way ANOVA: $F_{\text{4-MH (1, 8)}} = 10.47$, $p < 0.05$, $F_{\text{DEVD-FMK (1, 8)}} = 29.09$, $p < 0.01$), but not of live cell counts (clozapine: Fig 5I $F_{\text{clozapine (1, 19)}} = 7.44$, $p < 0.05$, $F_{\text{DEVD-FMK (1, 19)}} = 0.64$, $p = 0.43$; 4-MH: Fig 5J two-way ANOVA: $F_{\text{4-MH (1, 8)}} = 30.19$, $p < 0.01$, $F_{\text{DEVD-FMK (1, 8)}} = 379$, $p = 0.08$).

Discussion

Some clinical hematopoietic toxicity studies demonstrated that clozapine acts on myeloid precursor cells in bone marrow rather than on granulocytes in peripheral blood (Gerson *et al.*, 1994; Pirmohamed and Park, 1997). In the present *in vitro* study using HL-60 cells, exposure to clozapine ($> 50 \mu\text{M}$) decreased the survival rate in all state of the hematopoietic process. Clozapine has been demonstrated to decrease cell survival at $60 \mu\text{M}$, but not at $40 \mu\text{M}$ for 2 days in primary cultured hepatocytes from rats (Jia *et al.*, 2014). Thus, decreased cell survival induced by clozapine at concentrations above $50 \mu\text{M}$ is suspected to be the result of non-specific cell toxicity. Exposure to clozapine ($25 \mu\text{M}$) with ATRA ($1 \mu\text{M}$) for 5 days also decreased the survival rate of HL-60 cells. It has been demonstrated that cells undergoing ATRA-induced granulocytic differentiation are susceptible to clozapine toxicity. A decreased the survival rate was observed after 5 days, but not 2 days, after exposure of clozapine ($25 \mu\text{M}$) with ATRA. As hematopoietic toxicity is delayed in onset (Galderisi and Bucci, 1998; Munro *et al.*, 1999), it is noteworthy that clozapine toxicity is not observed acutely.

Clozapine also decreased cell proliferation in all states of the hematopoietic process. Specifically, lower doses of clozapine (1 and $10 \mu\text{M}$) decreased live cell counts of undifferentiated HL-60 cells that were under ATRA-induced granulocytic differentiation without affecting the survival rate. In the present study, exposure to clozapine ($25 \mu\text{M}$) with ATRA for 5 days did not affect nuclear segmentation by Giemsa staining or expression of CD11b. Decreased survival and proliferation of HL-60 cells induced by clozapine were specifically observed without affecting ATRA-induced granulocytic differentiation of HL-60 cells for 5 days. These results suggest that decreased cell survival and proliferation induced by clozapine contribute to its hematopoietic toxicity. Our investigations using HL-60 cells under granulocytic differentiation are appropriate for *in vitro* assay systems of hematopoietic toxicity.

Olanzapine has structural similarities to clozapine and was developed to reduce the risk of agranulocytosis (Steinwachs *et al.*, 1999). In the present study, at an equal dose did not decrease the cell survival rate in all state of the hematopoietic process. Thus, the present results support previous reports that clozapine shows more toxicity for myeloid precursor cells than olanzapine. A clinical case report

1 showed that olanzapine has a lower risk of agranulocytosis (Steinwachs *et al.*, 1999). In fact, it decreased
2 the proliferation of undifferentiated HL-60 cells. Olanzapine has therefore been shown to induce
3 agranulocytosis by decreasing cell proliferation. However, further investigation of the mechanisms
4 involved is needed.

5 Clozapine has unique affinity for the histamine H₄ receptor as an agonist (Buckland *et al.*, 2003).
6 Histamine H₄ receptor has been reported to be exclusively expressed in blood cells (de Esch *et al.*, 2005)
7 and is undetectable in the brain (Pereira *et al.*, 2003). In the present study, histamine H₄ receptor mRNA
8 was also found in undifferentiated HL-60 cells. 4-MH, a histamine H₄ receptor agonist, as well as
9 clozapine, caused a decrease in the survival rate and live cells counts under ATRA-induced granulocytic
10 differentiation. Thioperamide, a histamine H₄ receptor antagonist prevented a clozapine-induced decrease
11 in the survival rate, but not of live cell counts under ATRA-induced granulocytic differentiation. These
12 results suggest that the histamine H₄ receptor is involved primarily in the decreased cell survival rather than
13 that of cell proliferation induced by clozapine.

14 It remains unclear whether the histamine H₄ receptor is fully responsible for the decreased cell
15 proliferation induced by clozapine. Clozapine binds to many kinds of neurotransmitter receptors, such as
16 dopamine, adrenaline, serotonin and other histamine receptors, which are all expressed in HL-60 cells
17 (Klinker *et al.*, 1996). It is possible that not only the histamine H₄ receptor, but also other receptors, are
18 involved in the decreased cell proliferation induced by clozapine. Thioperamide has a lower affinity for
19 the histamine H₄ receptor (Wade *et al.*, 2012) and acts as not only an antagonist but also an inverse agonist
20 to the histamine H₄ receptor (Seifert *et al.*, 2011). Thus, thioperamide's antagonism of the histamine H₄
21 receptor is relatively mild, and therefore it did not prevent a clozapine- and 4-MH-induced decrease in live
22 cell counts.

23 Apoptosis and/or necrosis are eventually involved in cell death induced by hematopoietic toxicity
24 (Darzynkiewicz *et al.*, 1997). It is unknown whether apoptosis and/or necrosis are involved via the
25 histamine H₄ receptor in the cell death induced by clozapine. Annexin V detects PS exposed on the cell
26 surface of early apoptotic cells, and 7-AAD detects an alteration in the integrity of the cell membrane of

late apoptotic and necrotic cells. **We found that clozapine (25 μ M) increased the rate of Annexin V-positive cells higher than that of 7-AAD, suggesting that clozapine induces apoptosis rather than necrosis.** Clozapine (25 μ M) also increased the number of caspase-positive cells under ATRA-induced granulocytic differentiation, indicating that clozapine activates caspase, the key mediator of apoptosis (Nicholson and Thornberry, 1997). DEVD-FMK, a caspase-3 inhibitor, prevented a clozapine (25 μ M)-induced decrease in the survival rate. Taken together, these findings suggest that clozapine causes a decrease in the survival rate of HL-60 cells under ATRA-induced granulocytic differentiation through the induction of apoptosis rather than necrosis.

In conclusion, HL-60 cells under granulocytic differentiation are susceptible to clozapine toxicity as shown by *in vitro* assay systems for hematopoietic toxicity. The histamine H₄ receptor is involved in clozapine-induced hematopoietic toxicity of HL-60 cells which undergo apoptosis. The antipsychotic effects of clozapine could be independent from its agonism of the histamine H₄ receptor, which is undetected in the brain (Pereira *et al.*, 2003). Thus, the histamine H₄ receptor is a potential molecular therapeutic target, and could provide a basis for the design of new antipsychotics with the goal of preventing agranulocytosis in the future.

1 **Acknowledgments**

2 We would like to thank Mr. Minoru Tanaka of Division for Medical Research Engineering, Graduate
3 School of Medicine, Nagoya University for technical advice in this study, Dr. Tomohiro Mizuno of Faculty
4 of Pharmacy, Meijo University, for helpful advice in this study, Ms. Hiromi Ohguchi of Faculty of
5 Pharmacy, Meijo University, for technical assistance in this study.

6

Figure legends

Fig 1. The effect of clozapine on survival and proliferation of HL-60 cells for 2 days.

(A, B) Undifferentiated HL-60 cells were exposed to clozapine, olanzapine, or doxorubicin for 2 days. (C, D) HL-60 cells were exposed to clozapine, olanzapine, or doxorubicin with ATRA (1 μ M) for 2 days. (E) After exposure to ATRA (1 μ M) for 5 days, HL-60 cells were exposed to clozapine, olanzapine, or doxorubicin for 2 days. Cell survival and proliferation were evaluated by trypan blue staining. Values indicate the mean $\pm$ SEM (n = 6). *p < 0.05, **p < 0.01, compared to DMSO or DMSO/ATRA groups. DMSO, dimethyl sulfoxide; CLZ, clozapine; OLZ, olanzapine; DOX, doxorubicin; ATRA, *all-trans* retinoic acid.

Fig 2. The effect of clozapine on survival and proliferation of HL-60 cells for 5 days.

(A, B) Undifferentiated HL-60 cells were exposed to clozapine, olanzapine, or doxorubicin for 5 days. (C, D) HL-60 cells were exposed to clozapine, olanzapine, or doxorubicin with ATRA for 5 days. (E) After exposure to ATRA (1 μ M) for 5 days, HL-60 cells were exposed to clozapine, olanzapine, or doxorubicin for 5 days. Cell survival and proliferation were evaluated by trypan blue staining. Values indicate the mean $\pm$ SEM (n = 6). *p < 0.05, **p < 0.01, compared to DMSO or DMSO/ATRA groups. DMSO, dimethyl sulfoxide; CLZ: clozapine, OLZ, olanzapine; DOX, doxorubicin; ATRA, *all-trans* retinoic acid.

Fig 3. The effect of clozapine on ATRA-induced granulocytic differentiation of HL-60 cells.

HL-60 cells were exposed to clozapine with ATRA for 5 days. Granulocytic differentiation of HL-60 cells was evaluated by nuclear segmentation using May-Giemsa staining (A, B) and expression levels of CD11b by using flow cytometry (C, D). Values indicate the mean $\pm$ SEM (n = 3-6). **p < 0.01 Compared to DMSO/EtOH groups, ##p < 0.01 Compared to DMSO/ATRA groups. DMSO, dimethyl sulfoxide; CLZ, clozapine; EtOH, Ethanol; ATRA, *all-trans* retinoic acid.

Fig 4. Involvement of histamine H₄ receptor in the decreased survival and proliferation of HL-60 cells by

clozapine under ATRA-induced granulocytic differentiation.

HL-60 cells were exposed to clozapine with ATRA for 5 days. Expression of histamine H₄ receptor mRNA in HL-60 cells under ATRA-induced granulocytic differentiation was quantified by real-time RT-PCR (A). HL-60 cells were exposed to thioperamide (histamine H₄ receptor antagonist), clozapine (B, C) or 4-methyl histamine (histamine H₄ receptor agonist: 4-MH) with ATRA (D, E, F, G). Cell survival (B, D, F) and proliferation (C, E, G) were evaluated by trypan blue staining. Values indicate the mean $\pm$ SEM (n = 3-6). *p < 0.05, **p < 0.01, compared to DMSO/ATRA (-), H₂O/ATRA or H₂O/ATRA (-) groups, #p < 0.05, ##p < 0.01, compared to CLZ 25 μ M/ATRA (-) or 4-MH 25 μ M/ATRA (-) groups. DMSO, dimethyl sulfoxide; CLZ, clozapine; DOX, doxorubicin; ATRA, *all-trans* retinoic acid; 4-MH, 4-methyl histamine.

Fig 5. Involvement of apoptosis and necrosis in the decreased survival and proliferation of HL-60 cells by clozapine under ATRA-induced granulocytic differentiation.

HL-60 cells were exposed to clozapine or 4-methyl histamine (histamine H₄ receptor agonist: 4-MH) with ATRA for 5 days (A-J). Apoptosis and necrosis were evaluated by staining with Annexin V, 7-amino actinomycin D (7-AAD), and Hoechst 33258 (A-D). Caspase activity was analyzed using a Muse™ Cell Analyzer (E, F). HL-60 cells were exposed to DEVD-FMK (caspase-3 inhibitor), clozapine (G, I) or 4-MH (H, J) with ATRA for 5 days. Cell survival (G, H) and proliferation (I, J) were evaluated by trypan blue staining. Values indicate the mean $\pm$ SEM (n = 3-6). *p < 0.05, **p < 0.01, compared to DMSO/ATRA, DMSO/ATRA (-) or H₂O/ATRA (-) groups, ##p < 0.01, compared to CLZ 25 μ M/ATRA (-) or 4-MH/ATRA (-) groups. DMSO, dimethyl sulfoxide; CLZ, clozapine; ATRA, *all-trans* retinoic acid; 4-MH, 4-methyl histamine.

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35

2 days

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Fig 1

5 days

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Fig 2

Fig 3

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(G)

Fig 4

Fig 5