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Micromolar zinc modifies the cell death induced by clotrimazole in human leukemia K562 cells

Masako Kobayashi[1], Yoko Sakanashi[1], Hiroko Matsui[1], Yumiko Nishimura[1], Kaori Kanemaru[1], Yoshiro Okano[2], Yasuo Oyama[1],*

[1] *Laboratory of Cell Signaling, Faculty of Integrated Arts and Sciences, The University of Tokushima, Tokushima 770-8502, Japan and* [2] *Department of Pharmaceutical Care and Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima 770-8512, Japan*

* *Correspondence: Yasuo Oyama, Ph.D.*

ABSTRACT

Our recent study showed that the simultaneous application of clotrimazole with $CdCl_2$ or $PbCl_2$ exerted potent cytotoxic action in rat thymocytes although respective agents were ineffective. It was also the case of $ZnCl_2$ and clotrimazole in preliminary study using rat thymocytes. Since clotrimazole is supposed to be a candidate for anticancer drug, we examined the effects of clotrimazole, $ZnCl_2$, and their combination on human leukemia K562 cells. The combination of clotrimazole and $ZnCl_2$ exerted potent cytotoxic effects on the growth and lethality of K562 cells by presumably modifying the process of cell death. The result suggests the possibility that endogenous Zn^{2+} may modify the action of clotrimazole.

Keywords: clotrimazole; zinc; cytotoxicity; human leukemia K562 cells

1. INTRODUCTION

Clotrimazole, classified as an azole antifungal agent, possesses diverse cellular actions. The agent inhibits Ca^{2+}-dependent K^+ channels (Alvarez *et al.*, 1992; Brugnara *et al.*, 1993; Rettenhouse *et al.*, 1997), voltage-dependent Ca^{2+} channels (Welker and Drews, 1997; Wu *et al.*, 1999), and sarcoplasmic reticulum Ca^{2+}-ATPase (Snajdrova *et al.*, 1998; Bartolommei *et al.*, 2006). Thus, the agent may decide the fate of cells under certain conditions since clotrimazole is supposed to modulate Ca^{2+}-dependent processes. In fact, the agent induced antiproliferative action (or cytotoxic action) on several types of cancer cells (Benzaquen *et al.*, 1995; Khalid *et al.*, 1999; Smith *et al.*, 2002; Ito *et al.*, 2002; Penso *et al.*, 2002).

Our recent study showed that the application of clotrimazole with $CdCl_2$ or $PbCl_2$ exerted potent cytotoxic action in rat thymocytes although both agents at individual concentrations were ineffective (Oyama *et al.*, 2006). Therefore, it is reminiscent of a possibility that it is also the case for $ZnCl_2$ and clotrimazole. The use of zinc as a nutritional supplement has become common in many developing countries because its deficiency causes growth retardation, delayed sexual maturation, impairment of immunocompetence, neuromotor and cognitive malfunctions (Prasad, 1995; Sandstead and Alcock, 1997; Ploysangam *et al.*, 1997; Prasad *et al.*, 1997; Schwartz *et al.*, 2005; Frederickson *et al.*, 2005).

To test the possibility, we have examined the effects of clotrimazole, $ZnCl_2$, and their combination on human leukemia K562 cells by using a flow cytometer with appropriate fluorescent probes.

2. MATERIALS AND METHODS

2.1. Cell Preparation

Human leukemia K562 cells were cultured in 24-well Falcon tissue culture plates placed in a CO_2 incubator (Sanyo, Tokyo) at a temperature of 37°C. Each well contained 2 ml of RPMI 1640 medium with glutamine (300 mg/l) and 10 % fetal bovine serum (Sigma Chemical, St. Louis, MO, USA).

2.2. Cell Viability

Cytograms (forward-scatter intensity *versus* side-scatter intensity) were obtained from a programmed number (2000 or 2500 cells) of K562 cells with a flow cytometer (Cyto-ACE150, JASCO, Tokyo). Cell viability was estimated using the propidium iodide (Molecular Probes Inc., Eugene, OR, USA) staining method (Chikahisa *et al.*, 1998). Since propidium iodide is unable to stain living cells, the cells exhibiting propidium fluorescence are dead. The fluorescence was measured at 2 min after adding propidium iodide to medium to achieve a final concentration of 5 μM. Excitation wavelength for propidium was 488 nm. Emission was monitored at

wavelength of 600 ± 20 nm. The cell lethality in this study indicates the percentage population of cells stained with propidium to total cells.

2.3. Estimation of Growth

The growth of K562 cells was estimated by the use of flow cytometer. The number of cells counted during a programmed time (30 s) with a flow cytometer was correlated with those estimated by erythrocytometer with a coefficient of 0.98 (Chikahisa *et al.*, 1998). Thus, the flow cytometric technique with propidium iodide also allowed the simultaneous estimation on drug effects on the cell growth and lethality of cells.

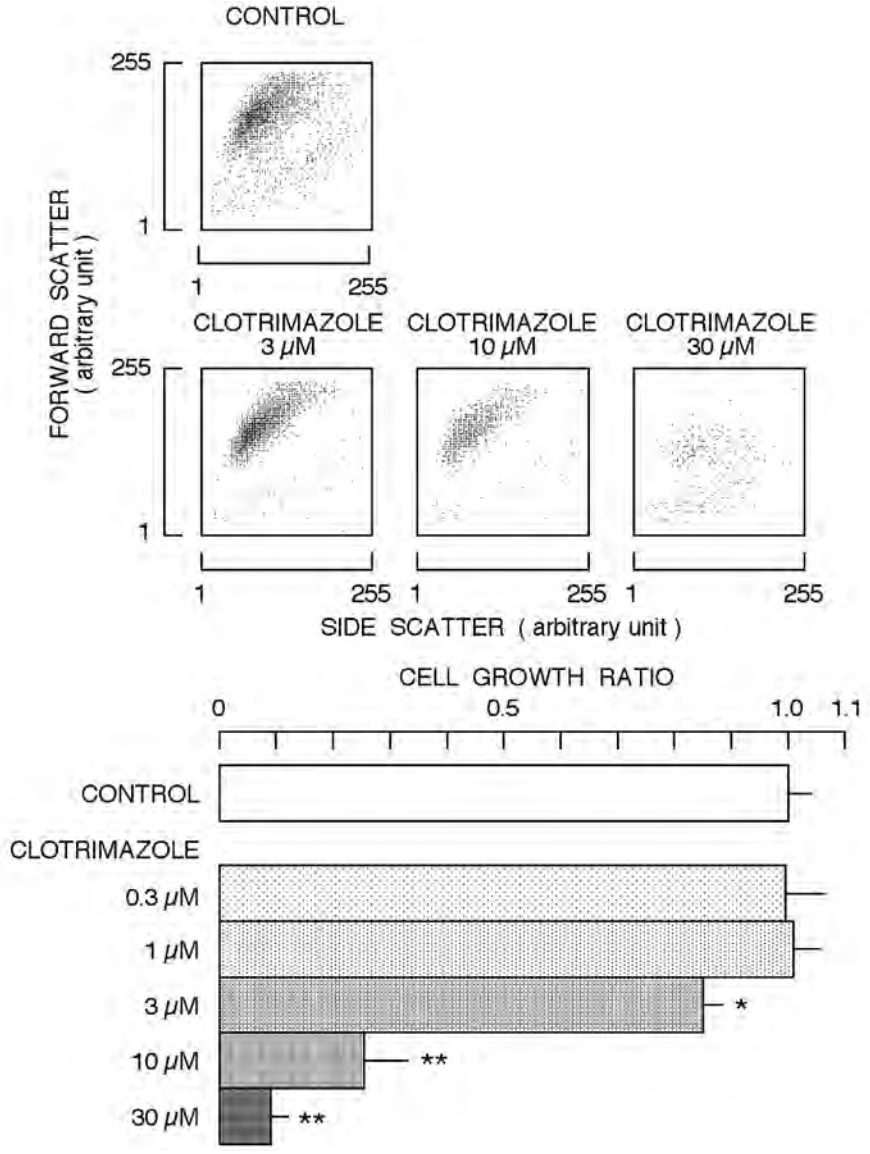

Figure 1

Effect of clotrimazole on growth of K562 cells. Upper panel: the cytograms in absence or presence of clotrimazole. Lower panel: Effect of clotrimazole on cell growth. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbols (and **) show significant difference between control group and respective test groups. * P<0.05 and ** P<0.01.*

2.4. Analysis of Cell Death Process

The exposure of phosphatidylserine on outer membranes of K562 cells was detected by using annexin V-FITC (Sigma Chemical Co., St. Louis, MO, USA) (Oyama *et al.*, 1999; Nakata *et al.*, 1999). The cells were incubated with both annexin V-FITC (10 μl/ml) for 15-30 min and propidium for 2 min before the measurement. Excitation wavelength for the dyes was

also 488 nm. The emissions were detected at 530 ± 20 nm for FITC (annexin V binding to membranes) and 600 ± 20 nm for propidium.

2.5. Data Presentation and Statistics

Numerical values of experimental data are presented as the mean ± standard deviation (SD) in this study. Statistical analysis was performed using Tukey multivariate test. A P value of <0.05 was considered significant.

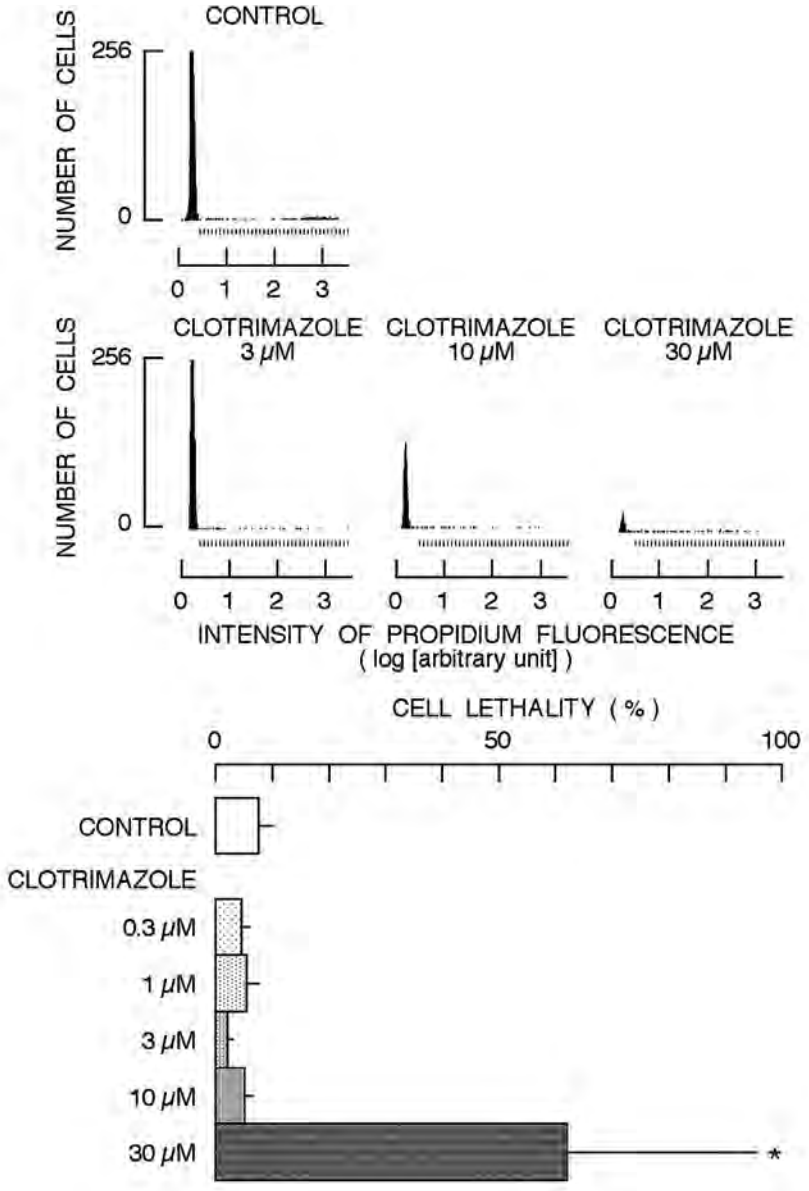

Figure 2

*Effect of clotrimazole on lethality of K562 cells. Upper panel: the histogram of propidium fluorescence in absence or presence of clotrimazole. Lower panel: Effect of clotrimazole on cell lethality. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbol (*) shows significant difference between control group and respective test groups. * P<0.05.*

3. RESULTS AND DISCUSSION

3.1. Effect of clotrimazole on cell growth and lethality

The cells were incubated with micromolar clotrimazole for 72 hr. As shown in Fig. 1, the number of cells shown in cytogram decreased as the concentration of clotrimazole increased. The threshold concentration of clotrimazole for inhibiting the growth was 3 μM.

Clotrimazole at 10 μM greatly decreased the number of cells counted during a programmed time (30 sec) without significantly affecting the forward scatter (cell size). Further increase in clotrimazole concentration to 30 μM significantly reduced the cell size, presumably inducing cell shrinkage, and almost-completely suppressed the growth of K562 cells. The concentration-dependent inhibition of cell growth by clotrimazole is summarized in Fig. 1.

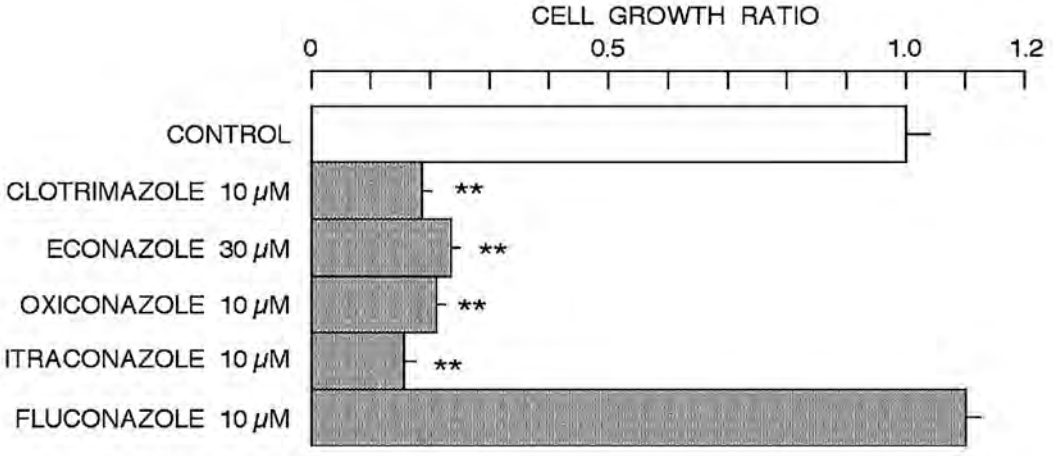

Figure 3

*Effects of azole antifungal agents on cell growth of K562 cells. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbol (**) shows significant difference between control group and respective test groups. ** P<0.01.*

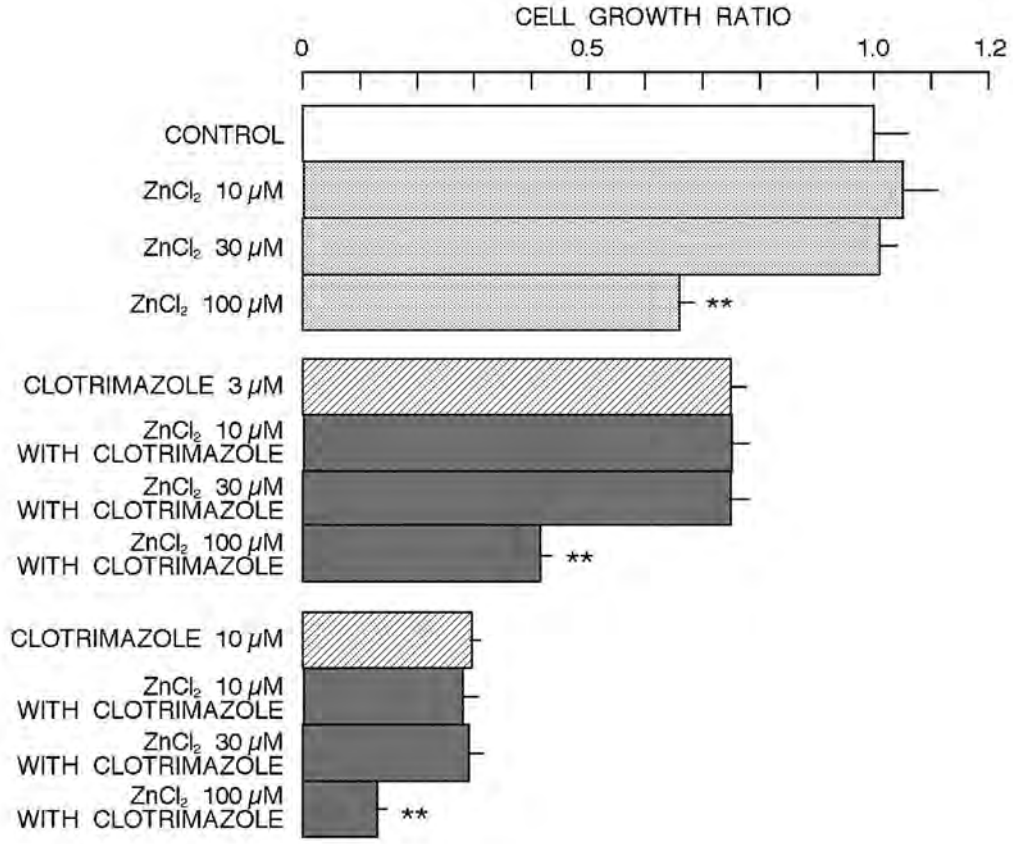

Figure 4

*Effects of ZnCl2, clotrimazole, and their combination on cell growth. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbol (**) shows significant difference between respective control group (control and clotrimazole-treated groups) and test groups. ** P<0.01.*

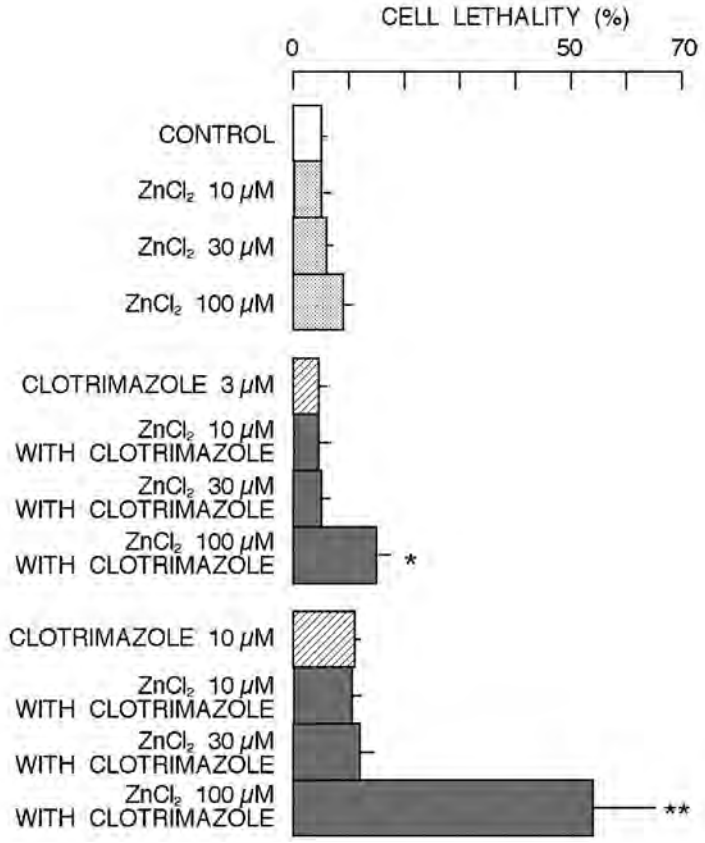

Figure 5

Effects of ZnCl2, clotrimazole, and their combination on cell lethality. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbols (and **) show significant difference between respective control group (control and clotrimazole-treated groups) and test groups. * P<0.05 and ** P<0.01.*

Clotrimazole at 30μM reduced the cell size, suggesting that the agent induced cell death. Fig. 2 shows the histogram of propidium fluorescence monitored from K562 cells incubated with and without clotrimazole. The population of cells stained with propidium, presumably dead cells, was increased in the case of 30μM. The concentration-dependent effect of clotrimazole on cell lethality is shown in Fig. 2. Thus, clotrimazole at 3 and 10 μM inhibited the cell growth without affecting the lethality while the growth inhibition by 30 μM clotrimazole was associated with cell death. The threshold concentration of clotrimazole to reduce the cell viability of K562 cells was more than 10 μM. It is also the case for rat thymocytes (Oyama *et al.*, 2006).

3.2. Effects of other azole antifungal agents on cell growth

Clotrimazole is classified as an azole antifungal agent. Other azole antifungal agents were also tested on K562 cells. The agents except for fluoconazole inhibited the cell growth (Fig. 3) when the cells were incubated with them for 72 hr. The potency of clotrimazole was similar to those of econazole, oxiconazole, and itraconazole. The lethality was not significantly affected by them (not shown). Therefore, further studies were done with 10 μM clotrimazole and micromolar ZnCl2.

3.3. Effect of ZnCl2 on cell growth and lethality

The cells were also incubated with ZnCl2 at concentrations of 10-100 μM for 72 hr. ZnCl2 at 10-30 μM did not affect the cell growth (Fig. 4) and viability (Fig. 5). In the case of 100 μM, ZnCl2 significantly decreased the cell growth without affecting the lethality (Figs. 4 and 5).

3.4. Effects of the combination on cell growth and lethality

As shown in Fig. 4, ZnCl2 at 10-30 μM did not affect the growth inhibition by 3-10 μM clotrimazole. The combination of 100 μM ZnCl2 with clotrimazole further inhibited the cell growth.

ZnCl2 (100 μM) and clotrimazole (10 μM) did not affect the cell lethality, respectively (Fig. 5). However, their combination greatly increased the cell lethality.

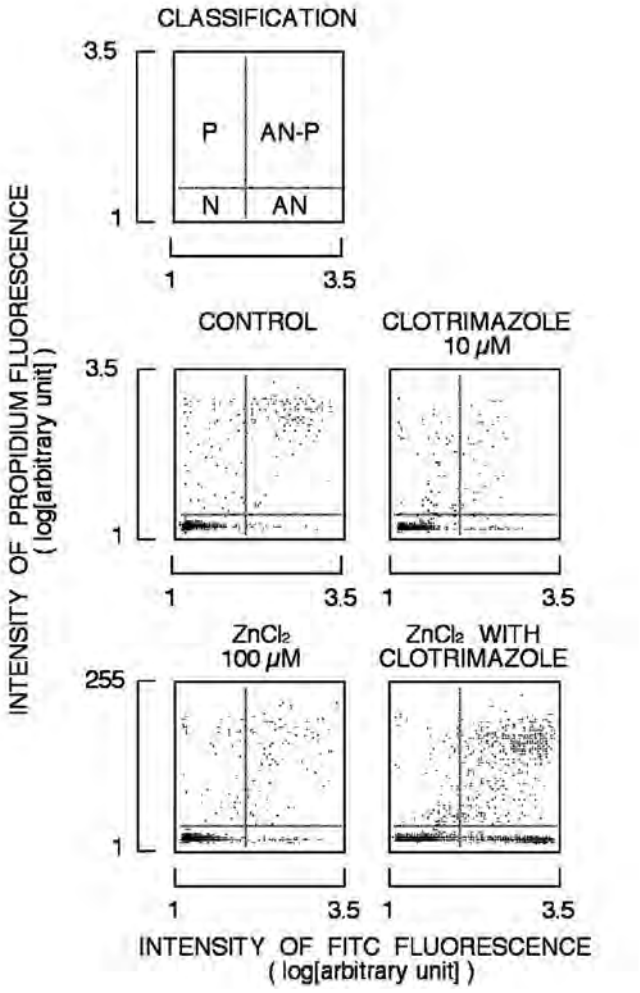

Figure 6

Effects of ZnCl2, clotrimazole, and their combination on fluorescence cytograms. Area N: intact living cells, area AN: the living cells stained with annexin V-FITC, area P: the cells stained with propidium, presumably necrotic dead cells, and area AN-P: the cells stained with annexin V-FITC and propidium, presumably apoptotic dead cells.

3.5. Effect of the combination on process of cell death

ZnCl2 (100 μM) and clotrimazole (10 μM) did not affect the intensity of forward scatter, indicating no change in cell size (not shown), respectively. However, the combination of clotrimazole and ZnCl2 decreased the intensity of forward scatter, indicating decrease in cell size. It was reminiscent of cell shrinkage during an early phase of apoptosis (Hughes and Cidlowski, 1998; Elliott and Higgins, 2003). Therefore, the exposure of phosphatidylserine on outer membrane surface of living cells, one of markers during an early stage of apoptosis, was detected by using the fluorescence cytogram with annexin V-FITC and propidium iodide. The cell population under the simultaneous treatment with ZnCl2 and clotrimazole was different from that under the presence of ZnCl2 or clotrimazole (Figs. 6 and 7). The combination of ZnCl2 and clotrimazole significantly decreased the population of area N, intact living cells. Therefore, the populations of annexin V-positive living cells (area AN) and dead cells (area AN-P) were also analyzed under the presence of ZnCl2, clotrimazole, and their combination. The population of cells stained with propidium, but not annexin V-FITC (area P) was not changed during the experiment. The cell population under the presence of ZnCl2 or clotrimazole was similar to that under the control condition (Fig. 7). On the contrary, the simultaneous application of ZnCl2 and clotrimazole significantly increased both populations (Fig. 7). Thus, the combination induced cell death in human leukemia K562 cells.

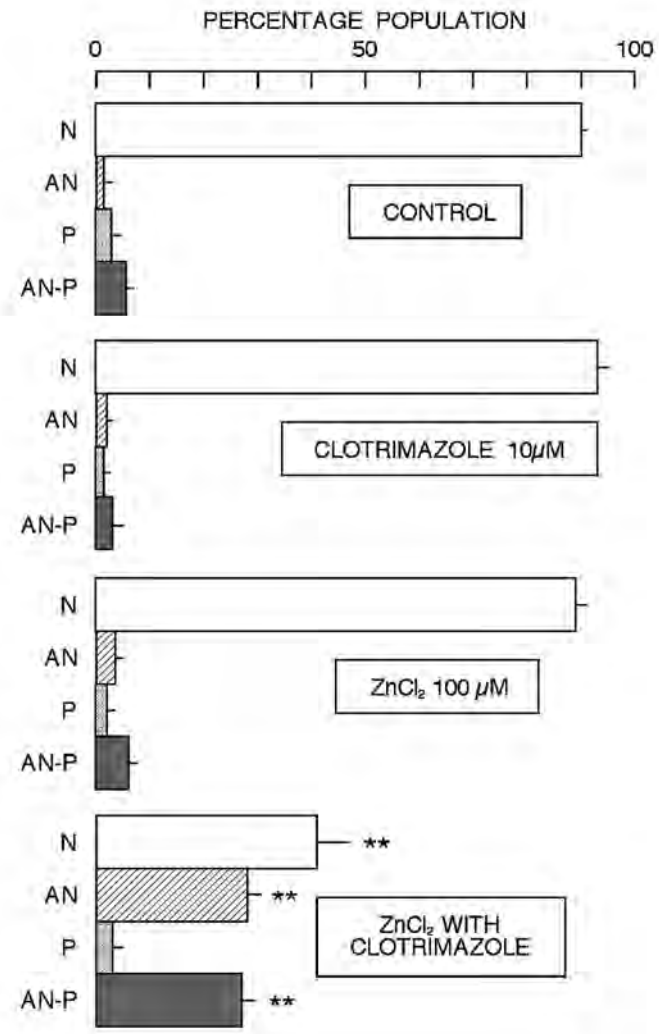

Figure 7

*Effects of ZnCl2, clotrimazole, and their combination on cell populations (N, AN, AN-P and P) classified by propidium and annexin V-FITC. Column and bar respectively indicate mean value and its standard deviation of 4-6 experiments. Symbol (**) shows significant difference between respective control group (control and clotrimazole-treated groups) and test groups. ** P<0.01.*

Zn^{2+} was reported to increase apoptosis at low concentrations (7.5-15 μM) and inhibit it at high concentrations (75-600 μM) in mouse thymocytes (Provinciali *et al.*, 1995). The application of Zn^{2+} chelator induced apoptosis in thymocytes (Hyun *et al.*, 2000) while ZnCl2 inhibited it (Mathieu *et al.*, 1996; Chukhlovi *et al.*, 2001). In this study, the population of cells positive to annexin V, apoptotic cells, was increased in the presence of 100 μM. Therefore, it is presumably suggested that ZnCl2 augments the cytotoxicity of clotrimazole.

There is no clinical report that clotrimazole and zinc are simultaneously used for treatment as far as our knowledge. If azole antifungal drugs and zinc supplement are simultaneously administered, their combination would be harmful for some patients. Clotrimazole is also used as an antimalarial drug. The use of zinc as a nutritional supplement has been necessary in many developing countries because it prevents acute lower respiratory tract infection and diarrhea, and reduces child mortality (Brooks *et al.*, 2005; Shah and Sachdev, 2006). The combination of azole antifungal drugs with zinc supplement may be avoided.

4. CONCLUSION

The present result provide a new insight into toxicological and/or pharmacological characteristics of clotrimazole. This antifungal agent possesses diverse cellular actions such as blockers for Ca^{2+}-dependent K^+ channels (Alvarez *et al.*, 1992; Brugnara *et al.*, 1993; Rettenhouse *et al.*, 1997), voltage-dependent Ca^{2+} channels (Welker and Drews, 1997; Wu *et al.*, 1999), and

sarcoplasmic reticulum Ca^{2+}-ATPase (Snajdrova *et al.*, 1998; Bartolommei *et al.*, 2006). Furthermore, the agent increased the permeation of some divalent cations such as Ca^{2+}, Zn^{2+} and Cd^{2+} (unpublished observations). The actions of zinc are also diverse, so that it is difficult to predict its synergistic and/or antagonistic action in simultaneous presence of drug(s). Thus, the actions of some clinically-used drugs may be modified by endogenous Zn^{2+}.

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