Ziram, a dithiocarbamate fungicide, exhibits pseudo-cytoprotective actions against oxidative stress in rat thymocytes: Possible environmental risks

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Highlights

• Ziram exerted reciprocal actions on H_2O_2-induced cell death.

• Ziram augmented H_2O_2-induced decrease in cellular glutathione levels.

• Ziram enhanced H_2O_2-induced increase in intracellular Zn^{2+} levels.

• Ziram attenuated H_2O_2-induced depolarization of mitochondrial membrane potential.

• Ziram exhibited pseudo-cytoprotective actions against oxidative stress induced by H_2O_2.

Abstract

Ziram, a dithiocarbamate fungicide, protects various vegetables and fruits against infections by fungus. Recently, there have been increasing anxieties about the risks in the use of dithiocarbamate fungicides. Our previous studies showed that Zn^{2+} was a determinant of Ziram cytotoxicity. In addition, Zn^{2+} is linked to H_2O_2 cytotoxicity. Therefore, in this study, we aimed to test the hypothesis that Ziram could augment the cytotoxicity of H_2O_2 by examining the changes induced by Ziram in some cellular parameters in rat thymic lymphocytes subjected to H_2O_2-induced oxidative stress using flow-cytometric methods with fluorescent dyes. Ziram significantly attenuated H_2O_2-induced cell death at sublethal concentrations. However, in the cells under oxidative stress elicited by H_2O_2, Ziram promoted the changing over from intact cells to living cells with exposed phosphatidylserine (PS) on plasma membranes, whereas it inhibited the transition from PS-exposed living cells to dead cells. Ziram significantly augmented H_2O_2 actions, including reduction of cellular glutathione levels and elevation of intracellular Zn^{2+} concentrations. Conversely, it attenuated H_2O_2-induced depolarization of mitochondrial membrane potential. Ziram at sublethal concentrations seems to exhibit promotive and suppressive actions on the process of cell death caused by H_2O_2. Ziram increased the number of living cells with exposed PS, a phenomenon characteristic of early stages of apoptosis. Thus, it is concluded that Ziram exhibits pseudo-cytoprotective actions against H_2O_2-induced oxidative stress.

*** A capsule of no more than two lines that summarizes the main finding**

Ziram at sublethal concentrations exerts promotive and suppressive actions on the process of cell death caused by oxidative stress

Keywords: Ziram; lymphocytes; cytoprotective action; oxidative stress; flow cytometer.

1. Introduction

Ziram, a dithiocarbamate fungicide, is widely used in agriculture to protect various fruits and vegetables against fungal infections (Berrada et al., 2010; Tsakiris et al., 2011; López-Fernández et al., 2012). Recently, there have been increasing anxieties about the environmental and health risks in Ziram use (Tsakiris et al., 2011; Lozowicka et al., 2015). In our previous studies (Kanemoto-Kataoka et al., 2015, 2017), we investigated the adverse actions of Ziram in rat thymocytes. Ziram markedly increased the intracellular Zn^{2+} concentrations ($[Zn^{2+}]i$) and decreased the cellular glutathione content ([GSH]i). In addition, the agent increased Zn^{2+}-dependently the cells that were positive to annexin V. Zn^{2+} chelators greatly attenuated the increase in cell lethality by Ziram. Furthermore, a synergistic increase in cell lethality was observed after simultaneous application of micromolar $ZnCl_2$ and Ziram at a sublethal concentration. Therefore, it was suggested that Zn^{2+} was a determinant of Ziram cytotoxicity (Kanemoto-Kataoka et al., 2017).

Ziram was reported to inhibit both superoxide dismutase and catalase activities (Sbrana et al., 1995). Zinc pyrithione, a zinc ionophore, and $ZnCl_2$ increased cell vulnerability to H_2O_2-induced oxidative stress by increasing $[Zn^{2+}]i$ levels (Matsui et al., 2010; Oyama et al., 2012). Therefore, Ziram might be suggested to increase cell vulnerability to H_2O_2-induced oxidative stress *via* an increase in $[Zn^{2+}]i$ levels and/or induction of oxidative stress. In this study, we tested this hypothesis by cytometrically investigating the adverse actions of sublethal concentrations of Ziram in rat thymic lymphocytes. However, Ziram, at sublethal (nanomolar) concentrations, exhibited a cytoprotective action against H_2O_2-induced oxidative stress. Therefore, we explored the possible mechanisms by which Ziram might decrease H_2O_2-induced cell death. The present study might provide some insights into the risks in the use of Ziram.

2. Materials and methods

2.1. Reagents

Ziram (purity, 99.9 %) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Fluorescent dyes used in this study are listed in Table 1. Other chemicals were purchased from Wako Pure Chemicals, unless otherwise stated.

(Table 1 near here)

2.2. Animals and cell preparation

The Committee for Animal Experiments at the University of Tokushima approved the present study (No. 05279). The cell suspension was prepared as previously described (Chikahisa et al., 1996; Sakanashi et al., 2009; Matsui et al., 2010). Briefly, thymus glands were dissected from anesthetized rats. The slices of thymus glands were ground in chilled Tyrode's solution. The cell suspension was incubated at 36–37 °C for 1 h before the experiment. The suspension contained 216.9 ± 14.4 nM zinc derived from the cell preparation. Ziram (0.003-1 mM in 2 μL of dimethyl sulfoxide) were added to the cell suspensions (2 mL) to achieve final concentrations of 0.003-1 μM, and incubated at 36-37 °C for 1-3 h, depending on experimental purposes. A sample (100 μL) was analyzed using a flow cytometry to evaluate the Ziram-induced changes in membrane and cellular parameters.

Oxidative stress was induced by 100 μM H_2O_2. The treatment with 100 μM H_2O_2 for 3 h induced cell death in about 20 % of cell population under present experimental conditions. The population of dead cells caused by H_2O_2 was increased by adding $ZnCl_2$ and decreased by adding a chelator of intracellular Zn^{2+} (Matsui et al., 2010).

2.3. Fluorescence measurements

Membrane and cellular changes were evaluated using a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan) and respective fluorescent dyes. Wavelength for dye excitation was 488 nM. Dye emissions were detected at a wavelength of 530 ± 20 nm for FITC, 5-CMF, Fluo-3, FluoZin-3, and JC-1 (green) fluorescence and 600 ± 20 nm for PI and JC-1 (red) fluorescence. Data analyses were performed with JASCO software (Version 3.06; JASCO).

Cells with PI fluorescence were dead. 5-CMF, Fluo-3, and FluoZin-3 fluorescence were

recorded from cells without PI fluorescence (living cells). Phosphatidylserine (PS) exposed on membrane surface, an event at initial phase of apoptosis, was revealed with annexin V-FITC (Koopman et al., 1994). The change in cellular glutathione content ([GSH]i) was estimated with 5-CMF-DA. The coefficient of correlation between 5-CMF fluorescence and [GSH]i was 0.965 (Chikahisa et al., 1996). Changes in mitochondrial membrane potentials were estimated with 1 μM JC-1 (Smiley et al., 1991). The cells were treated with 1 μM JC-1 for 1 h before the fluorescence measurement. Fluo-3-AM and FluoZin-3-AM at 1 μM were used to estimate the changes in intracellular Ca^{2+} and Zn^{2+} levels ($[Ca^{2+}]i$ and $[Zn^{2+}]i$), respectively.

2.4. Statistical analysis and data representation

Statistical differences were confirmed using analysis of variance followed by Tukey's *post-hoc* test. *P*-values < 0.05 were considered statistically significant. Data represent the means ± standard deviation of four samples. Each experiment was carried out in triplicate, unless otherwise specified.

3. Results

3.1. Attenuation of H_2O_2-induced increase in cell lethality by Ziram

Treatment of the cells with 100 μM H_2O_2 for 3 h increased the number of cells with PI fluorescence (dead cells). The increase was attenuated in the presence of 0.3 μM Ziram (Fig. 1A). Treatment with 0.3 μM Ziram for 3 h did not alter the number of dead cells. Therefore, Ziram attenuated H_2O_2-induced cell death at 0.003–0.3 μM (Fig. 1B).

(Figure 1 near here)

3.2. Effects of simultaneous treatment with H_2O_2 and Ziram

When the cells were incubated with PI and annexin V-FITC, the treatment with 100 μM H_2O_2 for 2.5 h increased the number of cells with FITC fluorescence but without PI fluorescence (annexin V-positive living cells / AVP living cells) and that of cells with PI fluorescence (dead cells). Ziram treatment at 0.3 μM did not have a significant effect. The

simultaneous treatment of Ziram and H_2O_2 increased the number of AVP living cells, whereas the increase in the number of dead cells by H_2O_2 was significantly attenuated (Fig. 2A). Thus, Ziram significantly promoted H_2O_2-induced change from intact cells to AVP living cells and attenuated that from AVP living cells to dead cells (Fig. 2B).

(Figure 2 near here)

3.3. Enhancement of H_2O_2-induced reduction of cellular non-protein thiol levels by Ziram

Ziram at sublethal levels was expected to exert cytoprotective actions on the cells suffering from oxidative stress by 100 μM H_2O_2. Treatment with 100 μM H_2O_2 for 1.5 h greatly reduced 5-CMF fluorescence intensity, indicating the reduction of [GSH]i. Threshold concentration of Ziram to augment 5-CMF fluorescence was 0.1–0.3 μM (Kanemoto-Kataoka et al., 2015, 2017). Slight increase in the fluorescence was observed in the presence of 0.3 μM Ziram (Fig. 3A and 3B). Simultaneous treatment of H_2O_2 and Ziram for 1.5 h induced a further reduction of the intensity (Fig. 3A). Thus, the simultaneous application of H_2O_2 and Ziram greatly decreased the [GSH]i (Fig. 3B). Therefore, Ziram was found to augment H_2O_2-induced oxidative stress despite its cytoprotective effects against oxidative stress (Fig. 1).

(Figure 3 near here)

3.4. Changes in $[Ca^{2+}]i$ by H_2O_2 and Ziram

We tested the hypothesis that Ziram could reduce H_2O_2-induced elevation of $[Ca^{2+}]i$, resulting in inhibition of H_2O_2-induced cell death by comparing the effects of H_2O_2, Ziram, and their combination on the cells loaded with Fluo-3-AM. Treatment with 100 μM H_2O_2 for 1 h augmented Fluo-3 fluorescence, whereas 0.3 μM Ziram did not affect it (Fig. 4A). Simultaneous application of 100 μM H_2O_2 and 0.1–0.3 μM Ziram slightly increased the intensity, compared to that observed in the presence of H_2O_2 alone (Fig. 4B). This increase by 0.3 μM Ziram was statistically significant. Thus, this hypothesis related to the $[Ca^{2+}]i$ was rejected.

(Figure 4 near here)

3.5. Changes in $[Zn^{2+}]i$ levels by H_2O_2 and Ziram

Both H_2O_2 and Ziram increased $[Zn^{2+}]i$ levels in this preparation (Matsui et al., 2010; Kanemoto-Kataoka et al., 2015). We evaluated the changes in $[Zn^{2+}]i$ levels in FluoZin-3-loaded cells treated with 100 μM H_2O_2, 0.1–0.3 μM Ziram, or both. Treatment with 100 μM H_2O_2 or 0.3 μM Ziram for 1 h significantly moved the histogram of FluoZin-3 fluorescence to higher direction (Fig. 5A). Simultaneous treatment with H_2O_2 and Ziram for 1 h greatly augmented the intensity. Thus, combination of H_2O_2 and Ziram markedly increased $[Zn^{2+}]i$. Results are summarized in Fig. 5B.

(Figure 5 near here)

3.6. Ca^{2+}-induced cell death in the presence of Ziram

Treatment of the cells with 100 nM A23187 for 3 h increased the number of cells with PI fluorescence (dead cells). The A23187-induced increase in cell lethality was not observed under external Ca^{2+}-free conditions, suggesting that A23187-induced cell death is Ca^{2+}-dependent (Sakanashi et al., 2008, 2009). Although Ziram at 0.1–0.3 μM did not increase cell lethality, combination of 100 nM A23187 and 0.1–0.3 μM Ziram synergistically increased cell lethality (Fig. 6). Both H_2O_2 and A23187 were shown to induce cell death (Figs. 1 and 6). Ziram attenuated H_2O_2-induced cell lethality (Fig. 1), whereas it enhanced A23187-induced cell death (Fig. 6). Combination of 100 μM H_2O_2 and 100 nM A23187 synergistically increased cell lethality.

(Figure 6 near here)

3.7. Changes in mitochondrial membrane potential by Ziram and H_2O_2

Treatment with 100 μM H_2O_2 for 2 h decreased the intensity of red JC-1 fluorescence intensity and increased that of green JC-1 fluorescence, which indicated that H_2O_2 induced depolarization of mitochondrial membrane potentials. Thus, the ratio of the green JC-1 fluorescence intensity to that of the red fluorescence increased. Ziram at 0.1–0.3 μM did not affect the ratio of JC-1 fluorescence. Treatment with 0.1–0.3 μM Ziram significantly attenuated H_2O_2-induced changes in the ratio of JC-1 fluorescence. Thus, Ziram was suggested to decrease

H_2O_2-induced depolarization of mitochondrial membrane potentials.

(Figure 7 near here)

4. Discussion

4.1. Pseudo-cytoprotective actions of Ziram against H_2O_2-induced oxidative stress

Ziram at 0.003–0.3 μM significantly attenuated cell death induced by H_2O_2 (Fig. 1 and 2). It was postulated that Ziram could exert protective actions on the cells under oxidative stress induced by H_2O_2. However, Ziram accelerated the 'dying' process before H_2O_2-promoted cell death because of following effects observed upon simultaneous treatment with H_2O_2 and Ziram. First, the number of intact cells greatly decreased, whereas H_2O_2-induced increase in the number of dead cells decreased (Fig. 2). Consequently, the population of living cells with exposed PS increased. The PS exposure on outer membranes is one of early events of apoptosis (Koopman et al., 1994). Thus, Ziram was suggested to prompt the initial process of H_2O_2-induced cell death. Second, Ziram profoundly augmented H_2O_2-induced increase in $[Zn^{2+}]$i levels and decrease in [GSH]i levels (Fig. 3 and 5). The correlation between H_2O_2-induced elevation of $[Zn^{2+}]$i concentrations and reduction of [GSH]i was −0.936 at H_2O_2 concentrations of 3–100 μM (Matsui et al., 2010). Thus, Ziram might potentiate some cytotoxic actions of H_2O_2. However, Ziram attenuated H_2O_2-induced depolarization of mitochondrial membrane potentials (Fig. 7). The mitochondrial membrane potential plays a crucial role in ATP synthesis (Dimroth et al., 2000), where loss of the mitochondrial membrane potential induces cell death (Kroemer et al., 2007). Therefore, the attenuation of H_2O_2-induced depolarization of mitochondrial membrane potential by Ziram might delay the change from PS-exposed living cells to dead cells (Fig. 2). Although Ziram is likely to exert reciprocal actions on the cells subjected to oxidative stress, the fate of the cells is not affected because the macrophages phagocytose the apoptotic lymphocytes after specific recognition of the exposed PS (Fadok et al., 1992). Thus, it was concluded that Ziram exhibited pseudo-cytoprotective actions against oxidative stress induced by H_2O_2.

One may argue the possibility that Ziram interferes apoptosis process, leading to delay the change from PS-exposed living cells to dead cells. Although Ziram itself at 0.3 μM did not increase the population of PS-exposed living cells, Ziram further increased the population of PS-exposed living cells but decreased the population of dead cells in the presence of H_2O_2. Therefore, the possibility can not ruled out because the elevation of cytosolic ATP level is a requisite to the apoptotic cell death process (Eguchi et al., 1997; Tsujimoto, 1997; Zamaraeva et al., 2005) and Ziram decreased the mitochondrial activity in this study. Ziram was reported to possess the genotoxic actions under in vivo and in vitro conditions (Scarabelli et al., 1993; Mosesso et al., 1994). Ziram is supposed to damage DNA. DNA damage triggers apoptosis because the cells prevent genotoxic mutation by either apoptosis or DNA repair (Ross and Kaina, 2006). However, apoptosis in some cells may be not completed in the presence of Ziram. Ziram causes various cellular actions (Rath et al., 2011). Of Ziram-induced cellular actions, intracellular Ca^{2+} dysregulation and oxidative stress are generally causes to induce cell death (Ermak and Davies, 2002; Orrenius et al., 2015). Therefore, the combination of Ziram and H_2O_2 may induce further complex actions on the cells. In addition, Mancozeb decreased mitochondrial membrane potential causing activation of caspase cascade, increasing oxidative stress (Srivastava et al., 2012). Thus, the observed effects are not exclusive to Ziram.

4.2. Potentiation of A23187-induced cytotoxicity by Ziram

Treatment of rat thymocytes with 100 nM A23187 induced cell death (Fig. 6). Removal of Ca^{2+} inhibits A23187-induced cell death, indicating Ca^{2+}-dependency (Sakanashi et al., 2008, 2009). Ziram at 0.1–0.3 μM promoted Ca^{2+}-dependent cell death induced by A23187 (Fig. 6). However, Ziram (0.1-0.3 μM) did not increase the $[Ca^{2+}]i$. Furthermore, Ziram (0.3 μM) slightly augmented H_2O_2-induced elevation of $[Ca^{2+}]i$ (Fig. 4). Therefore, Ziram is unlikely to significantly affect the intracellular Ca^{2+} homeostasis. In a previous study (Sakanashi et al., 2009), the chelator of intracellular Zn^{2+} did not inhibit A23187-induced cell lethality, indicating that Zn^{2+} was not involved. Therefore, Ziram was suggested to exert cytotoxic actions that

promote Ca^{2+}-dependent cell death.

4.3. Implications in environmental sciences

The effects of Ziram (molecular weight: 305.796) were observed at concentrations ranging from 0.003 to 0.3 μM (approximately from 0.92 to 91.7 μg/L) in this study. Dimethyldithiocarbamate, a dithiocarbamate fungicide metabolite, was identified in the coastal environments, Hiroshima Bay, Hiroshima Prefecture, Japan (Hano et al., 2015). The concentrations of dimethyldithiocarbamate in the sediment core of Hiroshima Bay ranged from 'not detected' to 1.7 μg/kg-dry (Hano et al., 2015). Although no monitoring data for Ziram or its degradate (Thiram) are available from the National Water-Quality Assessment Project of US Geological Survey in the US Department of the Interior, the predicted Ziram concentrations in the aquatic environment, near fruits farms in California, USA are 9.89–43.7 μg/L (Environmental Fate and Effects Division, 2008). In this study, Ziram at nanomolar concentrations was suggested to practically promote the process of cell death induced by oxidative stress and Ca^{2+} overload. Both insults are common in physiological and pathological states (Ermak and Davies, 2002; Filomeni et al., 2015; Orrenius et al., 2015). Cell death is important because it allows the removal of the unwanted cells in a timely manner. Therefore, Ziram might affect the processes of cell death, including apoptosis and autophagy, resulting in disturbance of the normal development of fetus and immature animals.

Conflict of interest

All authors have no conflicts of interest.

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Figure legends

Fig. 1. Changes in the population of cells exhibiting PI fluorescence by Ziram, H_2O_2, and their combination. Effects were examined 3 h after the respective application(s). (A) Changes in cytogram (forward scatter *versus* PI fluorescence) by Ziram, H_2O_2, and their combination. Each cytogram included 2500 cells. Dotted line under cytogram indicates the area of cells exhibiting PI fluorescence. (B) Cell lethality (percentage of cells exhibiting PI fluorescence) after treatment with Ziram or Ziram + H_2O_2. Asterisk (**) indicates significant difference ($P < 0.01$) between the control group (control) and drug-treated cells. Symbol (##) indicates significant difference ($P < 0.01$) between the H_2O_2-treated cells and cells co-treated with H_2O_2 and Ziram. Data represent the mean cell lethality and standard deviation of four samples. Dotted bars are placed for comparison with the respective control.

Fig. 2. Changes in the population of cells classified with annexin V-FITC and PI by Ziram, H_2O_2, and their combination. Effects were examined 2.5 h after drug application. (A) Changes in cytogram (PI fluorescence *versus* FITC fluorescence) by Ziram, H_2O_2, and their combination. N, Intact living cells; A, AVP living cells; P, Annexin V-negative dead cells, and AP: AVP dead cells. Each cytogram included 2500 cells. (B) Changes in the percentage of N (Intact living cells), A (AVP living cells), and P + AP (Dead cells). Values represent the mean cell lethality and standard deviation of four samples. Asterisk (**) indicates significant difference ($P < 0.01$) between the control group (control) and drug-treated cells. Symbol (##) indicates significant difference ($P < 0.01$) between the H_2O_2-treated cells and those co-treated with H_2O_2 and Ziram.

Fig. 3. Changes in 5-CMF fluorescence of the cells treated with Ziram, H_2O_2, and their combination. Effects were examined 1.5 h after drug application. (A) Changes in the histogram of 5-CMF fluorescence by the respective treatments. Histogram was constructed with 2500 cells.

(B) Changes in the mean intensity of 5-CMF fluorescence of the cells treated with Ziram, H_2O_2, and their combination. Values represent the mean intensity and standard deviation of four samples. Asterisks (*, **) indicate significant difference ($P < 0.05$, $P < 0.01$) between the control group (control) and drug-treated cells. Symbols (#, ##) indicate significant difference ($P < 0.05$, $P < 0.01$) between the H_2O_2-treated cells and those co-treated with H_2O_2 and Ziram.

Fig. 4. Changes in Fluo-3 fluorescence of the cells treated with Ziram, H_2O_2, and their combination. Effects were examined 1 h after drug application. (A) Changes in the histogram of Fluo-3 fluorescence by the respective treatments. Histogram was constructed with 2500 cells. (B) Changes in the mean intensity of Fluo-3 fluorescence of the cells treated with Ziram, H_2O_2, and their combination. Data represent the mean intensity and standard deviation of four samples. Asterisk (**) indicates significant difference ($P < 0.01$) between the control group (control) and drug-treated cells. Symbol (#) indicates significant difference ($P < 0.05$) between the H_2O_2-treated cells and those co-treated with H_2O_2 and Ziram.

Fig. 5. Changes in FluoZin-3 fluorescence of the cells treated with Ziram, H_2O_2, and their combination. Effects were examined 1 h after drug application. (A) Changes in the histogram of FluoZin-3 fluorescence by the respective treatments. Histogram was constructed with 2500 cells. (B) Changes in the mean intensity of FluoZin-3 fluorescence of the cells treated with Ziram, H_2O_2, and their combination. Values represent the mean intensity and standard deviation of four samples. Asterisk (**) indicates significant difference ($P < 0.01$) between the control group (control) and drug-treated cells. Symbol (##) indicates significant difference ($P < 0.01$) between the H_2O_2-treated cells and those co-treated with H_2O_2 and Ziram.

Fig. 6. Changes in lethality of cells treated with Ziram, A23187, and their combination. Effects were examined 3 h after drug application. Values represent the mean cell lethality and standard

deviation of four samples. Asterisk (**) indicates significant difference ($P < 0.01$) between the control group (control) and drug-treated cells. Symbols (#, ##) indicate significant difference ($P < 0.05$, $P < 0.01$) between the H_2O_2-treated cells and those co-treated with H_2O_2 and A23187.

Fig. 7. Changes in the ratio of green JC-1 fluorescence to red JC-1 fluorescence in the cells treated with Ziram, H_2O_2, and their combination. Effects were examined 2.5 h after drug application. Values represent the mean intensity and standard deviation of four samples. Asterisk (**) indicates significant difference ($P < 0.01$) between the control group (control) and drug-treated cells. Symbols (#, ##) indicate significant difference ($P < 0.05$, $P < 0.01$) between the H_2O_2-treated cells and those co-treated with H_2O_2 and Ziram.

FIGURE 1

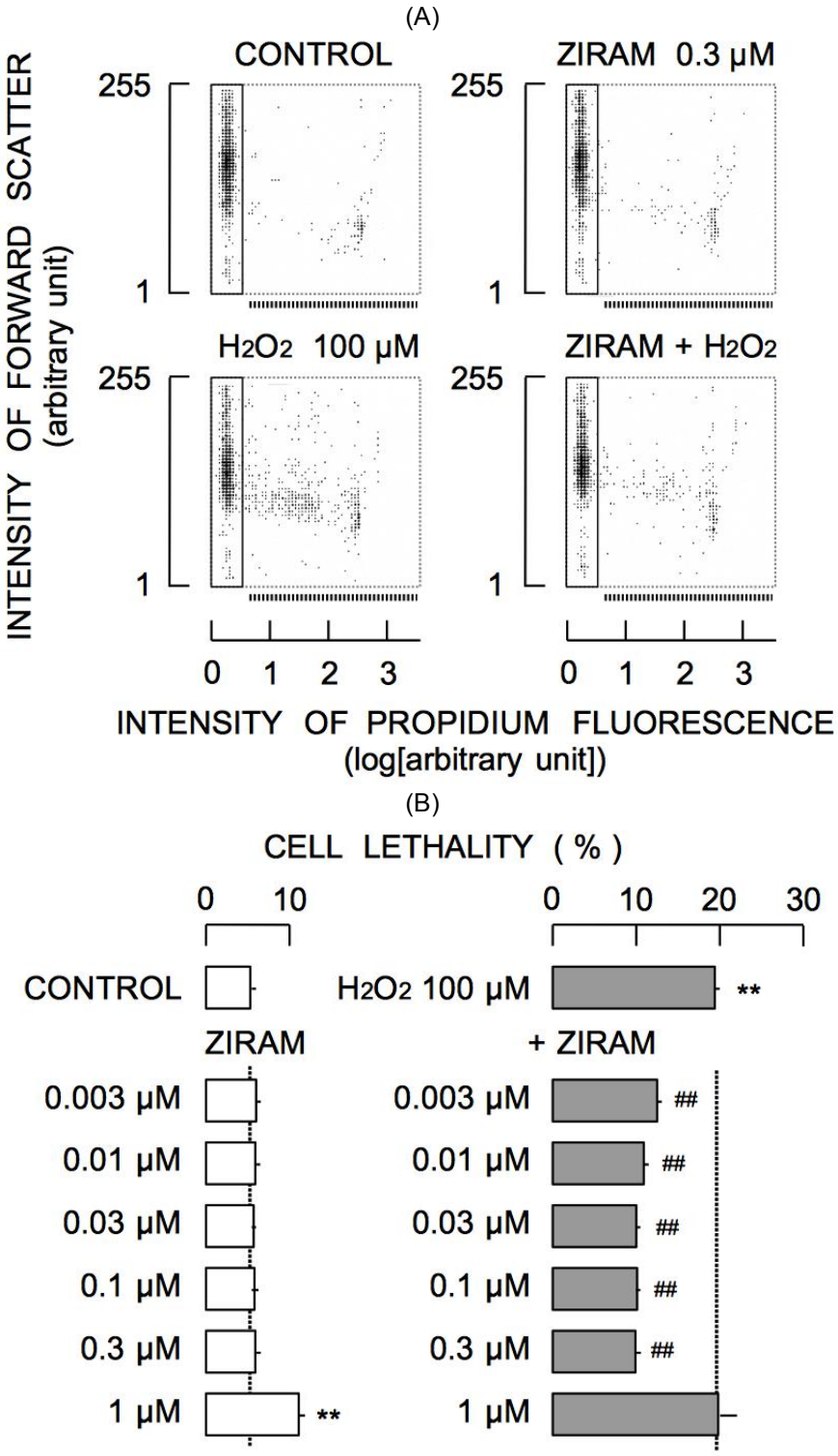
(A)
CONTROL
ZIRAM 0.3 μM
H2O2 100 μM
ZIRAM + H2O2
255
1
INTENSITY OF FORWARD SCATTER
(arbitrary unit)
0 1 2 3
INTENSITY OF PROPIDIUM FLUORESCENCE
(log[arbitrary unit])
(B)
CELL LETHALITY (%)
0 10
0 10 20 30
CONTROL
H2O2 100 μM
**
ZIRAM
+ ZIRAM
0.003 μM
0.01 μM
0.03 μM
0.1 μM
0.3 μM
1 μM
##

FIGURE 2

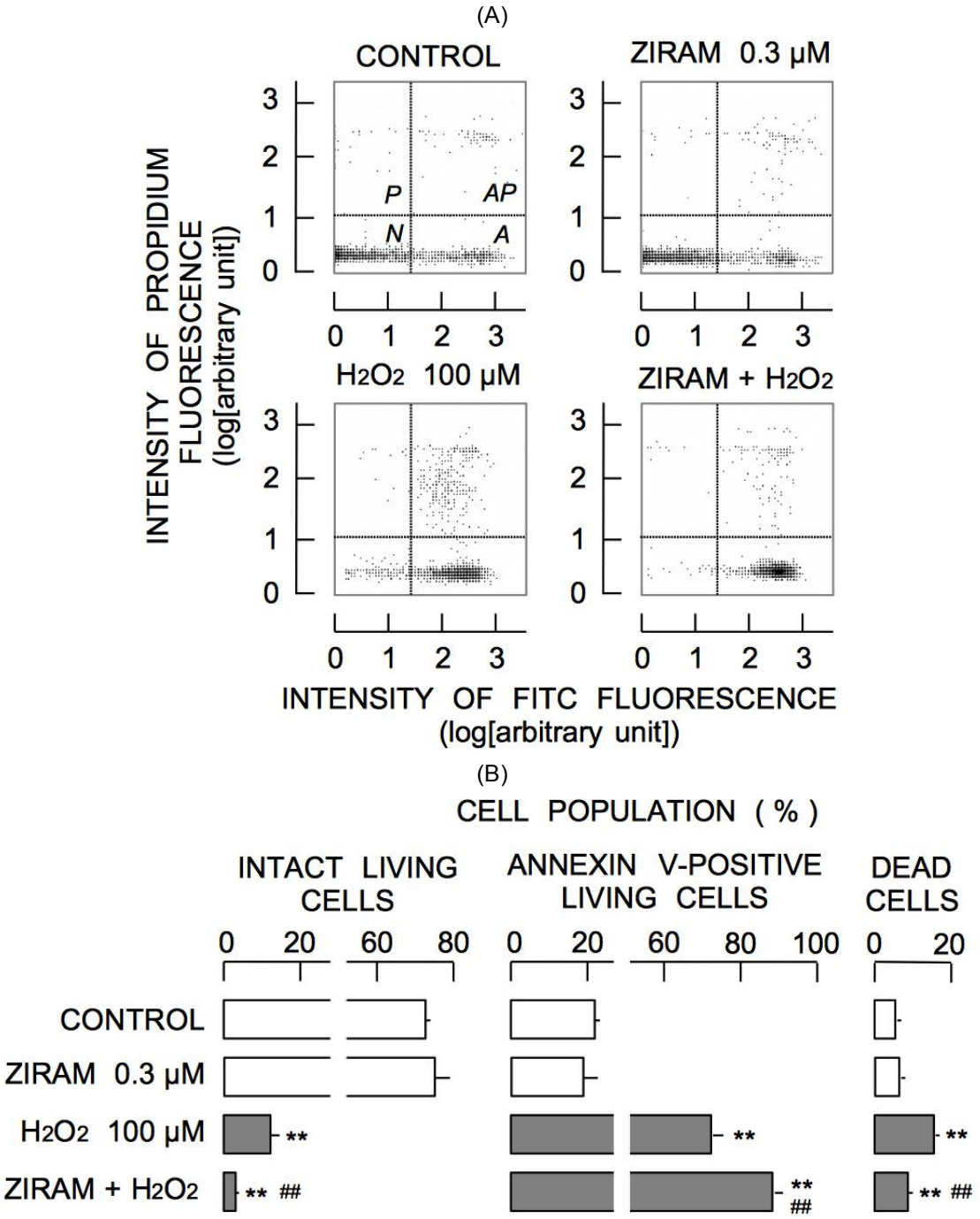
(A)
CONTROL
ZIRAM 0.3 μM
H2O2 100 μM
ZIRAM + H2O2
P
AP
N
A
INTENSITY OF PROPIDIUM FLUORESCENCE (log[arbitrary unit])
INTENSITY OF FITC FLUORESCENCE (log[arbitrary unit])
(B)
CELL POPULATION (%)
INTACT LIVING CELLS
ANNEXIN V-POSITIVE LIVING CELLS
DEAD CELLS
CONTROL
ZIRAM 0.3 μM
H2O2 100 μM
ZIRAM + H2O2
** ##

FIGURE 3

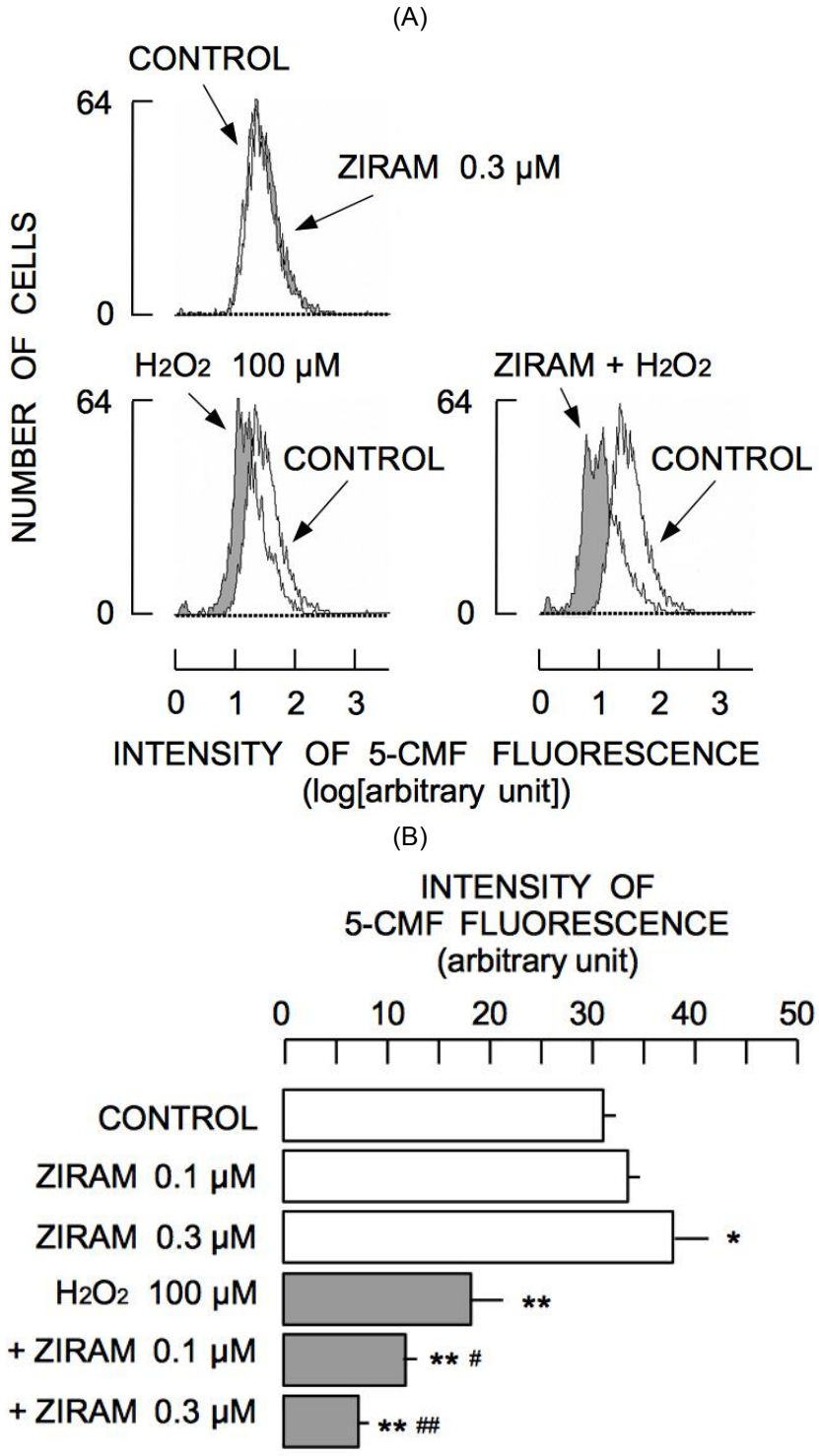
(A)
CONTROL
64
ZIRAM 0.3 μM
0
NUMBER OF CELLS
H2O2 100 μM
64
CONTROL
0
ZIRAM + H2O2
64
CONTROL
0
0 1 2 3
0 1 2 3
INTENSITY OF 5-CMF FLUORESCENCE
(log[arbitrary unit])
(B)
INTENSITY OF
5-CMF FLUORESCENCE
(arbitrary unit)
0 10 20 30 40 50
CONTROL
ZIRAM 0.1 μM
ZIRAM 0.3 μM
*
H2O2 100 μM
**
+ ZIRAM 0.1 μM
** #
+ ZIRAM 0.3 μM
** ##

FIGURE 4

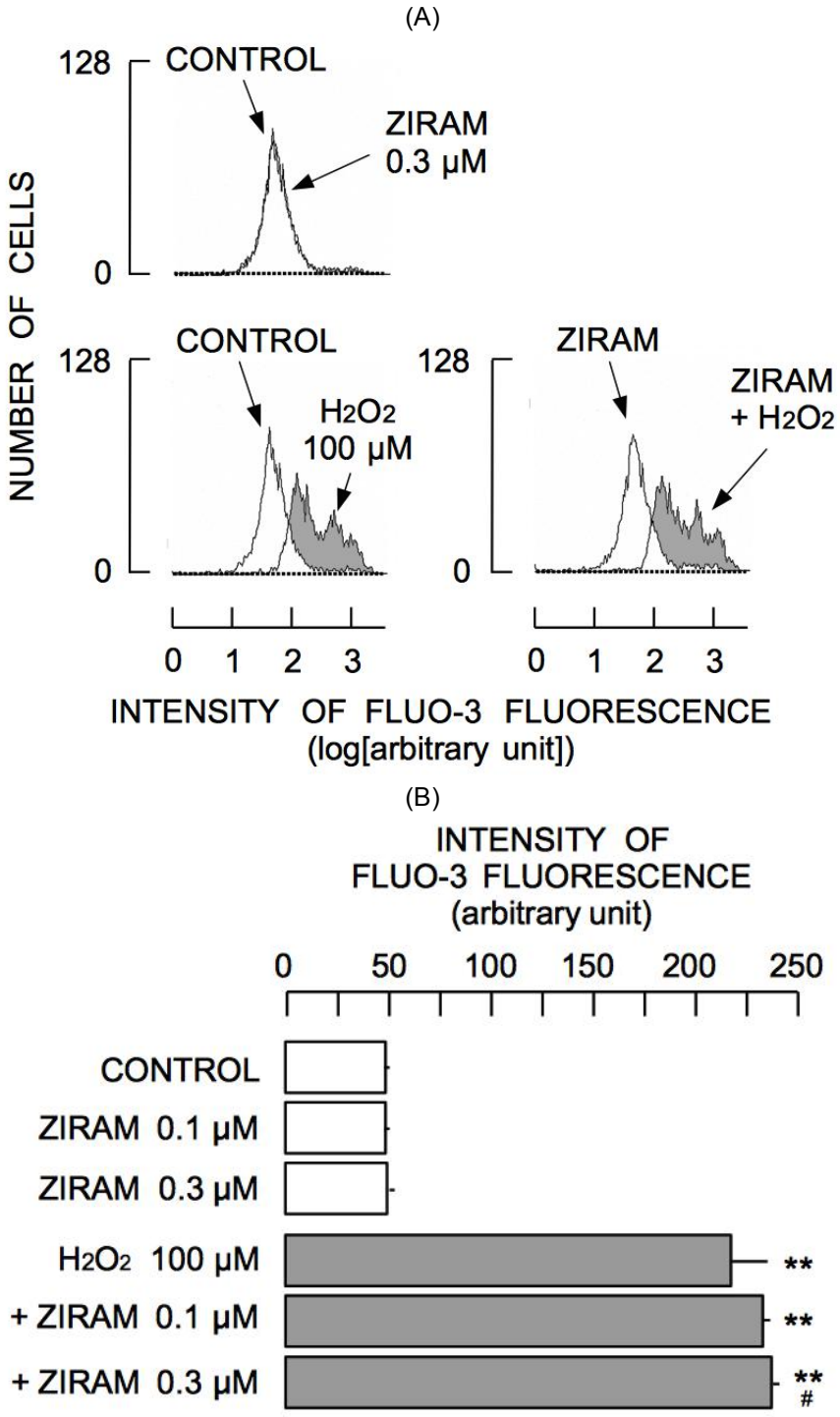
(A)
128
CONTROL
ZIRAM
0.3 μM
0
NUMBER OF CELLS
128
CONTROL
H2O2
100 μM
0
128
ZIRAM
ZIRAM
+ H2O2
0
0 1 2 3
0 1 2 3
INTENSITY OF FLUO-3 FLUORESCENCE
(log[arbitrary unit])
(B)
INTENSITY OF
FLUO-3 FLUORESCENCE
(arbitrary unit)
0 50 100 150 200 250
CONTROL
ZIRAM 0.1 μM
ZIRAM 0.3 μM
H2O2 100 μM
+ ZIRAM 0.1 μM
+ ZIRAM 0.3 μM
**
**
**
#

FIGURE 5

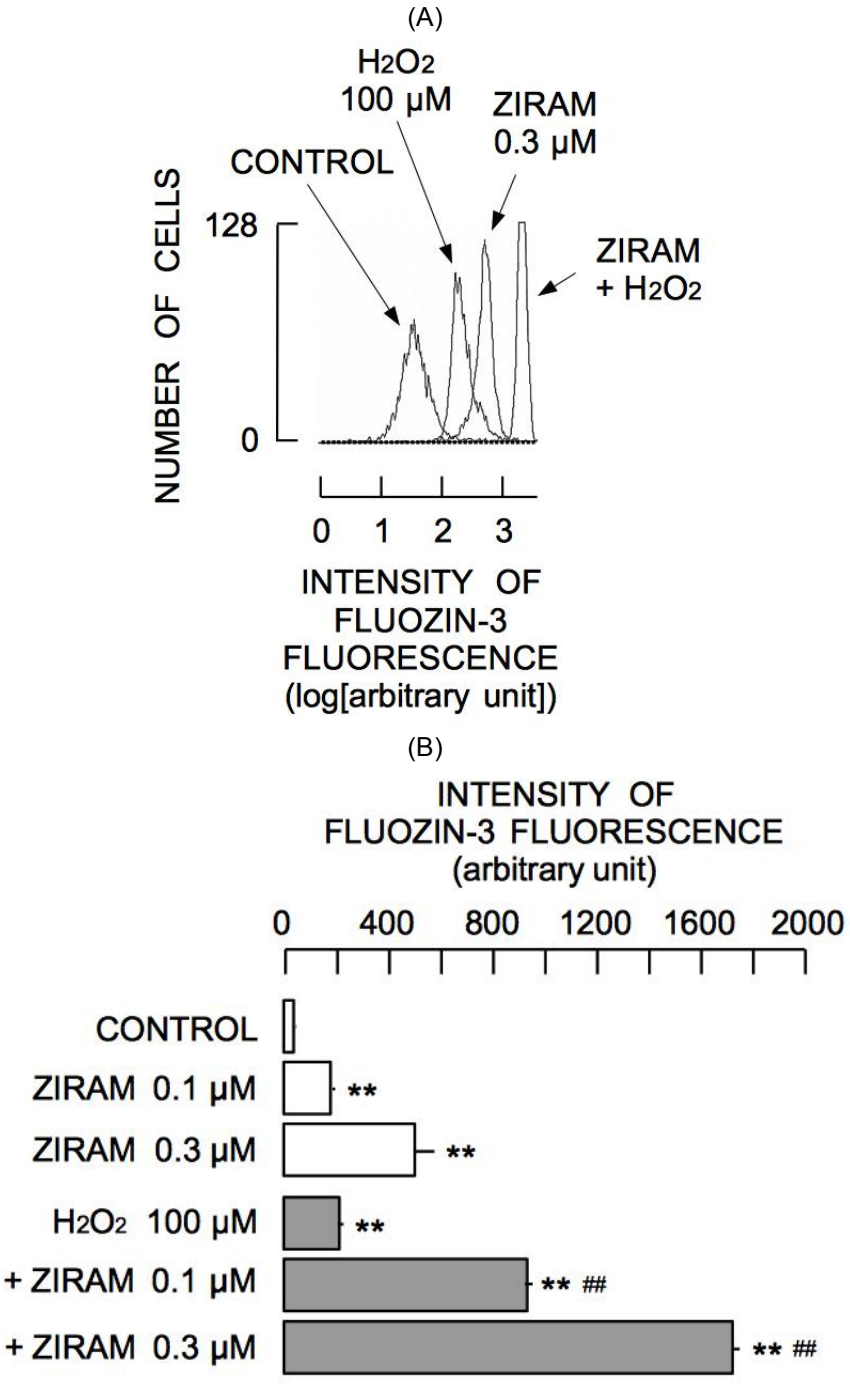
(A)
H2O2
100 μM
ZIRAM
0.3 μM
CONTROL
ZIRAM
+ H2O2
NUMBER OF CELLS
128
0
0 1 2 3
INTENSITY OF
FLUOZIN-3
FLUORESCENCE
(log[arbitrary unit])
(B)
INTENSITY OF
FLUOZIN-3 FLUORESCENCE
(arbitrary unit)
0 400 800 1200 1600 2000
CONTROL
ZIRAM 0.1 μM
ZIRAM 0.3 μM
H2O2 100 μM
+ ZIRAM 0.1 μM
+ ZIRAM 0.3 μM
** ##

FIGURE 6

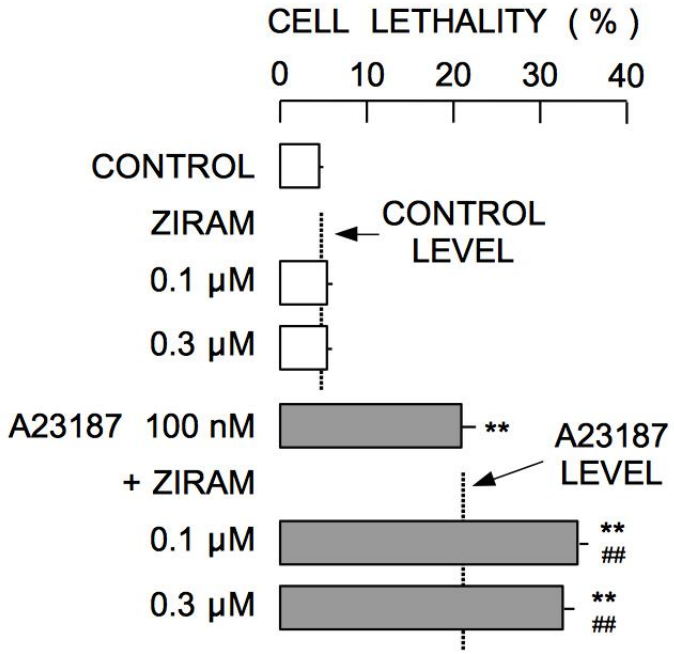
CELL LETHALITY (%)
0
10
20
30
40
CONTROL
ZIRAM
CONTROL LEVEL
0.1 μM
0.3 μM
A23187 100 nM
**
A23187 LEVEL
+ ZIRAM
0.1 μM
** ##
0.3 μM
** ##

FIGURE 7

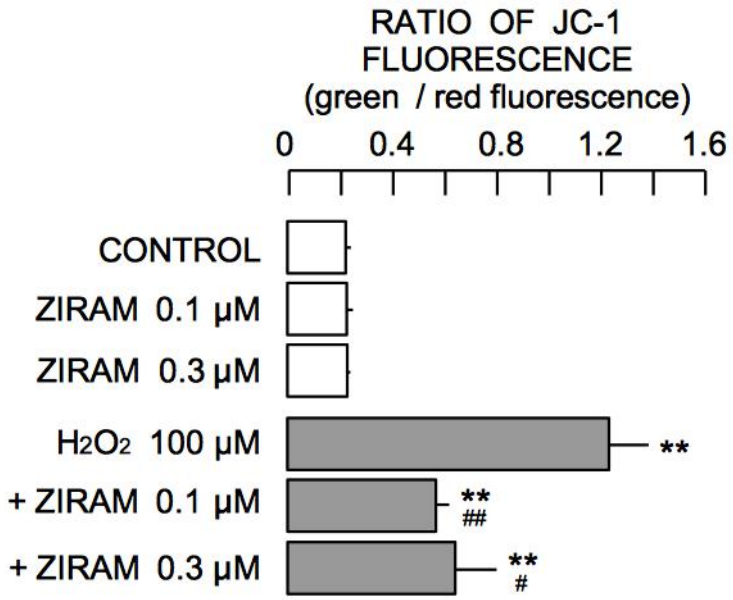
RATIO OF JC-1
FLUORESCENCE
(green / red fluorescence)
0
0.4
0.8
1.2
1.6
CONTROL
ZIRAM 0.1 µM
ZIRAM 0.3 µM
H2O2 100 µM
+ ZIRAM 0.1 µM
+ ZIRAM 0.3 µM
**
**
##
**
#

Table 1.

Fluorescent probes used in this study.

Fluorescent probes / Manufacture
Propidium iodide (PI) / Invitrogen (Eugene, OR, USA)
Annexin V-FITC / Invitrogen
FluoZin-3-AM / Invitrogen
Fluo-3-AM / Dojin Chemical Laboratory (Kumamoto, Japan)
5-Chloromethylfluorescein diacetate (5-CMF-DA) / Invitrogen
JC-1 / Invitrogen