Conflicting actions of 4-vinylcatechol in rat lymphocytes under oxidative stress induced by hydrogen peroxide

Takumi Kishida[*,1], Yurie Funakoshi[*,1], Yuya Fukuyama[2], Sari Honda[2], Toshiya Masuda[2], Yasuo Oyama[1]

[1] Faculty of Integrated Arts and Sciences, Tokushima University, Tokushima 770-8502, Japan

[2] Graduate School of Human Life Science, Osaka City University, Osaka 558-8585, Japan

[*] These authors equally contributed to this research work.

Running dead: Conflicting actions of 4-vinylcatechol

Corresponding author: Y. Oyama (oyamay@tokushima-u.ac.jp)

Highlights

• 4VC at 1–10 μM attenuated the cytotoxicity of 300 μM H_2O_2 in rat lymphocytes

• 4VC slowed the process of cell death induced by H_2O_2.

• 4VC prompted elevations in intracellular Zn^{2+} and Ca^{2+} levels under oxidative stress.

• 4VC exerted conflicting actions on the cells under oxidative stress.

ABSTRACT

4-Vinylcatechol (4VC) has been identified as an aroma compound in roasted foods, especially coffee. It is also a component in traditional herbal medicines. This compound may be subconsciously ingested through foods and herbs. Recent experimental evidence has shown that 4VC possesses an antioxidative action. However, the antioxidative action of 4VC at cellular levels is not well characterized. The effects of 4VC (0.1–100 μM) were examined on rat thymic lymphocytes without and with oxidative stress induced by 300 μM hydrogen peroxide (H_2O_2). Cell treatment with 100 μM 4VC alone for 4 hr significantly increased the population of dead cells. Thus, 4VC at 100 μM or above elicits cytotoxicity. However, 4VC at sublethal concentrations (1–10 μM) significantly attenuated the H_2O_2-induced increase in cell lethality in a concentration-dependent manner. While application of 10 μM 4VC slowed the process of cell death induced by H_2O_2, 4VC did not antagonize the H_2O_2-induced reduction of cellular nonprotein thiols. Although 4VC at 10 μM did not affect intracellular Ca^{2+} and Zn^{2+} levels, the agent potentiated the H_2O_2-induced increases in these levels. These actions of 10 μM 4VC are adverse to the cells under the oxidative stress. However, 10 μM 4VC partly attenuated the cell death induced by 100 nM A23187, a calcium ionophore. There are conflicting actions of 4VC at 1–100 μM on the cells under oxidative stress although the agent is used for an antioxidant. Thus, caution is required when using 4VC as a therapeutic agent.

Keywords: 4-vinylcatechol; lymphocytes; hydrogen peroxide; intracellular Zn^{2+}; intracellular Ca^{2+}; nonprotein thiol; cytotoxicity

Introduction

4-Vinylcatechol (4VC) was first identified as a component in an organic solvent extract of roasted coffee beans (Prescott et al., 1937). It has been identified as an aroma compound in roasted foods, especially coffee (Jiang and Peterson, 2010). 4VC, which is produced from caffeic acid and its derivatives during the roasting process, is an intermediate to other bitter compounds found in coffee (Frank et al. 2007). The high reactivity of 4VC has attracted the attention of scientists because it stabilizes the red color of wine in a condensation reaction with anthocyanin (Schwarz et al. 2003). Furthermore, 4VC is a component in traditional herbal medicines, such as the extracts of *Barleria lupulina* and *Morinda citrifolia* (Senger and Cao, 2016). This compound may be subconsciously ingested through foods and herbs. A recent study showed that 4VC possesses an antioxidant activity (Senger and Cao, 2016) and hastens the rate of diabetic wound healing (Long et al., 2016). The efficacy of 4VC as an antioxidant preservative has been proven also in edible oil models (Jai et al., 2015). However, the antioxidant properties and/or cytoprotective properties of 4VC in mammalian cells under oxidative stress are not well characterized. In this study, some characteristics of the actions of 4VC were examined on rat thymic lymphocytes under oxidative stress induced by H_2O_2 using flow cytometric techniques with the appropriate fluorescent probes. The results revealed the cytoprotective actions of 4VC, as well as some adverse actions, on the cells simultaneously incubated with H_2O_2. Such information is very crucial for drug safety when 4VC is used clinically.

Materials and methods

4-Vinylcatechol

Caffeic acid (purity > 98 % or more) was a product obtained from Tokyo Kasei (Tokyo, Japan). Dimethylformamide was obtained from Kanto Chemical (Tokyo, Japan). Silica gel 60 was obtained from Merck (Darmstadt, Germany). Other reagents, including solvents, were purchased from Nacalai Tesque (Kyoto, Japan).

4VC was synthesized, with slight modifications, according to the previous study of Jia et al. (2015). Sodium acetate (100 mg) as a catalyst was added to a dimethylformamide solution (40 mL) of caffeic acid (1 g). The solution was heated to 130°C for 15 min and extracted three times with diethylether (80 mL each time) after cooling to room temperature. The diethylether layers were combined and washed with saturated NaCl solution (400 mL H_2O). Evaporation of the diethylether solution was performed under reduced pressure. To obtain 4VC, the residue was purified by silica gel column chromatography eluted with ethyl acetate-n-hexane (1:2). The purified material was identified as 4VC by comparison with previously reported NMR data (Jia et al., 2016).

Fluorescent probes and other reagents

Propidium iodide, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CMDCF-DA), Annexin V-fluorescein isothiocyanate (annexin V-FITC), FluoZin-3-*tetra*(acetoxymethyl)ester (FluoZin-3-AM), and 5-chloromethylfluorescein diacetate (5-CMF-DA) were obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Fluo-3-AM was obtained from Dojin Chemical Laboratory (Kumamoto, Japan). A23187, a calcium ionophore, was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals were obtained from Wako Pure Chemicals unless mentioned.

Cell preparation

The present study on rats was approved by the Animal Experiment Committee of Tokushima University (T29-54).

The thymus glands were quickly dissected from 12 thiopental-anesthetized male Wistar rats (6–12 weeks). They were razor-sliced and triturated in Tyrode's solution, buffered with HEPES, to obtain a single-cell suspension (Chikahisa et al., 1996). The suspension was passed through a 50 μm filter before use in the experiments. The cells were incubated at 36–37°C at least 1 hr before any experiments.

Experimental procedures and cytometric measurements

All experiments using the cell suspension were carried out at 36–37°C. 4VC, dissolved and diluted (0.1–100 mM) in DMSO, was added to the cell suspension to achieve various final concentrations (0.1–100 μM) of 4VC. Final concentration of DMSO for testing cell lethality was 0.1 %. Control groups (without 4VC) contained 0.1 % DMSO. When CMDCF, 5-CMF, Fluo-3, and FluoZin-3 were used, final DMSO concentration was totally 0.2 % for testing cellular parameters because these fluorescent probes were initially dissolved in DMSO. The incubation of DMSO at 0.3% or less for 4 h did not affect cell viability and other cellular parameters estimated with fluorescent probes used in this study. Experiments were conducted in the presence of DMSO with and without 4VC.

300 μM H_2O_2 was used to give oxidative stress to the cells because of following observation. The incubation time with 100–300 μM H_2O_2 to induce cell death was 3–4 h. The cell lethality was 20–40 % after 4 h incubation with 300 μM H_2O_2 although the value varied from preparation to preparation. However, the incubation with 300 μM H_2O_2 for 1 h or shorter did not increase the cell lethality. A23187, a calcium ionophore, at 100 nM for intracellular Ca^{2+} overload induced cell death in 20–40% of rat thymocytes within 3–4 h after the application while it was not the case under Ca^{2+}-free conditions (Nishizaki et al., 2003; Sakanashi et al., 2008). A23187 and 4VC were simultaneously added to the cell suspension.

Fluorescence analysis was performed using a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan with JASCO software, Version 3.06). Cell lethality was assessed by adding 5 μM

propidium iodide at 2 min before the measurement (Darzynkiewicz et al., 1992). CM-DCF-DA was used to monitor reactive oxidants such as H_2O_2 (Redondo et al., 2005; Koopman et al., 2006). The cells were preloaded with 10 μM CM-DCF-DA for 60 min before the experiment (the measurement of CM-DCF fluorescence). Exposed phosphatidylserine on the outer surface of the cell membrane was detected by FITC fluorescence after treating the cells with 10 μL/mL of Annexin V-FITC and 5 μM propidium iodide for 30 min (Koopman et al., 1994). 5-Chloromethylfluorescein diacetate (5-CMF-DA) was employed to estimate the changes in cellular content of nonprotein thiols such as glutathione ([NPT]i) (Chikahisa et al., 1996). The cells were treated with 500 nM 5-CMF-DA for 30 min before measuring 5-CMF fluorescence. Fluo-3-AM and FluoZin-3-AM was used to monitor the changes in $[Ca^{2+}]i$ (Kao et al., 1996) and $[Zn^{2+}]i$ (Gee et al., 2002), respectively. The cells were treated with 1 μM Fluo-3-AM or FluoZin-3-AM for 1 hr prior to fluorescence measurement. Excitation wavelength for all fluorescent probes used in this study was 488 nm and emissions were detected at 530 ± 20 nm for CM-DCF, FITC, 5-CMF, Fluo-3, and FluoZin-3 fluorescence and at 600 ± 20 nm for propidium fluorescence.

Statistical analysis

The data were statistically analyzed using Tukey's multivariate analysis; $P < 0.05$ was considered significant. Experimental values are described as mean ± standard deviation (SD) of four samples. Each experiment was performed twice or thrice to validate the results.

Results

Cytotoxic concentrations of 4VC

Cell treatment with 30 μM 4VC for 4 hr did not significantly increase the population of cells stained with propidium (cells exhibiting propidium fluorescence), as shown in the left panel of

Figure 1A. However, in the case of 100 μM 4VC, the population of propidium-stained cells was significantly increased (right panel of Figure 1A). Because the cells exhibiting propidium fluorescence are dead cells, the increase in the population of cells displaying propidium fluorescence indicates an increase in cell lethality. Figure 1B shows the concentration-dependent increase in cell lethality by 4VC.

(Figure 1 near here)

Cytoprotective action of 4VC on the cells under oxidative stress induced by H_2O_2

4VC at 10 μM or lower was considered to be not cytotoxic, as shown in Figure 1B. The cytoprotective action of 4VC at 0.1–10 μM was examined on cells simultaneously treated with H_2O_2 for 3 hr or 4 hr. Cell treatment with 300 μM H_2O_2 for 4 hr significantly increased the population of cells with propidium fluorescence (left panel of Figure 2A). The increase in the population of propidium-stained cells by 300 μM H_2O_2 for 4 hr was significantly attenuated by simultaneous application with 10 μM 4VC (right panel of Figure 2A). A significant attenuation of the H_2O_2-induced increase in cell lethality by 4VC was observed when the cells were simultaneously treated with 1–10 μM 4VC for 3 hr or 4 hr (Figure 2B). Maximum cytoprotective action of 4VC against H_2O_2-induced oxidative stress was observed when the 4VC concentration was 10 μM because the treatment with 30 μM 4VC did not induce further cytoprotective action. The treatment of cells with 10 μM 4VC for 1 h reduced the intensity of CM-DCF fluorescence, a parameter for cellular content of reactive oxidants, in the absence and simultaneous presence of 300 μM H_2O_2 (Figure 2C). 4VC may reduce contents of endogenous and exogenous H_2O_2.

(Figure 2 near here)

Delayed action of 4VC on the process of cell death induced by H_2O_2

The effect of 10 μM 4VC on the process of cell death elicited by 300 μM H_2O_2 was studied using propidium iodide and Annexin V-FITC. The effects were examined at 2 hr following the application of 4VC, H_2O_2, and of both (Figure 3A). Cell treatment with 4VC for 2 hr did not change the cytogram (propidium fluorescence versus FITC fluorescence). In the case of H_2O_2, the populations of cells with FITC fluorescence (area A of cytogram) and with propidium fluorescence (areas P and PA of cytogram) were greatly increased. Thus, the cell treatment with H_2O_2 significantly increased the population of Annexin V-positive living cells and dead cells, resulting in a significant reduction to the population of intact living cells (Figure 3B). When 4VC and H_2O_2 were applied simultaneously, the H_2O_2-induced changes on the cell population stained with both Annexin V-FITC and propidium iodide (PA area) were slightly, but significantly attenuated. Thus, the population of intact living cells following simultaneous treatment with H_2O_2 and 4VC was greater than that with H_2O_2 treatment only. The H_2O_2-induced increase in dead cell population in the absence of 4VC was greater than that in presence of 4VC. Results are summarized in Figure 3B.

(Figure 3 near here)

H_2O_2-induced changes of some cellular parameters in cells treated with 4VC

Cell treatment with 300 μM H_2O_2 for 1 hr significantly decreased the intensity of 5-CMF fluorescence and increased both Fluo-3 and FluoZin-3 fluorescence intensity in rat thymocytes (Figure 4). To see whether 4VC as an antioxidant attenuates cellular changes under oxidative stress, the changes in fluorescence induced by H_2O_2 were examined in the simultaneous presence of 4VC. The simultaneous application with 4VC potentiated the H_2O_2-induced increases in both Fluo-3 and FluoZin-3 fluorescence intensities (Figure 4). Thus, it is unlikely that 4VC attenuates the H_2O_2-induced changes in [NPT]i, $[Ca^{2+}]$i, and $[Zn^{2+}]$i, although 4VC exerts cytoprotective action on cells under oxidative stress induced by H_2O_2 (Figure 2).

(Figure 4 near here)

Effect of 4VC on the cells simultaneously incubated with A23187, a calcium ionophore

Cell treatment with 300 μM H_2O_2 increased the $[Ca^{2+}]i$, as described above. To test the possibility that 4VC exerts cytoprotective action on the cells with an excessive increase in $[Ca^{2+}]i$, the effect of 10 μM 4VC on the cells simultaneously incubated with 100 nM A23187 was examined. The treatment with A23187 for 1.5 hr and 3 hr significantly increased the population of propidium-stained cells, indicating the A23187-induced increase in cell lethality. The lethality of cells incubated with A23187 alone was greater than that of cells simultaneously incubated with A23187 and 4VC (Figure 5). Thus, 4VC is considered to exert cytoprotective action on the cells with intracellular Ca^{2+} overload.

(Figure 5 near here)

Discussion

In this *in vitro* study using rat thymic lymphocytes, 4VC at low micromolar concentrations (10 μM or less) was not cytotoxic whereas the treatment with 100 μM 4VC induced a statistically significant increase in cell lethality. 4VC exerted the cytoprotective action against the H_2O_2-induced oxidative stress at concentrations ranging from one hundredth of its cytotoxic level to one tenth (1–10 μM). 4VC is effective as an antioxidant preservative in edible oils because of the scavenging action for reactive oxygen (Jai et al., 2015). In addition, recent reports argue that 4VC is a cofactor of Nrf2 that activates an endogenous defense system against oxidative stress (Long et al., 2016; Senger et al., 2016) and this compound is considered to be a promising compound for diabetic wound healing (Jindam et al., 2017). Therefore, the cytoprotective action of 4VC against oxidative stress induced by H_2O_2 is not surprising. However, 4VC did not attenuate the H_2O_2-induced changes in [NPT]i, $[Ca^{2+}]i$, and $[Zn^{2+}]i$. And, 4VC further increased $[Ca^{2+}]i$ and $[Zn^{2+}]i$ in the cells simultaneously treated with H_2O_2 although 4VC alone did not

change either. Thus, 4VC is assumed to potentiate some adverse cellular actions of H_2O_2. The process of cell death is facilitated by excessive increases in $[Ca^{2+}]i$ and $[Zn^{2+}]i$ (Matsui et al., 2010; Orrenius et al., 2015). However, 4VC slowed the cell death process induced by H_2O_2. 4VC was capable of attenuating the cell death induced by A23187 which causes an excessive increase in $[Ca^{2+}]i$. 4VC may simultaneously cause acceleration and deceleration of cell death process under oxidative stress induced by H_2O_2. The results reveal the conflicting actions of 4VC.

There are some experimental limitations in this *in vitro* study. Oxidative stress was induced by H_2O_2. H_2O_2 is less reactive than other reactive oxygen species because it is not a free radical. Oxidative stress by reactive oxygen species under *in vivo* conditions is complex. $ZnCl_2$ and ascorbic acid, known as antioxidants, potentiate the cytotoxicity of H_2O_2 in rat thymic lymphocytes (Kawanai et al., 2010). The combination of $ZnCl_2$ and H_2O_2 excessively increases $[Zn^{2+}]i$ (Matsui et al., 2010). Hydroxyl radical, a potent oxidant, is generated in the case of ascorbic acid and H_2O_2 (Smirnoff and Cumbes, 1989; Nappi and Vass, 2000). Therefore, the conflicting actions of 4VC may be specific for the H_2O_2-induced oxidative stress.

4VC is present in coffee beverages because it is produced from caffeic acid and its derivatives during the roasting process (Frank et al. 2007). Some herbs also contain this compound and its glycosides (Senger and Cao, 2016; Wang and Zhu, 2017). Although 4VC can be used as an antioxidative preservative in foods (Schwarz et al., 2003; Zhang et al., 2017), the cytoprotective action of 4VC against oxidative stress is not so potent. Curcumin, a well-known antioxidant, at 0.1–3 μM exerts cytoprotective action against oxidative stress induced by H_2O_2 in rat thymic lymphocytes (Nagano et al., 1997). Effective concentrations of 4VC for cytoprotection are higher than those of curcumin in the case of H_2O_2. Oxidative stress is induced by free radicals and their non-radical intermediates. Diseases may be caused by specific oxidant(s). It is necessary to examine the actions of 4VC on the cells respectively treated with reactive oxygen species.

Conclusion

4VC at lower micromolar concentrations (1–10 μM) possesses cytoprotective action on cells under oxidative stress induced by H_2O_2. However, the actions of 4VC on the $[Ca^{2+}]i$ and $[Zn^{2+}]i$ of cells treated with H_2O_2 are adverse. Therefore, cautions ought to be taken when using 4VC in a clinical setting because it is considered to be a promising compound for diabetic wound healing.

Disclosure statement

There is no potential conflict of interest in this study.

Funding

Experiments were carried out with a Grant-in-Aid for Scientific Research (C26340039) from the Japan Society for the Promotion of Science (Tokyo, Japan).

References

Chikahisa, L., et al., 1996. Fluorescent estimation of H_2O_2-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. Japanese Journal of Pharmacology, 71, 299–305.

Darzynkiewicz, Z., et al., 1992. Features of apoptotic cells measured by flow cytometry. Cytometry Part A, 13, 795–808.

Frank, O., et al., 2007. Structure determination and sensory analysis of bitter-tasting 4-vinylcatechol oligomers and their identification in roasted coffee by means of LC-MS/MS. Journal of Agricultural and Food Chemistry, 55, 1945–1954.

Gee, K.R. et al., 2002. Detection and imaging of zinc secretion from pancreatic β-cells using a new fluorescent zinc indicator. Journal of the American Chemical Society, 124, 776–778.

Jia, C.H., et al., 2015. Effect of caffeic acid phenethyl ester and 4-vinylcatechol on the stabilities of oil-in-water emulsions of stripped soybean oil, Journal of Agricultural and Food Chemistry, 63, 10280–10286.

Jia, C.H., et al., 2016. Antioxidant properties of caffeic acid phenethyl ester and 4-vinylcatechol in stripped soybean oil. Journal of Food Science, 81, C35–C41.

Jiang, D., and Peterson, D.G., 2010. Role of hydroxycinnamic acids in food flavor: a brief overview. Phytochemistry Reviews, 9, 187–193.

Jindam, A., et al., 2017. Nrf2: a promising trove for diabetic wound healing. Annals of Translational Medicine, 5, 469.

Kao, J.P., et al., 1989. Photochemically generated cytosolic calcium pulses and their detection by fluo-3. Journal of Biological Chemistry, 264, 8179–8184.

Kawanai, T., et al., 2010. $ZnCl_2$ and vitamin C, known as antioxidants, differently potentiate the cytotoxicity of H_2O_2 in rat thymocytes: Cytometric analysis using forward and side scatters. Natural Science Research, Tokushima University 24, 12–19.

Koopman, G., et al., 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84, 1415–1420.

Koopman, W.J., et al., 2006. Simultaneous quantification of oxidative stress and cell spreading using 5-(and-6)chloromethyl-2′,7′-dichlorofluorescein. Cytometry Part A, 69, 1184–1192.

Long, M., et al., 2016. An essential role of NRF2 in diabetic wound healing, Diabetes, 65, 780–793.

Nagano, T., et al., 1997. New curcuminoids isolated from Zingiber cassumunar protect cells suffering from oxidative stress: a flow-cytometric study using rat thymocytes and H_2O_2. Japanese Journal of Pharmacology, 75, 363–370.

Orrenius, S., et al., 2015. Calcium and mitochondria in the regulation of cell death. Biochemical and Biophysical Research Communications, 460, 72–81.

Prescott, S.C., et al., 1937. The staling of coffee II. Journal of Food Sciences, 2, 165–173.

Redondo, P.C., et al., 2005. Hydrogen peroxide and peroxynitrite enhance Ca^{2+} mobilization and aggregation in platelets from type 2 diabetic patients. Biochemical and Biophysical Research Communications, 333, 794–802.

Senger, D.R., and Cao, S., 2016. Diabetic wound healing and activation of Nrf2 by herbal medicine. Journal of Natural Sciences, 2(11), e247.

Senger, D.R., et al., 2016. Anti-inflammatory activity of Barleria lupulina: Identification of active compounds that activate the Nrf2 cell defense pathway, organize cortical actin, reduce stress fibers, and improve cell junctions in microvascular endothelial cells. Journal of Ethnopharmacology, 193, 397–407.

Smirnoff, N., and Cumbes, Q.J., 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28, 1057–1060.

Matsui, H., et al., 2010. Low micromolar zinc exerts cytotoxic action under H_2O_2-induced oxidative stress: excessive increase in intracellular Zn^{+} concentration. Toxicology, 276, 27–32.

Nappi, A. J., and Vass, E., 2000. Hydroxyl radical production by ascorbate and hydrogen peroxide. Neurotoxicity Research 2, 343–355.

Nishizaki, Y., et al., 2003. Increase in number of annexin V-positive living cells of rat thymocytes by intracellular Pb^{2+}. Environmental Toxicology and Pharmacology, 15, 45–51.

Sakanashi, Y., et al., 2008. Possible use of quercetin, an antioxidant, for protection of cells suffering from overload of intracellular Ca^{2+}: a model experiment. Life Sciences, 83, 164–169.

Schwarz, M., et al., 2013. Pathway leading to the formation of anthocyanin-vinylphenol adducts and related pigment in red wines. Journal of the American Chemical Society, 51, 3682–3687.

Zhang, J., et al., 2017. Inhibitory mechanism against oxidative stress and biological activities of canolol. Acta Poloniae Pharmaceutica, 74, 25–29.

Wang, S., and Zhu, F., 2017. Chemical composition and biological activity of staghorn sumac (*Rhus typhina*). Food Chemistry, 237, 431–443.

Figure legends

Figure 1. Changes in the population of cells exhibiting propidium fluorescence (dead cells), as induced by 4VC. (A) 4-vinylcatechol (4VC)-induced change in levels of propidium fluorescence. The histogram was constructed with 2500 cells. (B) Concentration-dependent change in cell lethality (percentage population of propidium-stained cells) by 4VC. Columns and bars show mean ± standard deviation of four samples. Asterisks (**) indicate significant differences between the control group and the group of cells treated with 4VC.

Figure 2. 4VC-induced change of dead cell population in cells simultaneously treated with H_2O_2. (A) Histograms of cells incubated without (CONTROL) and with H_2O_2 alone (H_2O_2 300 μM) and the combination of H_2O_2 and 4-vinylcatechol (4VC) (H_2O_2 + 4VC). (B) Concentration-dependent change in cell lethality of cells incubated with 300 μM H_2O_2 alone, and the combination of 300 μM H_2O_2 and 0.1–10 μM 4VC. Incubation times were 3 hr (upper panel) and 4 hr (lower panel). Columns and bars show mean ± standard deviation of four samples. Asterisks (**) indicate significant differences between the group of cells treated with H_2O_2 alone and the group of cells treated with H_2O_2 and 4VC. (C) Change in fluorescence intensity of CM-DCF in the cells treated without (CONTROL) and with 4VC alone (4VC 10 μM), H_2O_2 alone (H_2O_2 300 μM), and the combination (H_2O_2 + 4VC). Asterisks (**) indicate significant differences between the control group (CONTROL) and the groups of cells treated with H_2O_2, 4VC, and a combination of both. Pounds ($^{\#\#}$) indicate significant differences between the groups of H_2O_2-treated cells with and without 4VC.

Figure 3. Changes in the population of cells exhibiting propidium fluorescence (dead cells) and FITC fluorescence (Annexin V-positive cells). (A) Cytograms of cells incubated without

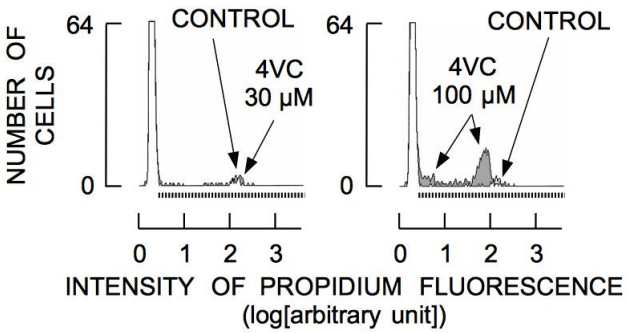
(CONTROL) and with 4VC alone (4VC 10 μM), H_2O_2 alone (H_2O_2 300 μM), and the combination (H_2O_2 + 4VC). Each cytogram was constructed with 2500 cells. (B) Changes in the percentage population obtained from the cytograms. Columns and bars show mean ± standard deviation of four samples. Asterisks (**) indicate significant differences between the control group (CONTROL) and the groups of cells treated with H_2O_2, 4VC, and a combination of both. Pounds ($^{\#\#}$) indicate significant differences between the groups of H_2O_2-treated cells with and without 4VC.

Figure 4. Changes in fluorescence intensities of 5-CMF (upper panel), Fluo-3 (middle panel), and FluoZin-3 (lower panel) in the cells treated without (CONTROL) and with 4VC alone (4VC 10 μM), H_2O_2 alone (H_2O_2 300 μM), and the combination (H_2O_2 + 4VC). Asterisks (**) indicate significant differences between the control group (CONTROL) and the groups of cells treated with H_2O_2, 4VC, and a combination of both. Pounds ($^{\#\#}$) indicate significant differences between the groups of H_2O_2-treated cells with and without 4VC.

Figure 5. Concentration-dependent change in cell lethality of cells incubated without (CONTROL) and with 4VC alone (4VC 10 μM), A23187 alone (A23187 100 nM), and the combination of 4VC and A23187 (4VC + A23187). Incubation times were 1.5 hr (upper panel) and 3 hr (lower panel). Columns and bars show mean ± standard deviation of four samples. Asterisks (**) indicate significant differences between the control group (CONTROL) and the groups of cells treated with A23187, 4VC, and a combination of both. Pounds ($^{\#\#}$) indicate significant differences between the groups of A23187-treated cells with and without 4VC.

Figure 1

(A)


NUMBER OF CELLS
64
0
CONTROL
4VC
30 μM
64
0
CONTROL
4VC
100 μM
0 1 2 3
0 1 2 3
INTENSITY OF PROPIDIUM FLUORESCENCE
(log[arbitrary unit])

(B)

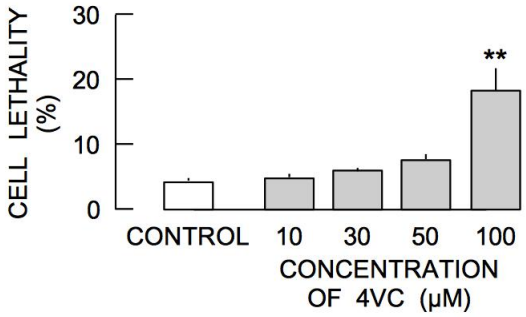
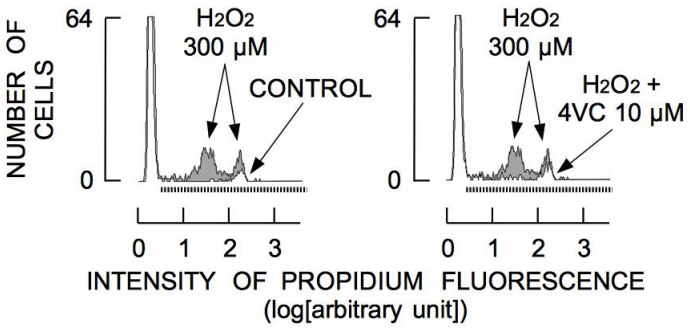
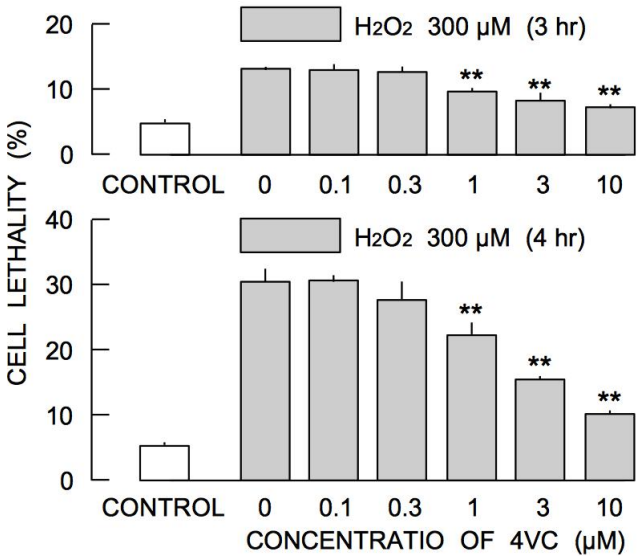

CELL LETHALITY (%)
30
20
10
0
**
CONTROL
10
30
50
100
CONCENTRATION OF 4VC (μM)

Figure 2

(A)


NUMBER OF CELLS
64
0
H2O2 300 μM
CONTROL
H2O2 + 4VC 10 μM
0 1 2 3
INTENSITY OF PROPIDIUM FLUORESCENCE
(log[arbitrary unit])

(B)


CELL LETHALITY (%)
H2O2 300 μM (3 hr)
H2O2 300 μM (4 hr)
CONTROL 0 0.1 0.3 1 3 10
CONCENTRATIO OF 4VC (μM)

(C)

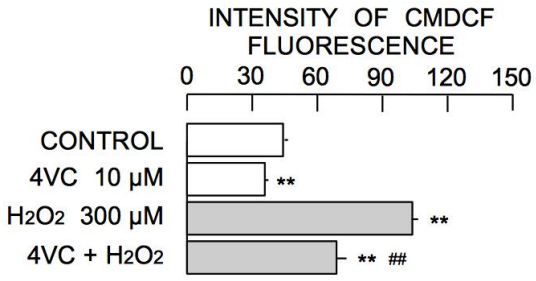
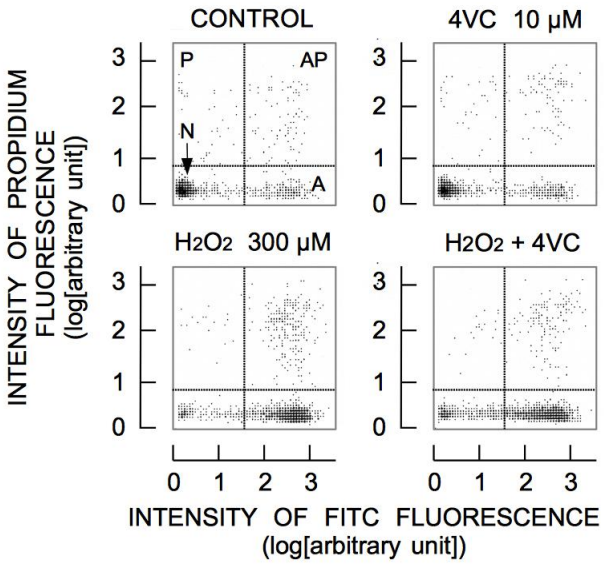

INTENSITY OF CMDCF FLUORESCENCE
0 30 60 90 120 150
CONTROL
4VC 10 μM
H2O2 300 μM
4VC + H2O2

Figure 3

(A)


CONTROL
4VC 10 μM
H2O2 300 μM
H2O2 + 4VC
P
AP
N
A
INTENSITY OF PROPIDIUM FLUORESCENCE (log[arbitrary unit])
INTENSITY OF FITC FLUORESCENCE (log[arbitrary unit])
0
1
2
3

(B)

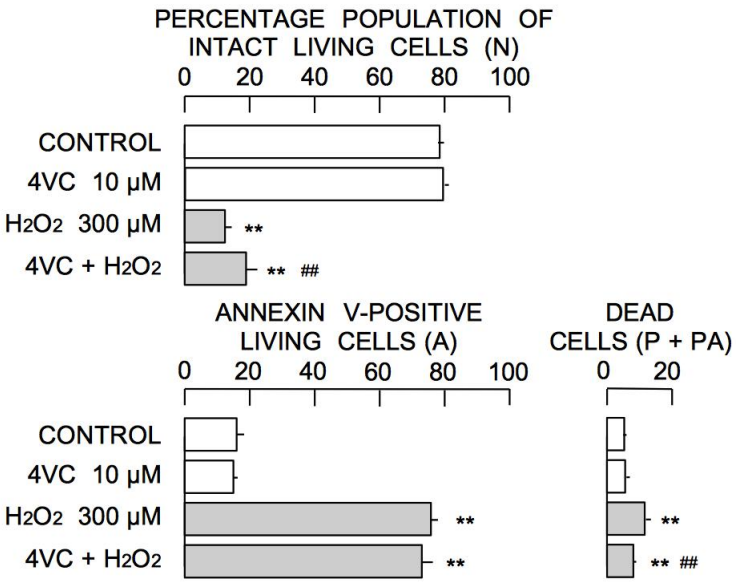

PERCENTAGE POPULATION OF INTACT LIVING CELLS (N)
0 20 40 60 80 100
CONTROL
4VC 10 μM
H2O2 300 μM
4VC + H2O2
**
** ##
ANNEXIN V-POSITIVE LIVING CELLS (A)
DEAD CELLS (P + PA)
0 20 40 60 80 100
0 20

Figure 4

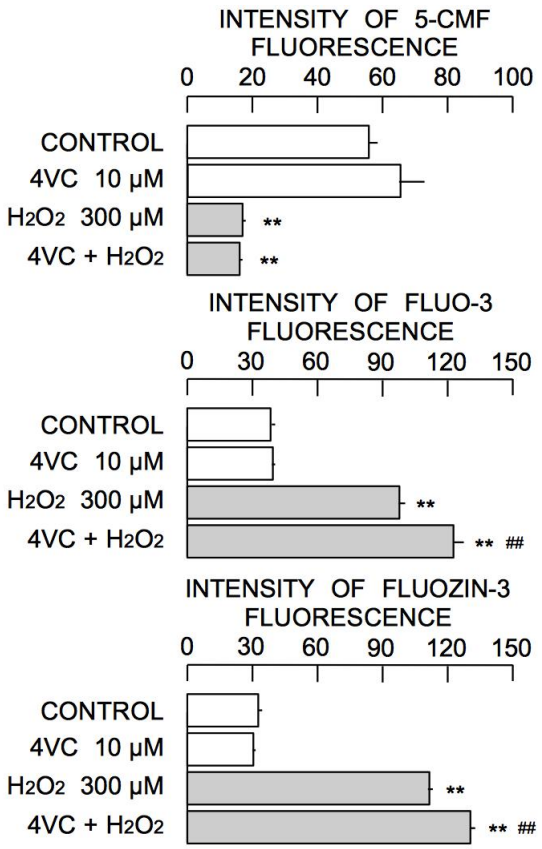
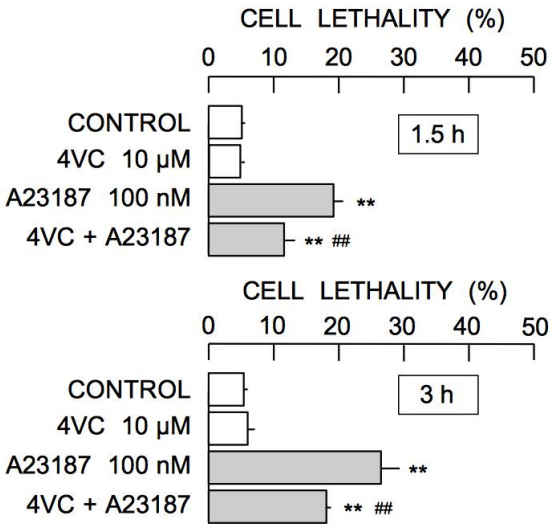

INTENSITY OF 5-CMF FLUORESCENCE
0 20 40 60 80 100
CONTROL
4VC 10 µM
H2O2 300 µM
4VC + H2O2
**
**
INTENSITY OF FLUO-3 FLUORESCENCE
0 30 60 90 120 150
CONTROL
4VC 10 µM
H2O2 300 µM
4VC + H2O2
**
** ##
INTENSITY OF FLUOZIN-3 FLUORESCENCE
0 30 60 90 120 150
CONTROL
4VC 10 µM
H2O2 300 µM
4VC + H2O2
**
** ##

Figure 5


CELL LETHALITY (%)
0 10 20 30 40 50
CONTROL
4VC 10 µM
A23187 100 nM
4VC + A23187
1.5 h
** ##
CELL LETHALITY (%)
0 10 20 30 40 50
CONTROL
4VC 10 µM
A23187 100 nM
4VC + A23187
3 h
** ##