Cetylpyridinium chloride at sublethal levels increases the susceptibility of rat thymic lymphocytes to oxidative stress

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ABSTRACT

Cetylpyridinium chloride (CPC) is an antimicrobial agent used in many personal care products, with subsequent release into the environment. Since CPC is found at low concentrations in river and municipal wastewater, its influence on wildlife is of concern. Therefore, in this study, we used flow cytometry to examine the effects of sublethal concentrations of CPC on rat thymic lymphocytes in order to characterize the cellular actions of CPC at low concentrations in the presence and absence of H_2O_2-induced oxidative stress. CPC treatment increased the population of living cells with phosphatidylserine exposed on the outer surface of their plasma membranes (a marker of early stage apoptosis), elevated intracellular Zn^{2+} levels, and decreased the cellular content of nonprotein thiols. CPC also potentiated the cytotoxicity of H_2O_2. Our results suggest that, even at environmentally relevant sublethal concentrations, CPC exerts cytotoxic effects under oxidative stress conditions by increasing intracellular Zn^{2+} concentration and decreasing the cellular content of nonprotein thiols. These findings indicate that, under some in vitro conditions, CPC is bioactive at environmentally relevant concentrations. Therefore, CPC release from personal care products into the environment may need to be regulated to avoid its adverse effects on wildlife.

Keywords: cetylpyridinium chloride; thymocytes; cytotoxicity; zinc; oxidative stress; hydrogen peroxide

1. Introduction

Cetylpyridinium chloride (CPC), a quaternary ammonium compound belonging to the group of cationic antimicrobial agents, is used in various types of mouthwashes, toothpastes, and other personal care products such as lozenges, throat sprays, breath sprays, and nasal sprays. It is considered a safe antimicrobial agent with broad-spectrum activity for preventing biofilm formation and gingivitis (Watanabe et al., 2008). CPC is also used as a disinfectant in private and public areas. These include swimming pools and aquariums; facilities storing water used for bathing and other purposes; air-conditioning systems; walls and floors in healthcare and other institutions; chemical toilets; and in organizations dealing with waste water, hospital waste, soil, or other similar substrates (EC Scientific Committee on Consumer Safety, 2015).

The reported toxicities of CPC under in vivo conditions were reviewed in the Revision of the Opinion on Cetylpyridinium Chloride (EC Scientific Committee on Consumer Safety, 2015). The use of CPC for oral or dermal applications is considered relatively safe. However, because CPC is used for the diverse purposes described above, a considerable amount is released into the environment. The CPC concentration in the river and municipal wastewater in Kaohsiung City, which is located in Southern Taiwan and has a population of approximately 2.77 million, was reported to be 52 μg/l and 47–88 μg/l, respectively (Shrivas and Wu, 2007). Therefore, the calculated molar concentration of CPC is 138–259 nM. CPC exhibited cytotoxic effects in human keratinocyte and murine fibroblast cell lines at concentrations of 0.0004–0.5 % (11.7 μM–14.7 mM) (Fromm-Dornieden et al., 2015) and developmental and acute toxicity in frog embryos at 0.5–3 μM (Park et al., 2016). In addition, low CPC concentrations, ranging from 3 nM to 30 nM, have been shown to inhibit nuclear factor-κB-induced osteoclast formation in mouse bone marrow cells in a concentration-dependent manner (Zheng et al., 2013). Thus, it is likely that CPC exerts some cellular actions even at sublethal environmentally relevant concentrations. This raises concerns regarding the effects of low concentrations of CPC on wildlife. Therefore, in this study, we used flow cytometry to explore the cellular effects of CPC at sublethal concentrations in rat thymic lymphocytes.

2. Materials and methods

2.1. Reagents

CPC was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). FluoZin-3-AM, 5-chloromethylfluorescein diacetate (5-CMF-DA), annexin V-FITC, and propidium iodide were obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Zn^{2+} chelators, diethylenetriamine-N,N,N',N'',N''-pentaacetic acid (DTPA) and N,N,N',N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN), were obtained from Dojin Chemical Laboratory (Kumamoto, Japan). DTPA chelates extracellular Zn^{2+} but not intracellular Zn^{2+} because the agent is not membrane-permeable. TPEN is a membrane-permeable Zn^{2+} chelator. TPEN chelates intracellular Zn^{2+}. Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless otherwise mentioned.

2.2. Cell preparation

This study was approved by the Committee for Animal Experiments of Tokushima University, Tokushima, Japan (No. 14124). The cell suspension was prepared as previously reported (Chikahisa et al., 1996; Sakanashi et al., 2009; Matsui et al., 2010). In brief, thymus glands dissected from ether-anesthetized rats were sliced under cold conditions. The slices were triturated in Tyrode's solution (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES at pH 7.3–7.4 adjusted with a small amount of NaOH) to dissociate the thymocytes. The cell suspension was incubated at 36–37°C for 1 h before the experiment. Importantly, the zinc concentration in the cell suspension was 216.9 ± 14.4 nM (Sakanashi et al., 2009). The cell suspension contained trace amounts of zinc derived from the cell preparation.

There was one technical limitation in present study. Thymocytes are the cells that spontaneously undergo apoptosis during the incubation for 24 h (Rinner et al., 1996; Nishimura et al., 2008). The incubation of rat thymocytes for 12 h or longer (up to 24 h) significantly increased the population of shrunken cells, one of parameters during early phase of apoptosis. Therefore, the experiments were performed within 6 h after the start of incubation.

2.3. Fluorescence measurements of cellular parameters

The methods employed for measurements of cellular and membrane parameters using a flow cytometer equipped with an argon laser (CytoACE-150, JASCO, Tokyo, Japan) and fluorescent probes were similar to those previously described (Chikahisa et al., 1996; Matsui et

al., 2010). The fluorescence was analyzed using JASCO software (JASCO). Excitation wavelength for fluorescent probes was 488 nm. FITC, FluoZin-3, and 5-CMF fluorescence were detected at 530 ± 20 nm. Propidium fluorescence was detected at 600 ± 20 nm.

To assess cell lethality, propidium iodide was added to cell suspension to achieve a final concentration of 5 μM. Since propidium stains dead cells (or the cells with compromised membranes), the measurement of propidium fluorescence from cells provides a clue to estimate the lethality. The fluorescence was measured at 5 min after the application of propidium iodide by a flow cytometer.

To detect phosphatidylserine, annexin V-FITC was used in a combination with propidium iodide. Annexin V interacts strongly and specifically with phosphatidylserine and can be used to detect apoptosis by targeting for the loss of plasma membrane asymmetry. Phosphatidylserine is normally exposed on inner side of plasma membranes and it becomes exposed on outer membrane surface of the cells undergoing apoptosis. Therefore, annexin V-FITC is able to detect the surface changes in membrane surface that occur early during apoptosis (Koopman et al., 1994). Annexin V-FITC (10 μl/ml) and propidium iodide (5 μM), respectively, were added to cell suspension at 30 and 2 min before the measurement.

FluoZin-3 is a Zn^{2+}-selective indicator and exhibits high Zn^{2+}-binding affinity that is unperturbed by Ca^{2+} concentrations up to at least 1 μM (Gee et al., 2002). To estimate the change in intracellular Zn^{2+} levels of rat thymocytes with intact membranes, FluoZin-3-AM and propidium iodide were simultaneously used. Both dyes were added to the solution to achieve final concentrations of 500 nM for FluoZin-3-AM and 5 μM for propidium iodide. Cells were incubated with FluoZin-3-AM for 50–60 min before the application of CPC. FluoZin-3 fluorescence was measured from the cells that were not stained with propidium iodide (living cells with intact membranes).

5-CMF-DA was used to estimate cellular content of glutathione. The correlation coefficient between 5-CMF fluorescence intensity and cellular glutathione content was 0.965 in rat thymocytes (Chikahisa et al., 1996). Oxidative stress decreases cellular content of glutathione. The CMF fluorescence was measured at 30 min after the application of 1 μM CMF-DA because it attains peak intensity within 30 min after the application. 5-CMF fluorescence was monitored from the cells that were not stained with propidium iodide.

2.4. Experimental protocol

The agents were added to the cell suspension with 2 ml of cell suspension in each 10 ml test tube. The cells were incubated with the agent(s) at 36°C for 1–3 h under room air condition. The incubation time was dependent on each experimental purpose. Fluorescence data acquisition by the flow cytometer from 2500 cells required a maximum of 30 s.

2.5. Statistical analysis

Statistical analyses were performed using ANOVA with post-hoc Tukey's multivariate analysis. P-values of less than 0.05 were considered significant. The results, including columns and error bars in the figures, are expressed as mean and standard deviation of 4–8 samples.

3. Results

3.1. Change in cell lethality after CPC treatment

As shown in Fig. 1, the incubation of rat thymocytes with 3 μM CPC for 3 h increased the population of cells exhibiting propidium fluorescence (the population of dead cells). The intensity of forward scatter in living cells decreased significantly ($P < 0.01$) from 150.3 ± 0.8 (arbitrary unit, mean ± SD of four samples) to 139.7 ± 0.9 by 3 μM CPC, indicating CPC-induced cell shrinkage. Treatment with 3 μM CPC resulted in a time-dependent increase in cell lethality (Fig. 2A). Significant increases were observed 0.5–3 h after the start of CPC application. The increase in cell lethality by CPC was also concentration-dependent (Fig. 2B). Significant increases were observed when the concentration of CPC was 2 μM or more (up to 5 μM).

(Figures 1 and 2 near here)

3.2. Increase in the population of living cells with exposed phosphatidylserine induced by a sublethal CPC concentration

Fig. 3 shows the increase of side scatter intensity in the cells treated with 1 μM CPC, a sublethal concentration, for 3 h. The mean intensity (arbitrary unit) of side scatter increased significantly from 86.2 ± 1.3 to 91.9 ± 0.1 (mean and standard deviation of four samples; P <

0.01) in living cells, indicating that CPC significantly affected the cells even at a sublethal concentration (1 μM). Therefore, in subsequent experiments, we examined the effects of CPC at a concentration of 1 μM or less. Incubation with 1 μM CPC, but not 0.3 μM CPC, for 3 h significantly increased the population of living cells with exposed phosphatidylserine (area A of Fig. 4A) and decreased that of intact living cells (area N of Fig. 4A). The percent changes in cell population after 0.3 and 1 μM CPC treatment for 3 h are shown in Fig. 4B.

(Figures 3 and 4 near here)

3.3. Effects of sublethal concentrations of CPC on 5-CMF and FluoZin-3 fluorescence

Incubation of cells with 0.3 μM and 1 μM CPC, but not 0.1 μM CPC, for 3 h equally and significantly decreased the intensity of 5-CMF fluorescence (Fig. 5), indicating a decrease in the cellular content of nonprotein thiols ($P < 0.01$). The intensity of FluoZin-3 fluorescence was significantly increased by incubation with 0.3 μM and 1 μM CPC, but not 0.1 μM CPC, for 2 h (Fig. 6A), indicating the elevation of intracellular Zn^{2+} levels by CPC. Although the removal of extracellular Zn^{2+} by DTPA significantly decreased the CPC-induced augmentation of FluoZin-3 fluorescence, CPC still increased the intensity of FluoZin-3 fluorescence significantly in the presence of DTPA (Fig. 6B) ($P < 0.01$ for both effects). TPEN completely suppressed the CPC-induced change in FluoZin-3 fluorescence (Fig. 6B).

(Figures 5 and 6 near here)

3.4. Simultaneous application of CPC and hydrogen peroxide (H_2O_2)

The results shown in Figs. 5 and 6 indicated the possibility that, even at sublethal concentrations, CPC induced oxidative stress. To determine if CPC, at concentrations ranging from 0.1 μM to 1 μM, increases cell susceptibility to oxidative stress, the effects of CPC, H_2O_2 (100 μM), and their combination on the cells were examined. As shown in Fig. 7, the incubation with 0.1–1 μM CPC for 3 h did not change cell lethality and H_2O_2 at 100 μM slightly, but significantly, increased cell lethality from 6.8 ± 0.6 % to 10.8 ± 0.5 % (mean ± SD of four samples; $P < 0.01$). Simultaneous incubation with CPC (0.3 and 1 μM) and H_2O_2 for 3 h induced a significant increase in cell lethality (Fig. 7).

(Figure 7 near here)

4. Discussion

In this study, we found that CPC at sublethal concentrations (300 nM–1 μM) increased the susceptibility of cells to H_2O_2-induced oxidative stress. Previous studies have shown that Zn^{2+} potentiates the cytotoxicity of H_2O_2 (Matsui et al., 2010) while TPEN, a chelator of intracellular Zn^{2+}, greatly attenuates it (Matsui et al., 2009). Our results revealed that CPC decreased the cellular content of non-protein thiols such as glutathione, as indicated by the decrease in the fluorescence of 5-CMF. Decrease in the cellular content of nonprotein thiols induces an increase in intracellular Zn^{2+} concentration in this preparation (Kinazaki et al., 2011). Consistent with this, we also found that CPC increased the intensity of FluoZin-3 fluorescence, indicating that CPC increases intracellular Zn^{2+} concentration, which may potentiate the cytotoxicity of H_2O_2. Moreover, since cellular nonprotein thiols protect cells against oxidative stress (Hayes and McLellan, 1999), the CPC-induced decrease in cellular glutathione content may also augment the cytotoxicity of H_2O_2.

One may be concerned about the source of Zn^{2+} in the CPC-induced increase in intracellular Zn^{2+} concentration. CPC still increased the intensity of FluoZin-3 fluorescence in the presence of DTPA. DTPA chelates extracellular Zn^{2+}, producing external Zn^{2+}-free condition. Thus, the increase in the intensity of FluoZin-3 fluorescence by CPC under external Zn^{2+}-free condition is supposed to be due to an intracellular release of Zn^{2+} by CPC. The cell suspension contained small amount (about 230 nM) of zinc that was derived from the cell preparation. The augmentation of FluoZin-3 fluorescence by CPC in the absence of DTPA was significantly higher than that in the presence of DTPA. Therefore, the possibility is not ruled out that CPC prompts Zn^{2+} permeation through membranes.

The incubation of thymocytes with H_2O_2 increases the population of living cells with phosphatidylserine exposed on the outer surface of plasma membranes (Oyama et al., 1999). Phosphatidylcholine is zwitterionic while phosphatidylserine is anionic. Therefore, the electrochemical property of cell membranes is changed in cells exposed to H_2O_2. If positively charged CPC binds with high preference to anionic phosphatidylserine over zwitterionic phosphatidylcholine, CPC would be selectively attracted by the membranes of cells exposed to H_2O_2 rather than those of normal cells, possibly resulting in the increase in CPC cytotoxicity.
CPC itself at a concentration of 1 μM or less did not increase cell lethality under the in vitro conditions used in this study. However, CPC at 300 nM–1 μM significantly potentiated the

cytotoxicity of H_2O_2. Thus, CPC at nanomolar concentrations may exert more toxicity under conditions of oxidative stress. Although CPC is used in various types of personal care products and is released into the environment, there are no reports on the adverse effect of CPC on wildlife. In this study, CPC at 300 nM significantly decreased the cellular content of glutathione and increased intracellular Zn^{2+} concentration in rat thymocytes.

5. Conclusion

Since the results indicate that an environmentally relevant CPC concentration is bioactive under certain experimental conditions, CPC release from personal care products into the environment may need to be regulated to avoid its adverse effects on wildlife.

Conflict of interest

All authors affirm that there are no conflicts of interest to declare.

Acknowledgements

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Figure legends

Fig. 1. Change in the cytogram (propidium fluorescence versus forward scatter) after 3 μM CPC treatment. The cytogram was obtained from 2500 cells. The dotted line under the cytogram indicates the population of cells exhibiting propidium fluorescence, dead cells, and/or the cells with compromised membranes.

Fig. 2. Time- and concentration-dependent changes (A and B, respectively) in the percentage of cells exhibiting propidium fluorescence (cell lethality) after 3 μM CPC treatment. The columns and error bars show the mean value and standard deviation of four samples, respectively. ** indicate significant differences ($P < 0.01$) between the control (CONTROL) and CPC-treated groups.

Fig. 3. Change in the cytogram (side scatter versus forward scatter) after 1 μM CPC treatment. The cytogram was obtained from 2500 cells.

Fig. 4. CPC-induced change in the population of cells classified with propidium iodide and annexin V-FITC. (A) Changes in the fluorescence cytogram (propidium fluorescence versus FITC fluorescence) after 1 μM CPC treatment. Areas of N, A, P, and AP show the population of intact living cells, annexin V-positive living cells, dead cells, and annexin V-positive dead cells, respectively. Each cytogram consisted of 2500 cells. (B) Percentage changes in the cell population after 0.3 and 1 μM CPC treatment. ** indicate significant differences ($P < 0.01$) between the control (CONTROL) and CPC-treated groups.

Fig. 5. Change in the intensity of 5-CMF fluorescence after CPC treatment. The columns and error bars show the mean value and standard deviation of four samples, respectively. ** indicate significant differences ($P < 0.01$) between the control (CONTROL) and CPC-treated groups.

Fig. 6. Change in the intensity of FluoZin-3 fluorescence after CPC treatment. (A) Concentration-dependent effect of CPC on FluoZin-3 fluorescence. (B) Effects of Zn^{2+} chelators on the CPC-induced increase in FluoZin-3 fluorescence intensity. The columns and error bars show the mean value and standard deviation of four samples, respectively. ** indicates significant differences ($P < 0.01$) between the control (CONTROL) and DTPA- or

TPEN-treated groups. ## indicates significant differences between respective control groups (CONTROL and DTPA 10 μM) and the 1 μM CPC-treated group.

Fig. 7. Changes in the percentage population of cells exhibiting propidium fluorescence (cell lethality) after treatment with CPC, H_2O_2, and their combination. The columns and error bars show the mean value and standard deviation of four samples, respectively. ** indicates significant differences ($P < 0.01$) between the control group (CONTROL) and the group treated with CPC, H_2O_2, or their combination. ## indicates significant differences between the group treated with H_2O_2 alone and the group treated with the combination of H_2O_2 and CPC.

Figure 1

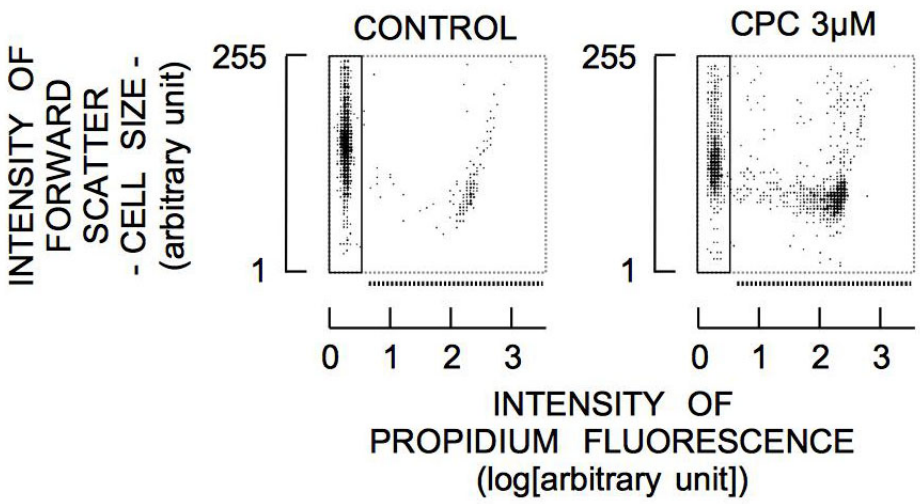
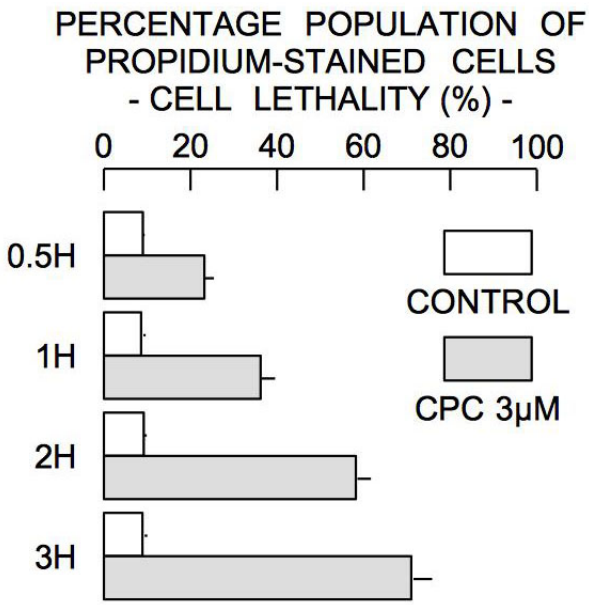

CONTROL
CPC 3μM
INTENSITY OF FORWARD SCATTER - CELL SIZE - (arbitrary unit)
255
1
0 1 2 3
INTENSITY OF PROPIDIUM FLUORESCENCE (log[arbitrary unit])

Figure 2

A.


PERCENTAGE POPULATION OF
PROPIDIUM-STAINED CELLS
- CELL LETHALITY (%) -
0
20
40
60
80
100
0.5H
1H
2H
3H
CONTROL
CPC 3μM

B.

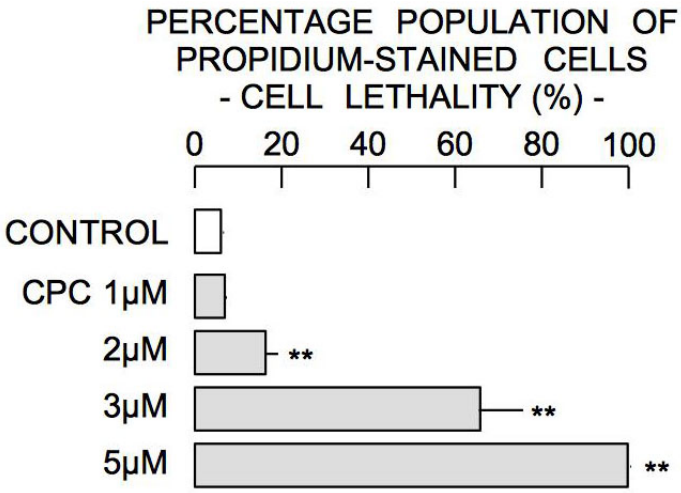

PERCENTAGE POPULATION OF
PROPIDIUM-STAINED CELLS
- CELL LETHALITY (%) -
0
20
40
60
80
100
CONTROL
CPC 1μM
2μM
3μM
5μM
**
**
**

Figure 3

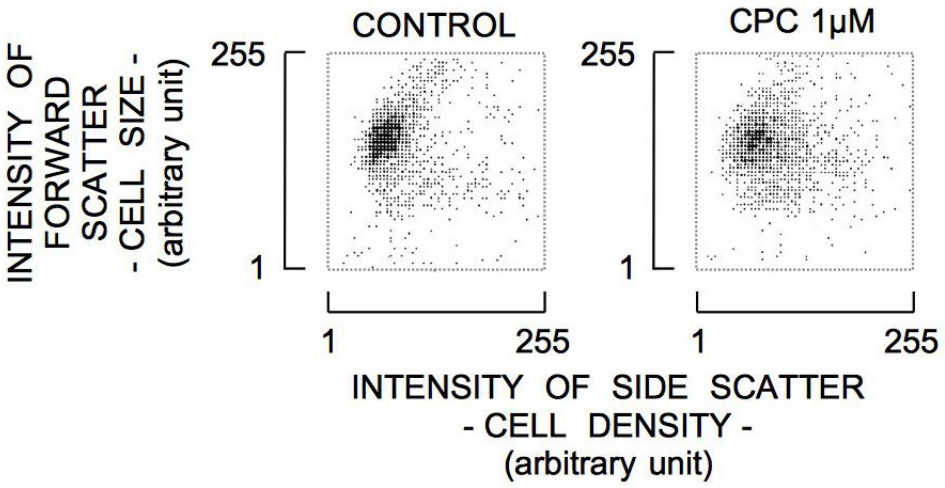

CONTROL
CPC 1μM
255
1
255
1
1
255
1
255
INTENSITY OF FORWARD SCATTER - CELL SIZE - (arbitrary unit)
INTENSITY OF SIDE SCATTER - CELL DENSITY - (arbitrary unit)

Figure 4

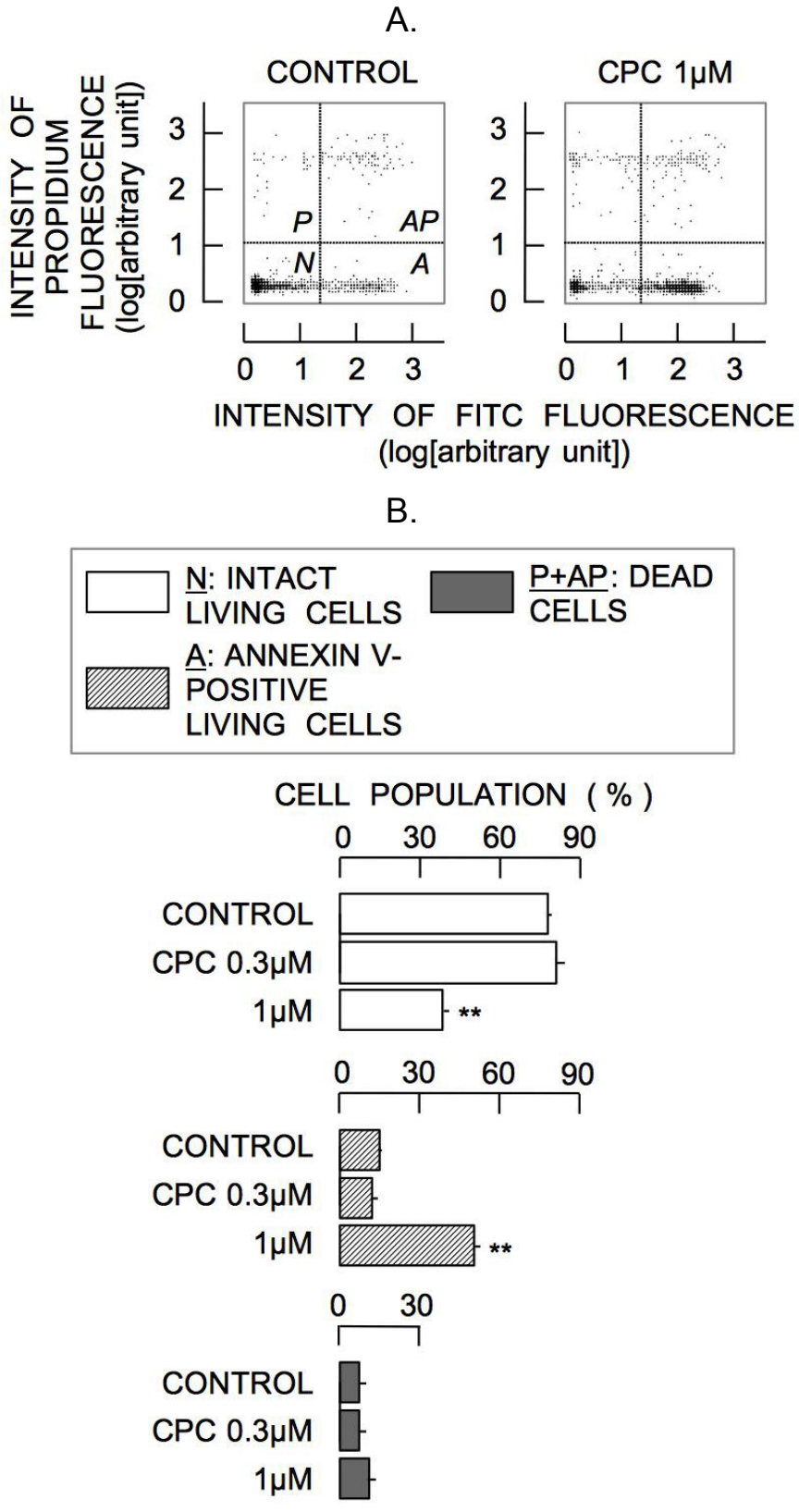

A.
CONTROL
CPC 1μM
INTENSITY OF PROPIDIUM FLUORESCENCE (log[arbitrary unit])
P
AP
N
A
0
1
2
3
INTENSITY OF FITC FLUORESCENCE (log[arbitrary unit])
B.
N: INTACT LIVING CELLS
P+AP: DEAD CELLS
A: ANNEXIN V-POSITIVE LIVING CELLS
CELL POPULATION (%)
0
30
60
90
CONTROL
CPC 0.3μM
1μM
**

Figure 5

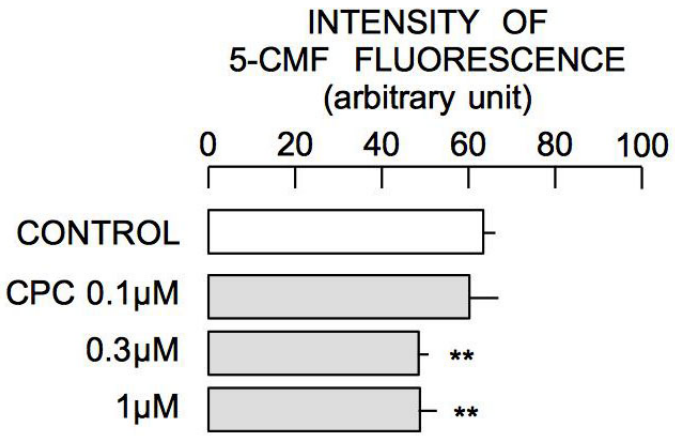
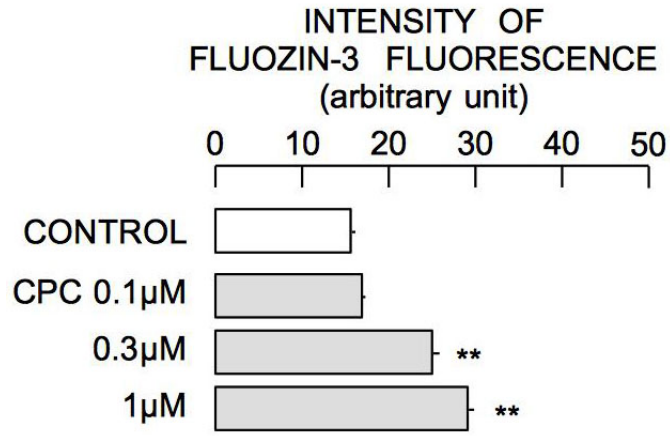

INTENSITY OF
5-CMF FLUORESCENCE
(arbitrary unit)
0
20
40
60
80
100
CONTROL
CPC 0.1μM
0.3μM
1μM
**
**

Figure 6

A.


INTENSITY OF
FLUOZIN-3 FLUORESCENCE
(arbitrary unit)
0
10
20
30
40
50
CONTROL
CPC 0.1μM
0.3μM
1μM
**
**

B.

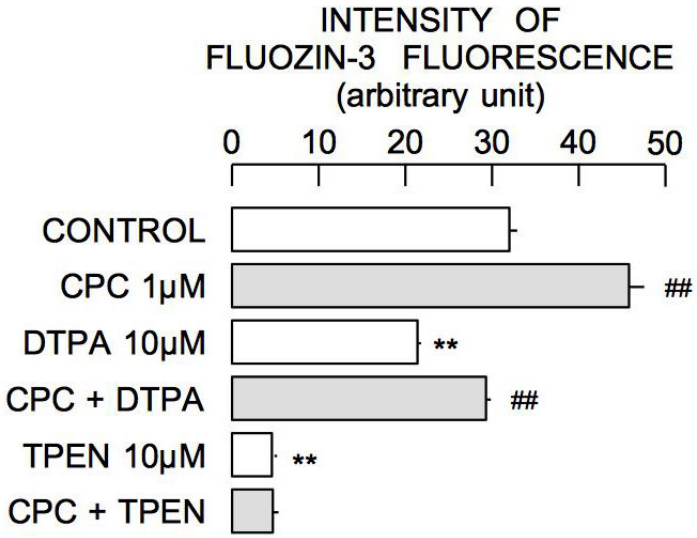
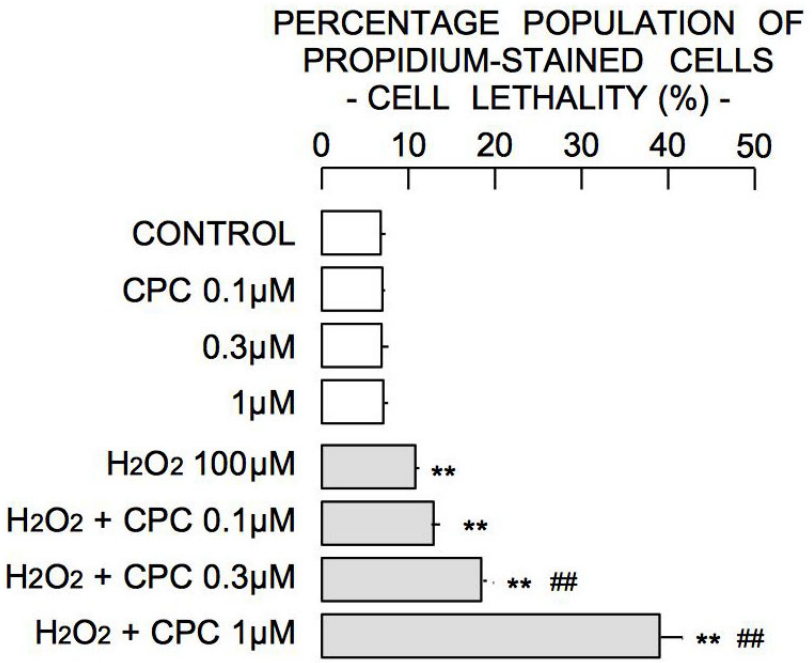

INTENSITY OF
FLUOZIN-3 FLUORESCENCE
(arbitrary unit)
0
10
20
30
40
50
CONTROL
CPC 1μM
DTPA 10μM
CPC + DTPA
TPEN 10μM
CPC + TPEN
##
**
##
**

Figure 7


PERCENTAGE POPULATION OF
PROPIDIUM-STAINED CELLS
- CELL LETHALITY (%) -
0
10
20
30
40
50
CONTROL
CPC 0.1μM
0.3μM
1μM
H2O2 100μM
H2O2 + CPC 0.1μM
H2O2 + CPC 0.3μM
H2O2 + CPC 1μM
**
**
** ##
** ##