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Some adverse actions of chlorothalonil at sublethal levels in rat thymic lymphocytes: Its relation to Zn^{2+}

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Highlights

• Cellular actions of chlorothalonil at sublethal levels were examined in rat thymocytes.

• Chlorothalonil disrupted cellular levels of nonprotein thiols.

• Chlorothalonil increased intracellular Zn^{2+} concentration.

• Chlorothalonil and pentachloronitrobenzene potentiated the cytotoxicity of H_2O_2.

• Increase in Zn^{2+} levels by chlorothalonil was partly responsible for its actions.

Abstract

Chlorothalonil, a polychlorinated aromatic fungicide, is considered non-toxic to small mammals. However, chlorothalonil inactivates sulfhydryl enzymes and depletes cellular glutathione. Chlorothalonil increases intracellular Zn^{2+} concentration ($[Zn^{2+}]i$) in mammalian cells possibly because intracellular Zn^{2+} is released via zinc-thiol/disulfide interchange. The effects of chlorothalonil at sublethal concentrations on the cellular content of nonprotein thiols ([NPT]i) and $[Zn^{2+}]i$ were examined using flow cytometry in rat thymocytes. Low concentrations (0.3–1 μM) of chlorothalonil increased, but high concentrations (3–10 μM) decreased [NPT]i. These effects of chlorothalonil were partly attenuated by an intracellular Zn^{2+} chelator. Chlorothalonil at 0.3–10 μM increased $[Zn^{2+}]i$ in a concentration-dependent manner, which was largely dependent on the release of intracellular Zn^{2+}. Both the decrease in [NPT]i and increase in $[Zn^{2+}]i$ increase the vulnerability of cells to oxidative stress. Chlorothalonil at 1–10 μM potentiated the cytotoxicity of H_2O_2 (300 μM). It was also the case for 10 μM pentachloronitrobenzene, but not 10 μM pentachlorophenol. In conclusion, chlorothalonil at low (sublethal) micromolar concentrations is cytotoxic to mammalian cells under oxidative stress.

Keywords: Chlorothalonil; Intracellular Zn^{2+}; Nonprotein thiol; Cytotoxicity; Lymphocyte

1. Introduction

Chlorothalonil, a polychlorinated aromatic fungicide for vegetables and fruit, was synthesized at the Diamond Shamrock Corporation (OH, USA) in 1962 and was first registered in 1966 as a fungicide on turf grass. The National Water-Quality Assessment program estimated its average annual use to be over 10 million pounds in the USA between 2011 and 2015 (United States Geological Survey, 2017). Chlorothalonil is considered non-toxic to small mammals because it has an LD_{50} of > 10,000 mg/kg in rats (United States Environmental Protection Agency, 1999). Its toxicity profile in mammals, birds, plants, and fungi has been reviewed by Van Scoy and Tjeerdema (2014). It is also nontoxic to birds and exhibits high LD_{50} values in quails after oral administration (2,000–10,000 mg/kg). However, in early studies (Vincent and Sisler, 1968; Gallagher et al., 1992; Yamano and Morita, 1995), chlorothalonil inactivated sulfhydryl enzymes and depleted cellular glutathione (GSH). This action is supposed to be one of the action modes of chlorothalonil. Chlorothalonil-induced cytotoxicity in rat hepatocytes was reported to be due to its GSH-depleting effects (Tamano and Morita, 1995). Thiol contains a carbon-bonded sulfhydryl group. Zn^{2+} is released via zinc-thiol/disulfide interchange (Maret, 1994), resulting in an increase in intracellular Zn^{2+} concentration ($[Zn^{2+}]i$). It is possible that chlorothalonil increases $[Zn^{2+}]i$ in mammalian cells. Because Zn^{2+} serves as a signal transducer and performs several physiological functions (Murakami and Hirano, 2008; Haase and Rink, 2009; Prasad, 2009), an abnormal elevation in $[Zn^{2+}]i$ could cause cytotoxicity. Therefore, using flow cytometry with fluorescent probes, we evaluated the effects of chlorothalonil on the cellular content of nonprotein thiols [NPT]i and $[Zn^{2+}]i$ in rat thymic lymphocytes to verify whether Zn^{2+} contributes to the cytotoxicity of chlorothalonil. This study highlights novel toxicological aspects of chlorothalonil. Thymocytes were used for this model study of chemical cytotoxicity for the following reasons. Thymus is most active during human neonatal and pre-adolescent periods and this organ begins to atrophy by early teens. The equivalent occurs in rats (Kuper et al., 1990). Since many people are very concerned about the adverse effects of

compounds on the health of their children, the results obtained from rat thymocytes in the current work are scientifically relevant.

2. Materials and methods

2.1. Chemicals

Tetrachloroisophthalonitrile (chlorothalonil), pentachlorophenol (PCP), and pentachloronitrobenzene (PCNB) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Fluorescent probes used to measure various cellular parameters are listed in Table 1. Other chemical reagents were obtained from Wako Pure Chemicals (Osaka, Japan). Specific reagents, such as Zn^{2+} chelators, are also listed in Table 1.

(Table 1 near here)

2.2. Cell preparation

This study on rats was approved by the Animal Experiment Committee of Tokushima University (T29-54). The thymus glands were quickly dissected from 12 pentobarbital-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 36–37ºC for 1 h at least before the use because they were isolated under cold conditions.. Thereafter, the fluorescent probes were applied to the cells as described below.

2.3. Experimental procedures and cytometric measurements

Chlorothalonil, dissolved and diluted (0.1–10 mM) in DMSO, was added to the cell suspension to achieve various final concentrations (0.1–30 μM) of chlorothalonil. H_2O_2 was used to induce oxidative stress in the cells. All experiments using the cell suspension were carried out at 36–37ºC.

Final concentration of DMSO was 0.1–0.3 % in the cell suspension because DMSO was used to prepare the stock solution of chlorothalonil, Fluo-3-AM, FluoZin-3-AM, 5-CMF-DA,

and Zn^{2+} chelators. DMSO at 0.3 % did not affect the cell viability and the fluorescence measurement.

Fluorescence analysis was performed using a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan with the JASCO software, Version 3.06). Cell lethality was assessed by adding 5 μM propidium iodide. 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 [NPT]i (Chikahisa et al., 1996). The cells were treated with 500 nM 5-CMF-DA for 30 min before the measurement of 5-CMF fluorescence. FluoZin-3-AM was used to monitor the changes in $[Zn^{2+}]i$ (Gee et al., 2002). The cells were treated with 1 μM FluoZin-3-AM for 1 h prior to fluorescence measurement. Excitation and emission wavelengths for the fluorescent probes are also listed in Table 1.

2.4. 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 series of experiments (4–8 samples per one concentration) was performed twice or thrice to validate the results.

3. Results

3.1. Sublethal concentrations of chlorothalonil

Preliminary studies (data not shown) indicated that treatment of rat thymocytes with 0.1–10 μM chlorothalonil for 3 h did not affect cell lethality. However, chlorothalonil at 30 μM concentration significantly increased cell lethality. Therefore, all experiments were performed with chlorothalonil at sublethal concentrations (0.1–10 μM).

3.2. Chlorothalonil-induced changes in [NPT]i

Treatment with 1 μM chlorothalonil for 3 h increased 5-CMF fluorescence intensity; however, it was reduced for 3 μM and up to 10 μM chlorothalonil (Figure 1A and 1 B). A plot of chlorothalonil concentration against 5-CMF fluorescence is shown in Figure 1B. The ability of chlorothalonil (3–10 μM) to reduce the fluorescence intensity was comparative to that of H_2O_2 (100 μM). Kinazaki et al. (2011) previously reported that elevated $[Zn^{2+}]i$ increases [NPT]i. Therefore, to examine whether Zn^{2+} contributed to the chlorothalonil-induced augmentation of 5-CMF fluorescence, changes in the fluorescence intensity by chlorothalonil (0.3–3 μM) were examined in the presence of the zinc chelator, *N,N,N′,N′*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN, 10 μM). TPEN did not affect control fluorescence intensity. However, it attenuated the chlorothalonil-induced changes in fluorescence intensity, suggesting that intracellular Zn^{2+} contributes to chlorothalonil-induced toxicity (Figure 1C).

(Figure 1 near here)

Cotreatment with H_2O_2 (100 μM) and chlorothalonil (0.3–10 μM) significantly reduced the intensity of 5-CMF fluorescence in a concentration-dependent manner (Figure 2A). Treatment with $ZnCl_2$ (3–10 μM) alone increased the intensity of 5-CMF fluorescence (Figure 2B). However, cotreatment with chlorothalonil (3 μM) and $ZnCl_2$ (3–10 μM) did not increase the fluorescence intensity.

(Figure 2 near here)

3.3. Chlorothalonil-induced elevation in $[Zn^{2+}]i$

The treatment of cells with chlorothalonil (3 μM) for 1 h increased FluoZin-3 fluorescence intensity (Figure 3A), suggesting an elevated $[Zn^{2+}]i$. Treatment with chlorothalonil (0.3–10 μM) for 1 h also increased the intensity of FluoZin-3 fluorescence in a concentration-dependent manner (Figure 3B). The ability of chlorothalonil (3–10 μM) to increase $[Zn^{2+}]i$ was comparative to that of H_2O_2 (100 μM). To examine if extracellular Zn^{2+} contributes to the chlorothalonil-induced augmentation of FluoZin-3 fluorescence, the fluorescence intensity was monitored after cotreating the cells with chlorothalonil (3 μM) and diethylenetriamine-

N,N,N′,N″,N″-pentaacetic acid (DTPA, 10 μM). Treatment with DTPA alone reduced the control fluorescence of FluoZin-3, suggesting that extracellular Zn^{2+} contributes to steady state $[Zn^{2+}]i$. However, chlorothalonil increased FluoZin-3 fluorescence in the presence of DTPA (Figure 4A). This suggests that chlorothalonil releases intracellular Zn^{2+}, and the contribution of extracellular Zn^{2+} is insignificant. Treatment with $ZnCl_2$ (3 μM) also increased the steady-state $[Zn^{2+}]i$, verifying the contribution of extracellular Zn^{2+} to the steady state $[Zn^{2+}]i$. The extent of chlorothalonil-induced increase in FluoZin-3 fluorescence in the presence of $ZnCl_2$ (3 μM) was similar to that under control condition (Figure 4B). TPEN diminished chlorothalonil-induced changes in FluoZin-3 fluorescence, indicating that $[Zn^{2+}]i$ contributed to FluoZin-3 fluorescence (Figure 4C).

(Figures 3 and 4 near here)

3.4. Effects of chlorothalonil on the cells under H_2O_2-induced oxidative stress

Both the decrease in [NPT]i and increase in $[Zn^{2+}]i$ potentiate the cytotoxicity of H_2O_2 (Matsui et al., 2010). In this study, chlorothalonil (3–10 μM) decreased [NPT]i and increased $[Zn^{2+}]i$. To verify if these effects of chlorothalonil potentiate the cytotoxicity of H_2O_2, the cells were cotreated with chlorothalonil (0.1–10 μM) and H_2O_2 (300 μM). Treatment with chlorothalonil alone (≤ 10 μM) for 3 h did not affect cell lethality, whereas incubation with H_2O_2 (300 μM) alone increased cell lethality (8.8 ± 0.6% vs 5.0 ± 0.7% control) as shown in Figure 7. Simultaneous treatment with chlorothalonil (1–10 μM) enhanced the H_2O_2-induced increase in cell lethality (Figure 5A). Of polychlorinated aromatic fungicides, 10 μM PCNB was also the case, but not 10 μM PCP (Figure 5B).

(Figure 5 near here)

4. Discussion

4.1. Mechanism of chlorothalonil-induced changes in [NPT]i

The complex concentration-response relation of chlorothalonil-induced change in 5-CMF fluorescence can be explained as follows: Kinazaki et al. (2011) demonstrated a concentration-dependent increase in $[Zn^{2+}]i$ and [NPT]i upon external $ZnCl_2$ treatment with correlation coefficients of 0.99 in rat thymocytes. Chlorothalonil is reported to deplete cellular GSH (Vincent and Sisler, 1968; Gallagher et al., 1992; Yamano and Morita, 1995). In this study, chlorothalonil (3–10 μM) significantly reduced 5-CMF fluorescence intensity (Figure 1) in rat thymocytes, indicating that chlorothalonil decreases [NPT]i as reported. However, chlorothalonil at lower concentrations (0.3–1 μM) increased 5-CMF fluorescence intensity (Figure 1), indicating an increase in [NPT]i. A decrease in [NPT]i, especially GSH content, increases $[Zn^{2+}]i$ via a zinc-thiol/disulfide interchange that releases intracellular Zn^{2+} (Maret, 1994). Zn^{2+} increases [NPT]i either by activating the *de novo* synthesis pathway of GSH or by increasing the transcription of the catalytic subunit of glutamate-cysteine ligase and GSH synthetase (Ha et al., 2006; Cortese et al., 2008). Therefore, the increase in [NPT]i by Zn^{2+} may downplay the decrease in [NPT]i by chlorothalonil. However, high concentrations of chlorothalonil further decrease [NPT]i (Figure 1) and increase $[Zn^{2+}]i$ (Figure 3). An excessive increase in $[Zn^{2+}]i$ augments oxidative stress (Kim et al., 1999; Matsui et al., 2010). Thus, the decrease in [NPT]i by chlorothalonil at high concentrations may downplay the increase in [NPT]i by Zn^{2+}. Consequently, chlorothalonil induced a complex change in [NPT]i.

TPEN partly attenuated the chlorothalonil (1 μM)-induced increase in 5-CMF fluorescence; however, it significantly decreased the intensity of FluoZin-3 fluorescence. Thus, it can be verified that chlorothalonil (1 μM) increases [NPT]i in the presence of TPEN, suggesting a Zn^{2+}-independent increase in [NPT]i by chlorothalonil. However, this is not conclusive because FluoZin-3 fluorescence reflects $[Zn^{2+}]i$, but not Zn^{2+} bound to protein and nonprotein thiols. Further, a decrease in 5-CMF fluorescence was observed when the cells were cotreated with chlorothalonil along with $ZnCl_2$ or H_2O_2 (Figure 2), indicating an excessive increase in $[Zn^{2+}]i$ and the augmentation of oxidative stress (Kim et al., 1999; Matsui et al., 2010).

4.2. Toxicological implication

Although chlorothalonil at 0.1–10 μM did not affect cell lethality, it potentiated H_2O_2 cytotoxicity at 1–10 μM (Figure 5). Micromolar concentrations of $ZnCl_2$ are also reported to potentiate H_2O_2 cytotoxicity by increasing $[Zn^{2+}]i$ (Matsui et al., 2010). Chlorothalonil significantly increased $[Zn^{2+}]i$ at concentrations of 1–10 μM (Figure 3B), which were consistent with those required to potentiate H_2O_2 cytotoxicity (Figure 5). Although chlorothalonil (1 μM) alone increased [NPT]i (Figure 1), it decreased [NPT]i further at the same concentration in the presence of H_2O_2 (Figure 2A). Thus, chlorothalonil at 1 μM may induce further oxidative stress in the cells in the presence of H_2O_2. Therefore, it can be concluded that sublethal concentrations of chlorothalonil exert significant cytotoxicity in cells under oxidative stress.

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Figure legends

Figure 1. Chlorothalonil-induced changes in 5-CMF fluorescence. (A) Change in histogram. Each histogram was constructed from 2500 cells after 3 h of chlorothalonil exposure. (B) Concentration-dependent changes in 5-CMF fluorescence intensity induced by chlorothalonil; each column and error bar indicate the mean and SD, respectively, of four samples; the dotted line indicates control level. Asterisks (**) indicate a significant difference between control cells (CONTROL) and cells treated with chlorothalonil or H_2O_2. (C) Chlorothalonil-induced changes in 5-CMF fluorescence of cells treated with or without TPEN; each column and error bar indicate the mean and SD of four samples; the dotted line indicates control level. Asterisks (**) indicate a significant difference ($P < 0.01$) between control cells (without chlorothalonil treatment) and TPEN-treated, and chlorothalonil-treated cells. Symbols (##) indicate a significant difference ($P < 0.01$) between cells treated without and with TPEN (CONTROL and TPEN).

Figure 2. Chlorothalonil-induced changes in 5-CMF fluorescence of cells simultaneously treated with H_2O_2 or $ZnCl_2$. (A) Concentration-dependent decrease in 5-CMF fluorescence of cells treated with 100 μM H_2O_2; each column and bar indicates the mean and SD, respectively, of four samples; the dotted line indicates control level (100 μM H_2O_2 alone). Asterisks (**) indicate a significant difference ($P < 0.01$) between control cells and chlorothalonil-treated cells. (B) Effect of $ZnCl_2$ on 5-CMF fluorescence of cells treated with or without chlorothalonil; each column and bar indicate the mean and SD, respectively, of four samples; the dotted line indicates control level. Asterisks (**) indicate a significant difference ($P < 0.01$) between control cells (without $ZnCl_2$ or chlorothalonil) and cells treated with $ZnCl_2$ and/or chlorothalonil.

Figure 3. Chlorothalonil-induced changes in FluoZin-3 fluorescence. (A) Change in histogram

by chlorothalonil. Each histogram was constructed from 2000 cells after 1 h of chlorothalonil exposure. (B) Concentration-dependent increase in the intensity of FluoZin-3 fluorescence by chlorothalonil; each column and bar indicate the mean and SD, respectively, of four samples; the dotted line indicates control level (CONTROL). Asterisks (**) indicate a significant difference ($P < 0.01$) between control cells and cells treated with chlorothalonil or H_2O_2.

Figure 4. Chlorothalonil-induced increase in FluoZin-3 fluorescence in the presence of (A) DTPA, (B) $ZnCl_2$, or (C) TPEN; each column and bar indicate the mean and SD, respectively, of four samples; the dotted line indicates control level. Asterisks (* and **) indicate a significant difference ($P < 0.05$ and 0.01, respectively) between control cells and cells treated with chlorothalonil or H_2O_2. Symbols (##) indicate a significant difference ($P < 0.01$) between cells treated without and with DTPA, $ZnCl_2$, or TPEN.

Figure 5. Effects of chlorothalonil, PCP, and PCNB on the cells suffering from oxidative stress. (A) Potentiation of H_2O_2 cytotoxicity by chlorothalonil; each column and bar indicate the mean cell lethality and SD, respectively, of four samples; the dotted line indicates control level. Asterisks (**) indicate a significant difference ($P < 0.01$) between control cells and cells treated with chlorothalonil, H_2O_2, or their combination. Symbols (##) indicate a significant difference ($P < 0.01$) between cells treated with H_2O_2 alone and cells treated with H_2O_2 and chlorothalonil. The concentration (300 μM) and incubation time (3 h) of H_2O_2 were pre-adjusted to induce cell death in about 10% of rat thymocytes to reduce the variability in cell susceptibility to H_2O_2. (B) Comparison with PCP and PCNB. Asterisks (**) indicate a significant difference ($P < 0.01$) between control cells and cells treated with test fungicide, H_2O_2, or their combination. Symbols (#, ##) indicate a significant difference ($P < 0.05$, 0.01) between cells treated with H_2O_2 alone and cells treated with H_2O_2 and test fungicide.

Table 1. Reagents used in this study

A. Fluorescent probes

Excitation wavelength was 488 nm for all fluorescent probes.

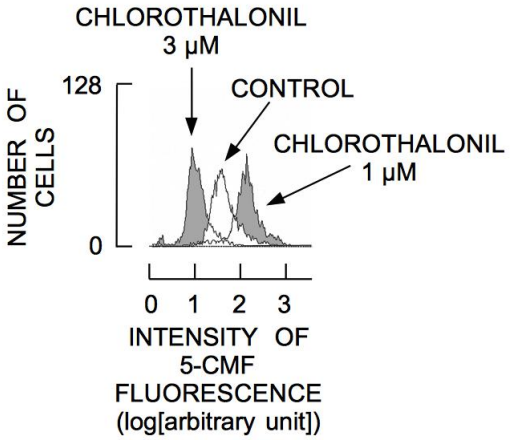
Probe [Manufacturer]	Emission wavelength (nm)
Propidium iodide [Molecular Probes, Inc., Eugene, OR, USA]	600 ± 20
5-Chloromethylfluorescein diacetate (5-CMF-DA) [Molecular Probes]	530 ± 20
FluoZin-3-AM [Molecular Probes]	530 ± 20

B. Specific reagents

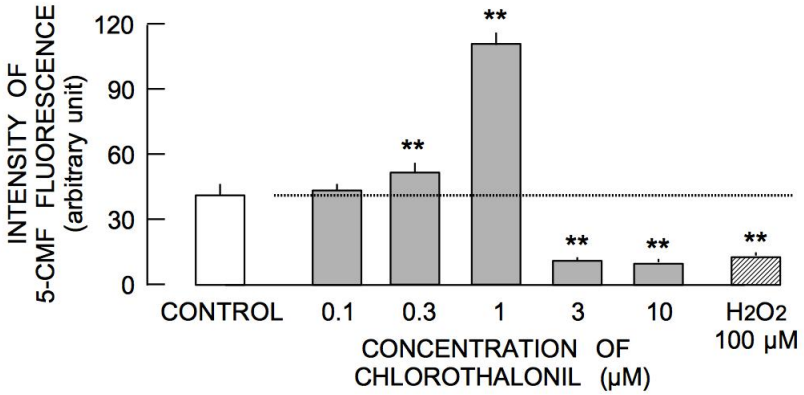
Reagent [Manufacturer]	Purpose
Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) [Dojin Chemical, Kumamoto, Japan]	Extracellular Zn^{2+} chelator
N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) [Dojin Chemical]	Intracellular Zn^{2+} chelator

Figure 1

(A)


CHLOROTHALONIL
3 μM
CONTROL
CHLOROTHALONIL
1 μM
128
0
NUMBER OF CELLS
0 1 2 3
INTENSITY OF 5-CMF FLUORESCENCE
(log[arbitrary unit])

(B)


INTENSITY OF 5-CMF FLUORESCENCE (arbitrary unit)
120
90
60
30
0
**
CONTROL
0.1
0.3
1
3
10
H2O2 100 μM
CONCENTRATION OF CHLOROTHALONIL (μM)

(C)

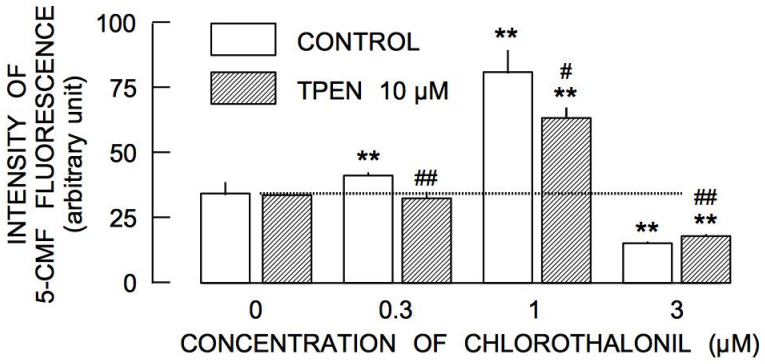
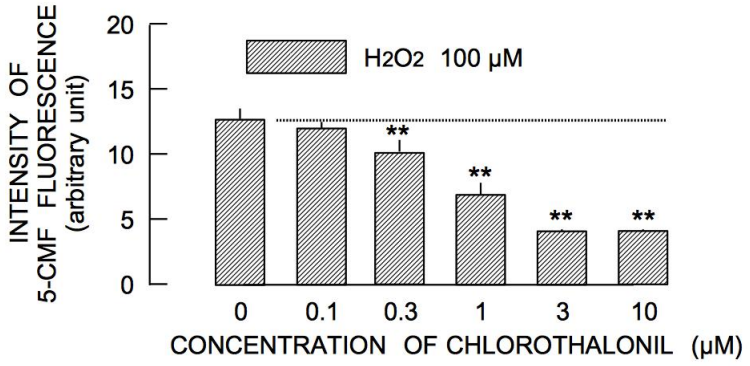

INTENSITY OF 5-CMF FLUORESCENCE (arbitrary unit)
100
75
50
25
0
CONTROL
TPEN 10 μM
**
##
#
0
0.3
1
3
CONCENTRATION OF CHLOROTHALONIL (μM)

Figure 2

(A)


INTENSITY OF
5-CMF FLUORESCENCE
(arbitrary unit)
20
15
10
5
0
H2O2 100 μM
**
**
**
**
0
0.1
0.3
1
3
10
CONCONCENTRATION OF CHLOROTHALONIL (μM)

(B)

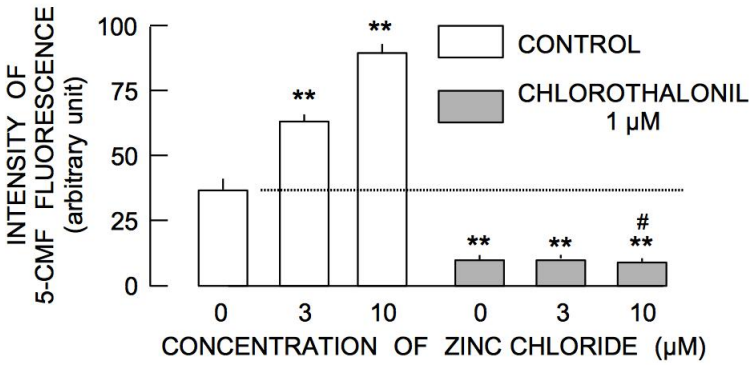
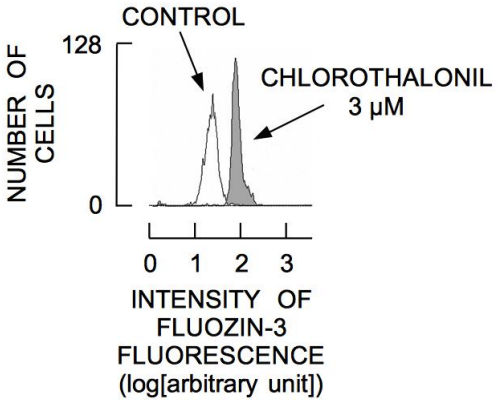

INTENSITY OF
5-CMF FLUORESCENCE
(arbitrary unit)
100
75
50
25
0
**
**
**
**
#
CONTROL
CHLOROTHALONIL
1 μM
0
3
10
0
3
10
CONCENTRATION OF ZINC CHLORIDE (μM)

Figure 3

(A)


CONTROL
128
NUMBER OF CELLS
CHLOROTHALONIL
3 μM
0
0 1 2 3
INTENSITY OF FLUOZIN-3 FLUORESCENCE (log[arbitrary unit])

(B)

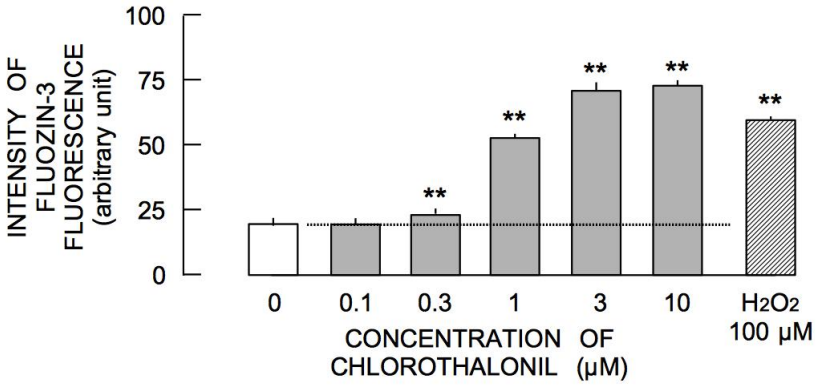
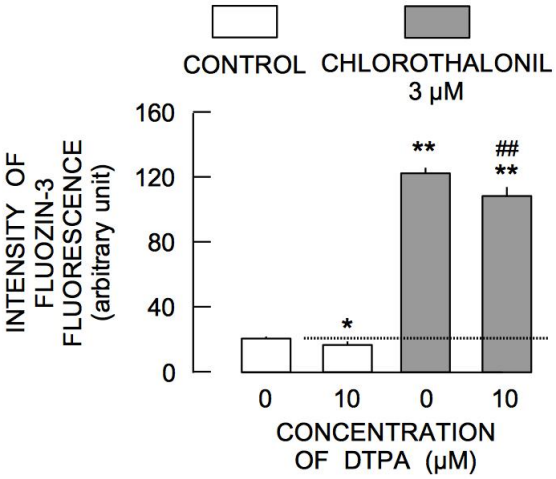
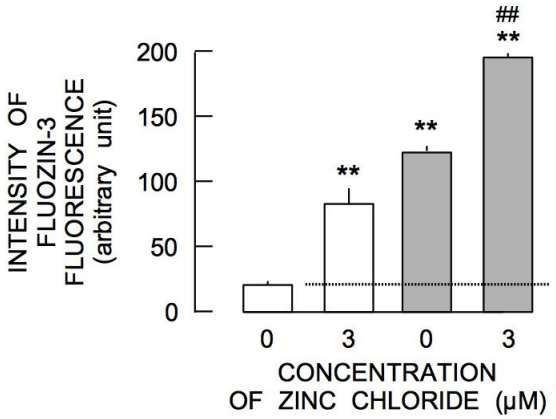

100
75
50
25
0
INTENSITY OF FLUOZIN-3 FLUORESCENCE (arbitrary unit)
**
**
**
**
**
0 0.1 0.3 1 3 10 H2O2 100 μM
CONCENTRATION OF CHLOROTHALONIL (μM)

Figure 4

(A)


CONTROL
CHLOROTHALONIL 3 μM
INTENSITY OF FLUOZIN-3 FLUORESCENCE (arbitrary unit)
160
120
80
40
0
*
**
##
**
0
10
0
10
CONCENTRATION OF DTPA (μM)

(B)


INTENSITY OF FLUOZIN-3 FLUORESCENCE (arbitrary unit)
200
150
100
50
0
**
**
##
**
0
3
0
3
CONCENTRATION OF ZINC CHLORIDE (μM)

(C)

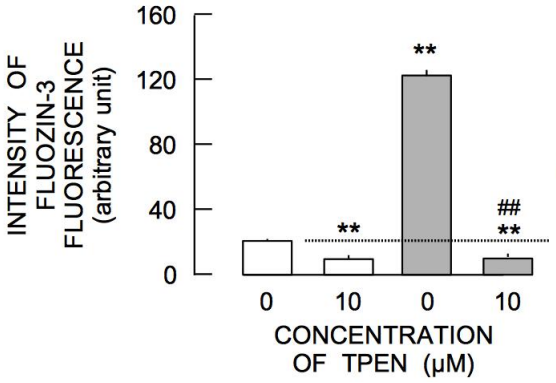
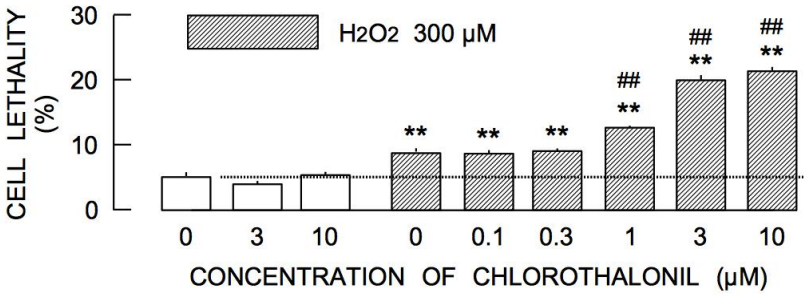
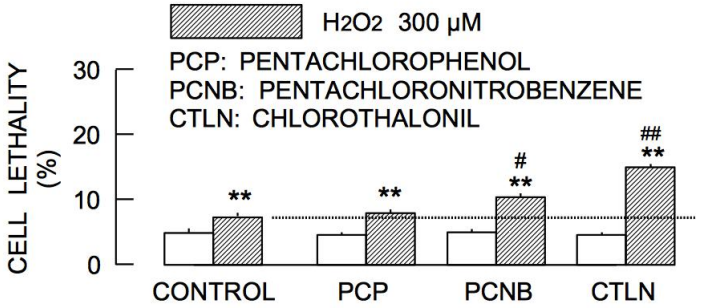

INTENSITY OF FLUOZIN-3 FLUORESCENCE (arbitrary unit)
160
120
80
40
0
**
**
##
**
0
10
0
10
CONCENTRATION OF TPEN (μM)

Figure 5

(A)


CELL LETHALITY (%)
30
20
10
0
H2O2 300 μM
**
##
0
3
10
0
0.1
0.3
1
3
10
CONCENTRATION OF CHLOROTHALONIL (μM)

(B)


H2O2 300 μM
PCP: PENTACHLOROPHENOL
PCNB: PENTACHLORONITROBENZENE
CTLN: CHLOROTHALONIL
CELL LETHALITY (%)
30
20
10
0
**
#
##
CONTROL
PCP
PCNB
CTLN