

Zinc-related actions of sublethal levels of benzalkonium chloride: Potentiation of benzalkonium cytotoxicity by zinc

Tsuyoshi Mitani¹, Ahmed Ibrahim Elhossany Elmarhomy², Luvsandorj Dulamjav², Enkhtumur Anu², Shohei Saito³, Shiro Ishida⁴, Yasuo Oyama^{2,5}

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

² Graduate School of Integrated Arts and Sciences, Tokushima University, Tokushima 770-8502, Japan

³ Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan

⁴ Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima 770-8512, Japan

⁵ Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima 770-8513, Japan

*Corresponding author: Yasuo Oyama, Ph.D.

E-mail: oyamay@tokushima-u.ac.jp

Highlights

- Zn^{2+} -related cytotoxic actions of benzalkonium (BZK) were examined in rat thymocytes.
- BZK at sublethal levels increased intracellular Zn^{2+} concentration.
- Low micromolar levels of Zn^{2+} potentiated the cytotoxicity of sublethal levels of BZK.
- Co-treatment with Zn^{2+} and BZK accelerated an early process of apoptosis.
- Zn^{2+} is one of determinants of the cytotoxicity of BZK.

Abstract

Benzalkonium chloride (BZK) is a common preservative used in pharmaceutical and personal care products. ZnCl_2 was recently reported to significantly potentiate the cytotoxicity of some biocidal compounds. In the present study, therefore, we compared the cytotoxic potency of BZK and then further studied the Zn^{2+} -related actions of the most cytotoxic agent among BZK, using flow cytometric techniques with appropriate fluorescent probes in rat thymocytes. Cytotoxicity of benzylcetyldimethylammonium (BZK-C16) was more potent than those of benzyldodecyldimethylammonium and benzyldimethyltetradecylammonium. ZnCl_2 (1–10 μM) significantly potentiated the cytotoxicity of BZK-C16 at a sublethal concentration (1 μM). The co-treatment of cells with 3 μM ZnCl_2 and 1 μM BZK-C16 increased the population of both living cells with phosphatidylserine exposed on membrane surfaces and dead cells. BZK-C16 at 0.3–1.0 μM elevated intracellular Zn^{2+} levels by increasing Zn^{2+} influx, and augmented the cytotoxicity of 100 μM H_2O_2 . Zn^{2+} is concluded to facilitate the toxicity of BZK. We suggest that the toxicity of BZK is determined after taking extracellular (plasma) and/or environmental Zn^{2+} levels into account. (170 words)

Keywords: benzalkonium chloride; cytotoxicity; lymphocytes; zinc

1. Introduction

Benzalkonium chloride (BZK) is a common preservative used in pharmaceutical and personal care products (Xia et al., 2005). The concentration of BZK in such products is adjusted so that the minimum amount necessary to achieve antimicrobial actions is added. Some reports indicate an increased incidence of adverse effects after long-term use of products containing BZK (for a review, Marple et al., 2004). Additionally, BZK has been found to be genotoxic under some experimental conditions (Ferk et al., 2007; Antunes et al., 2016). It may be impossible to recommend a safe limit for BZK in the general population because of conflicting data (Marple et al., 2004). Recently, two inpatients were murdered with BZK that was mixed into their intravenous bags in Yokohama, Japan (The Japan Times, 2016). It is necessary to further study the cellular actions of BZK in order to accumulate basic information on its toxicity.

Zinc is involved in the cytotoxicity of some biocidal compounds. In brief, the chelator of intracellular Zn^{2+} greatly attenuates the increase in cell lethality induced by H_2O_2 (Matsui et al., 2009), while $ZnCl_2$ augments the cytotoxicity of H_2O_2 (Matsui et al., 2010). Furthermore, $ZnCl_2$ potentiates the cytotoxicity of biocides such as 2-n-octyl-4-isothiazolin-3-one (Fukunaga et al., 2015), 4,5-dichloro-2-octyl-4-isothiazolin-3-one (Saitoh et al., 2015), and 2,2-dibromo-3-nitrilopropionamide (Ishikawa et al., 2016). BZK is also one type of biocide. Therefore, we hypothesized that the cytotoxicity of BZK is potentiated by zinc, and evaluated the Zn^{2+} -related actions of BZK using flow cytometric techniques with appropriate fluorescent probes in rat thymocytes. Commercially available BZK consists of benzyldodecyldimethylammonium chloride, benzyldimethyltetradecylammonium chloride, and benzylcetyldimethylammonium chloride. In the present study, we compared their cytotoxic potency and then further studied the Zn^{2+} -related effect of the most potent agent among them.

2. Materials and methods

2.1. Chemicals

Benzylododecyldimethylammonium chloride dihydrate (BZK-C12, purity 99.7% estimated by HPLC), benzyldimethyltetradecylammonium chloride hydrate (BZK-C14, purity 98.9%), and benzylcetyldimethylammonium chloride hydrate (BZK-C16, purity 98.3%) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Propidium iodide (PI), annexin V-FITC, FluoZin-3-AM, and 5-chloromethylfluorescein diacetate (5-CMF-DA) were obtained from Invitrogen (Eugene, OR, USA). WST assay kit and Zn²⁺ chelators (diethylenetriamine-N,N,N',N'',N''-pentaacetic acid and N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine; DTPA and TPEN, respectively) were obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Other chemicals were supplied from Wako Pure Chemicals unless mentioned.

2.2. Animals and cell preparation

This study (Registered number 05279) was approved by the Tokushima University Committee for Animal Experiments, Tokushima, Japan. The cell suspension was prepared as previously reported (Chikahisa et al., 1996; Matsui et al., 2010). In brief, thymus glands were obtained from rats that were anesthetized with ether. The slices were gently triturated in cold Tyrode's solution (2–4°C, 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, pH 7.4 adjusted by 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and appropriate amount of NaOH) to dissociate single thymocytes. The solution containing dissociated cells was then passed through a mesh (56 µm in diameter) to prepare cell suspension. It is noted that the cell suspension contained 216.9 ± 14.4 nM zinc that was derived from cell preparation (Sakanashi et al., 2009). The cell suspension was incubated at 36–37°C for 1 h before any experimentation. This preparation has experimental limitation. Thymocytes spontaneously undergo apoptosis during a prolonged incubation (Nishimura et al., 2008; Fujimoto et al., 2010). Therefore, the experiments were completed within 8 h after the

dissection of thymus glands from rats.

Various concentrations of BZK (0.1–10 mM in distilled water) were added to the cell suspensions (2 mL per test tube) and thereafter incubated at 36–37°C for 2–4 h. A sample from each cell suspension (100 μ L) was analyzed by a flow cytometry to estimate the BZK-induced changes in cellular and membrane parameters. It took about 10 s to acquire data from 2500 cells.

2.3. Fluorescence measurements

Cellular and membrane parameters were measured using a flow cytometer equipped with an argon laser (CytoACE-150; JASCO, Tokyo, Japan) and fluorescent probes as previously described (Chikahisa et al., 1996; Matsui et al., 2008). The fluorescence was analyzed by JASCO software in operating system (Version 3.06; JASCO, Tokyo, Japan). Under experimental conditions, no fluorescence from the reagents used was observed, except for the fluorescent probes. The excitation wavelength for all fluorescent probes used in this study was 488 nm, while emissions wavelengths were 530 ± 20 nm for FITC, FluoZin-3, and 5-CMF and 600 ± 20 nm for PI.

To assess cell lethality, PI was added to the cell suspensions to a final concentration of 5 μ M. Because PI stains dead cells, the measurement of PI fluorescence provides information on cell lethality. The fluorescence was measured using a flow cytometer 2 min after adding PI. Exposure of phosphatidylserine on the outer surface of cell membranes, a phenomenon that occurs during the early stage of apoptosis, was detected using annexin V-FITC (Koopman et al., 1994). Cells were treated with annexin V-FITC (10 μ l/mL) for 30 min before evaluation. FluoZin-3-AM was used to monitor changes in the intracellular Zn^{2+} levels ($[Zn^{2+}]_i$) (Gee et al., 2002). The cells were treated with 1 μ M FluoZin-3-AM for 60 min prior to any fluorescence measurements. 5-CMF-DA was used to estimate the cellular content of glutathione ($[GSH]_i$) in rat thymocytes (Chikahisa et al., 1996). Oxidative stress decreases $[GSH]_i$. The 5-CMF fluorescence was measured 30 min after adding 1 μ M 5-CMF-DA because it attains peak

intensity within 30 min after application. 5-CMF fluorescence was monitored in the living cells that were not stained with PI.

2.4. WST assay

Cells were incubated with the WST-1 reagent for 2 h in a 96-well tissue culture plate after the cells were treated with BZK-C16 at 0.3–10 μM for 2 h. Thereafter, the formation of formazan was quantitated with a microplate reader (MTP-310Lab, Corona Electric, Hitachinaka, Japan). The measured absorbance at 450 nm correlates with the number of viable cells.

2.5. Statistical analysis and presentation

Statistical analyses were done using ANOVA, with post-hoc Tukey's multivariate analysis. P-values of 0.05 or less were statistically considered significant. In the results, values (columns and bars in figures) were expressed as the mean and the standard deviation of 4–8 samples. Each experiment was repeated three times unless noted otherwise.

3. Results

3.1. Cytotoxic actions of BZK-C12, BZK-C14, and BZK-C16

As shown in Fig. 1A, the treatment of rat thymocytes with 3 μM BZK-C14 or BZK-C16 for 2 h increased the cells exhibiting PI fluorescence. However, as almost all cells showed PI fluorescence in the presence of BZK-C16, its cytotoxicity was considered more potent than that of BZK-C14. The intensity of forward scatter, a parameter of cell size, was decreased in a large population of cells with PI fluorescence, suggesting shrunken dead cells. Dose-dependent changes in cell lethality by BZK-C12, BZK-C14, and BZK-C16 are summarized in Fig. 1B. The potency order for cytotoxicity was BZK-C16 > BZK-C14 > BZK-C12 when the cells were treated with 3 μM of the respective agents for 2 h. The experiments described below were carried out using 0.1–1.0 μM of BZK-C16. The change in cell viability, estimated with WST assay, by BZK-C16 was shown in Fig. 1C. The treatment of cells with 0.1–3 μM BZK-C16 for

2 h did not decrease the absorbance caused by formazan dye while significant reduction of absorbance in the case of 3 μM BZK-C16. Result indicates the significant decrease in cell viability by 3 μM BZK-C16.

(Figure 1)

3.2. Effects of BZK-C16 on forward scatter and side scatter

The changes of cell lethality (Fig. 1B) and viability (Fig. 1C) by BZK-C16 were very steep. Therefore, the effects of BZK-C16 at sublethal concentrations (1 μM or less) on the cells were examined. The treatment of cells with 0.1–1 μM for 4 h did not significantly increase the number of cells exhibiting PI fluorescence (dead cells). However, BZK at 0.1–1 μM slightly, but significantly, decreased the intensity of side scatter without affecting forward scatter (Fig. 2). Both forward and side scatter were decreased by 1 μM BZK.

(Figure 2)

3.3. Potentiation of BZK-C16 cytotoxicity by ZnCl_2

Zinc is involved in the cytotoxicity of some chemical compounds (Matsui et al., 2008, 2010; Saitoh et al., 2015; Fukunaga et al., 2015). To determine whether the cytotoxicity of BZK-C16 is similarly affected, the effects of 1–10 μM ZnCl_2 on cells simultaneously treated with 1 μM BZK-C16 for 2 and 4 h were examined. The changes in cell lethality after exposure to 0.1–1.0 μM BZK-C16 were not significant 2 h after the initiation of experimentation. The treatment of cells with DTPA (10 μM) or ZnCl_2 (1–10 μM) for 4 h did not affect cell lethality, while the co-treatment with ZnCl_2 (1–10 μM) with BZK-C16 (1 μM) significantly increased cell lethality in a dose-dependent manner (Fig. 3). Thus, we concluded that zinc potentiates the cytotoxicity of BZK-C16.

Effects of 0.1–1.0 μM BZK-C16 were examined at 2–3 h after the start of application in order to assess the process of cell death induced by BZK-C16 in the experiments described below.

(Figure 3)

3.4. Change in the population of living cells with exposed phosphatidylserine by BZK-C16 and ZnCl₂

Zinc accelerates the process of cell death induced by some biocidal compounds (Oyama et al., 2010; Kanemoto-Kataoka et al., 2015; Niwa et al., 2016). Therefore, to assess the possibility that zinc potentiates the cytotoxicity of BZK-C16, changes in the cell population as detected with annexin V-FITC and PI were examined in cells treated with ZnCl₂, BZK-C16, and both for 3 h. As shown in Fig. 4A, four populations were observed: area *N*, *A*, *P*, and *AP*. Co-treatment with 3 μM ZnCl₂ and 1 μM BZK-C16 for 3 h increased the populations of living cells with exposed phosphatidylserine (area *A*) and dead cells with exposed phosphatidylserine (area *AP*). The process of cell death induced by BZK-C16 seems to be prompted by ZnCl₂. Results are summarized in Fig. 4B.

(Figure 4)

3.4. Change in [Zn²⁺]_i by BZK-C16 and ZnCl₂

The treatment of cells with 0.1–1.0 μM BZK-C16 for 2 h increased the intensity of FluoZin-3 fluorescence in a dose-dependent manner (Fig. 5A). The increase produced by 0.3 and 1 μM BZK-C16 was statistically significant. A small increase in the intensity of FluoZin-3 fluorescence by 1 μM BZK-C16 was observed under an external Zn²⁺-free condition produced by adding 10 μM DTAP into the cell suspension (Fig. 5B). The addition of 3 μM ZnCl₂ into the cell suspension significantly increased the control intensity of FluoZin-3 fluorescence, and 1 μM BZK-C16 greatly augmented the FluoZin-3 fluorescence (Fig. 5B). Therefore, the elevation of [Zn²⁺]_i by BZK-C16 seems to be due to both intracellular Zn²⁺ release and external Zn²⁺ influx.

(Figure 5)

3.5. Change in [GSH]_i by BZK-C16 and ZnCl₂

Intracellular Zn²⁺ affects the [GSH]_i in similar preparations (Kinazaki et al., 2011). Therefore, the effects of 3 μM ZnCl₂, 1 μM BZK-C16, and both on 5-CMF fluorescence in cells

were assessed after treatment for 2 h. As shown in Fig. 6, all agents significantly increased the intensity of 5-CMF fluorescence, indicating an increase in the $[GSH]_i$.

(Figure 6)

3.6. Effect of BZK-C16 on the cells suffering from oxidative stress

Zn^{2+} increases the vulnerability of cells to oxidative stress induced by H_2O_2 (Matsui et al., 2010). Therefore, there is a possibility that BZK-C16 potentiates the cytotoxicity of H_2O_2 , because BZK-C16 increases $[Zn^{2+}]_i$ (Fig. 7). We treated cells with BZK-C16, H_2O_2 , and both for 4 h. As shown in Fig. 7, as the concentration of BZK-C16 increased, cell lethality increased in the simultaneous presence of H_2O_2 . The co-treatment with 100 μM H_2O_2 and 1 μM BZK-C16 greatly increased cell lethality (Fig. 7). Thus, we concluded that BZK-C16 seems to increase cell vulnerability to oxidative stress induced by H_2O_2 .

(Figure 7)

4. Discussion

4.1. Cellular actions of BZK

The cytotoxicity of BZK-C16 was more profound than those of BZK-C12 and BZK-C14 (Fig. 1B). It is widely accepted that the length of hydrocarbon chain is one of determinants for the cytotoxicity of same series of chemical compounds (Sikkema et al., 1995). This concept is also the case of BZK. In this study, the property of most potent cytotoxic BZK-C16 was examined. Important observation was the attenuation of forward scatter by the treatment with 1 μM BZK-C16 for 4 h (Fig. 2). The attenuation of forward scatter indicates cell shrinkage that is one of early prerequisites to apoptosis (Maeno et al., 2000). The attenuation of side scatter was also observed in the case of 0.3–1.0 μM BZK-16 (Fig. 2). Since side scatter is a parameter for cellular granularity, the result shows that BZK decreased the granularity. This was unexpected because the attenuation of forward scatter (cell shrinkage) is usually associated with the

augmentation of side scatter. However, the connotative meaning of attenuated intensity of side scatter is not elucidated. Further analysis will be necessary in this aspect. As shown in Fig. 3, the co-treatment of BZK-C16 and ZnCl₂ increased cell lethality as the concentration of ZnCl₂ increased. The co-treatment with BZK-C16 and ZnCl₂ greatly increased [Zn²⁺]_i. Excessive elevation of [Zn²⁺]_i induces oxidative stress (Sensi et al., 2011; Wu et al., 2013; McCord and Aizenman, 2014), due to the decrease in [GSH]_i. However, this was not the case with the co-treatment with BZK-C16 and ZnCl₂ in the present study, as the co-treatment with BZK-C16 and ZnCl₂ increased the [GSH]_i (Fig. 6). Therefore, we speculate that in the case of co-treatment with ZnCl₂ and BZK-C16, the excessive increase in [Zn²⁺]_i induces cell death without oxidative stress. Since this mechanism has not been elucidated, further study will be required.

BZK-C16 at sublethal concentrations (0.3–1.0 μM) increased the susceptibility of cells to H₂O₂-induced oxidative stress (Fig. 7). BZK-C16 at these concentrations elevated [Zn²⁺]_i (Fig. 5). BZK-C16 at 1 μM slightly increased the [Zn²⁺]_i under external Zn²⁺-free conditions, indicating intracellular Zn²⁺ release. In the extracellular presence of 3 μM ZnCl₂, BZK-C16 greatly increased [Zn²⁺]_i. This increase was much more profound than that under control conditions (Fig. 5), indicating an increase in external Zn²⁺ influx. Zinc pyrithione, a zinc ionophore, increases cell susceptibility to oxidative stress by elevating [Zn²⁺]_i (Oyama et al., 2012). This also seems to be the case for BZK-C16. However, another explanation for the increase in cell lethality by co-treatment with H₂O₂ and BZK-C16 is also possible, considering the following: BZK is a cationic molecule. Treatment with H₂O₂ increases the population of cells with exposed phosphatidylserine (Oyama et al., 1999). Phosphatidylcholine, normally exposed on the outer surface of membranes, is zwitterionic, while phosphatidylserine is anionic. Positively charged BZK-C16 may bind with high preference to anionic phosphatidylserine over zwitterionic phosphatidylcholine, resulting in an increase in the toxicity of BZK-C16.

4.2. Implications

The plasma zinc concentration is normally 10-20 μM and varies under nutritional and pathological conditions (Kuvibidila et al., 2006; Potocnik et al., 2006). Zinc is applied clinically for treatment of malaria, diarrhea, and respiratory infections (Shankar and Prasad, 1988; Richard et al., 2006). Potentiation of BZK-C16 cytotoxicity by ZnCl_2 in this study was observed when the concentrations of ZnCl_2 were between 1 and 10 μM (Figs. 3 and 4). Extracellular/plasma Zn^{2+} concentration could thus play a critical role in the toxicity of BZK. We caution however that the *in vitro* estimation of BZK cytotoxicity may be different from that under *in vivo* conditions.

Zinc is used in many industries, with wastewater especially polluted with zinc (Harte et al., 1991; Gakwisiri et al., 2012). Thus, environmental Zn^{2+} is also important for the estimation of the toxicity of BZK, while the concentration of ZnCl_2 used in this study was equivalent to 0.065–0.65 mg/L of zinc; the maximum concentrations of zinc in river water can range from 0.14 to 19.7 mg/L in the Przemsza River, Poland (Pistelok and Galas, 1999), from 0.71 to 1.76 mg/L in the Gombak and Penchala Rivers, Malaysia (Ismail et al., 2013), and from 4.2 to 6.7 mg/L in the Godavari River, India (Sayyed and Bhosle, 2010). BZK may be more toxic in rivers with high zinc levels. Of note, Zn^{2+} -induced potentiation of cytotoxicity is not specific to BZK, as the simultaneous application of ZnCl_2 is also known to augment the cytotoxicity of imidazole antifungals (Matsui et al., 2008).

Conflict of interest

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

Acknowledgements

This study was supported by Grant-in-Aids for Scientific Research (C26340039) from the

Japan Society for the Promotion of Science.

References

- Antunes, S.C., Nunes, B., Rodrigues, S., Nunes, R., Fernandes, J., Correia, A.T. 2016. Effects of chronic exposure to benzalkonium chloride in *Oncorhynchus mykiss*: cholinergic neurotoxicity, oxidative stress, peroxidative damage and genotoxicity. *Environmental Toxicology and Pharmacology*, 45, 115–122.
- Chikahisa, L., Oyama, Y., Okazaki, E., Noda, K. 1996. Fluorescent estimation of H₂O₂-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. *Japanese Journal of Pharmacology*, 71, 299–305.
- Ferk, F., Misík, M., Hoelzl, C., Uhl, M., Fuerhacker, M., Grillitsch, B., Parzefall, W., Nersesyan, A., Micieta, K., Grummt, T., Ehrlich, V., Knasmüller, S. 2007. Benzalkonium chloride (BAC) and dimethyldioctadecyl-ammonium bromide (DDAB), two common quaternary ammonium compounds, cause genotoxic effects in mammalian and plant cells at environmentally relevant concentrations. *Mutagenesis*, 22, 363–370.
- Fukunaga, E., Oyama, T.M., Oyama, Y. 2015. Elevation of the intracellular Zn²⁺ level by 2-n-octyl-4-isothiazolin-3-one in rat thymocytes: an involvement of a temperature-sensitive Zn²⁺ pathway. *Toxicology Research*, 4, 65–70.
- Fujimoto, A., Shingai, Y., Oyama, T.B., Kawanai, T., Hashimoto, E., Koizumi, K., Kimura, K., Masuda, T., Oyama, Y. 2010. Apoptosis-inducing action of two products from oxidation of sesamol, an antioxidative constituent of sesame oil: a possible cytotoxicity of oxidized antioxidant. *Toxicology in Vitro*, 24, 1720–1726.
- Gakwisiri, C., Raut, N., Al-Saadi, A., Al-Aisri, S., Al-Ajmi, A. 2012. A critical review of removal of zinc from wastewater. *Proceedings of World Congress on Engineering*, 1, 627–630.
- Gee, K.R., Zhou, Z.L., Qian, W.J., Kennedy, R. 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.
- Harte, J., Holdren, C., Schneider, R., Shirley, C. 1991. A guide to commonly encountered toxics. In: Harte, J., Holdren, C., Schneider, R., Shirley, C. (Eds.) *Toxics A to Z – a guide to everyday pollution hazards*. Uni. California Press, Berkeley, 244–247 and 436–438.
- Ishikawa, M., Muraguchi, R., Azuma, A., Nawata, S., Miya, M., Katsuura, T., Naito, T. Oyama, Y. 2016. Cytotoxic actions of 2, 2-dibromo-3-nitrilopropionamide, a biocide in hydraulic fracturing fluids, on rat thymocytes. *Toxicology Research*, 5, 1329–1334.
- Ismail, Z., Salim, K., Othman, S.Z., Ramli, A.H., Shirazi, S.M., Karim, R., Khoo, S.Y. 2013. Determining and comparing the levels of heavy metal concentrations in two selected urban river water. *Measurement*, 46, 4135–4144.
- Kanemoto-Kataoka, Y., Oyama, T.M., Ishibashi, H., Oyama, Y. 2015. Dithiocarbamate fungicides increase intracellular Zn^{2+} levels by increasing influx of Zn^{2+} in rat thymic lymphocytes. *Chemico-Biological Interactions*, 237, 80–86.
- Kinazaki, A., Chen, H., Koizumi, K., Kawanai, T., Oyama, T.M., Satoh, M., Ishida, S., Okano, Y., Oyama, Y. 2011. Putative role of intracellular Zn^{2+} release during oxidative stress: a trigger to restore cellular thiol content that is decreased by oxidative stress. *Journal of Physiological Sciences*, 61, 403–409.
- Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T., Van Oers, M.H. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood*, 84, 1415–1420.
- Kuvibidila, S.R., Sandoval, M., Lao, J., Velez, M., Yu, L., Ode, D., Gardner, R., Lane, G., Warrier, R.P. 2006. Plasma zinc levels inversely correlate with vascular cell adhesion molecule-1 concentration in children with sickle cell disease. *Journal of the National Medical Association*, 98, 1263–1272.
- Maeno, E., Ishizaki, Y., Kanaseki, T., Hazama, A., Okada, Y. 2000. Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proceedings*

- of the National Academy of Sciences of the United States of America, 97, 9487–9492.
- Marple, B., Roland, P., Benninger, M. 2004. Safety review of benzalkonium chloride used as a preservative in intranasal solutions: an overview of conflicting data and opinions. *Otolaryngology–Head and Neck Surgery*, 130, 131–141.
- Matsui, H., Oyama, T. M., Okano, Y., Hashimoto, E., Kawanai, T., Oyama, Y. 2010. Low micromolar zinc exerts cytotoxic action under H₂O₂-induced oxidative stress: excessive increase in intracellular Zn²⁺ concentration. *Toxicology*, 276, 27–32.
- Matsui, H., Sakanashi, Y., Nishimura, Y., Kawanai, T., Oyama, Y., Ishida, S., Okano, Y. 2009. Effects of Zn²⁺ chelators, DTPA and TPEN, and ZnCl₂ on the cells treated with hydrogen peroxide: a flow-cytometric study using rat thymocytes. *Natural Science Research University of Tokushima*, 23, 12–18.
- Matsui, H., Sakanashi, Y., Oyama, T.M., Oyama, Y., Yokota, S. I., Ishida, S., Okano, Y., Oyama, T.B., Nishimura, Y. 2008. Imidazole antifungals, but not triazole antifungals, increase membrane Zn²⁺ permeability in rat thymocytes: Possible contribution to their cytotoxicity. *Toxicology*, 248, 142–150.
- McCord, M.C., Aizenman, E. 2014. The role of intracellular zinc release in aging, oxidative stress, and Alzheimer's disease. *Frontiers in Aging Neuroscience*, 6, 77.
- Nishimura, Y., Oyama, T.B., Sakanashi, Y., Oyama, T.M., Matsui, H., Okano, Y., Oyama, Y. 2008. Some characteristics of quercetin-induced cytotoxicity on rat thymocytes under in vitro condition. *Toxicology in Vitro*, 22, 1002–1007.
- Niwa, E., Mitani, T., Saitoh, S., Kanemaru, K., Ishida, S., Yokoigawa, K., Oyama, Y. 2016. Zinc increases vulnerability of rat thymic lymphocytes to arachidonic acid under in vitro conditions. *Food and Chemical Toxicology*, 96, 177–182.
- Oyama, T.M., Oyama, K., Oyama, T.B., Ishida, S., Okano, Y., Oyama, Y. 2010. Zinc at clinically-relevant concentrations potentiates the cytotoxicity of polysorbate 80, a non-ionic surfactant. *Toxicology in Vitro*, 24, 737–744.

- Oyama, T.M., Saito, M., Yonezawa, T., Okano, Y., Oyama, Y. 2012. Nanomolar concentrations of zinc pyrithione increase cell susceptibility to oxidative stress induced by hydrogen peroxide in rat thymocytes. *Chemosphere*, 87, 1316–1322.
- Oyama, Y., Noguchi, S., Nakata, M., Okada, Y., Yamazaki, Y., Funai, M., Chikahisa, L., Kanemaru, K. 1999. Exposure of rat thymocytes to hydrogen peroxide increases annexin V binding to membranes: inhibitory actions of deferoxamine and quercetin. *European Journal of Pharmacology*, 384, 47–52.
- Pistelok, F., Galas, W., 1999. Zinc pollution of the Przemsza River and its tributaries. *Polish Journal of Environmental Studies*, 8, 47–54.
- Potocnik, F.C., van Rensburg, S.J., Hon, D., Emsley, R.A., Moodie, I.M., Erasmus, R.T. 2006. Oral zinc augmentation with vitamins A and D increases plasma zinc concentration; implications for burden of disease. *Metabolic Brain Disease*, 21, 139–147.
- Richard, S.A., Zavaleta, N., Caulfield, L.E., Black, R.E., Witzig, R.S., Shankar, A.H. 2006. Zinc and iron supplementation and malaria, diarrhea, and respiratory infections in children in the Peruvian Amazon. *American Journal of Tropical Medicine and Hygiene*, 75, 126–132.
- Saitoh, S., Fukunaga, E., Ohtani, H., Oyama, Y. 2015. Zn^{2+} -dependence of the synergistic increase in rat thymocyte cell lethality caused by simultaneous application of 4, 5-dichloro-2-octyl-4-isothiazolin-3-one (DCOIT) and H_2O_2 . *Chemosphere*, 135, 447–452.
- Sakanashi, Y., Oyama, T.M., Matsuo, Y., Oyama, T.B., Nishimura, Y., Ishida, S., Imai, S., Okano, Y., Oyama, Y. 2009. Zn^{2+} , derived from cell preparation, partly attenuates Ca^{2+} -dependent cell death induced by A23187, calcium ionophore, in rat thymocytes. *Toxicology In Vitro*, 23, 338–345.
- Sayed, J.A., Bhosle, A.B. 2010. The study of zinc metal concentration by spectrophotometric method from Godavari River at Nanded, Maharashtra. *Der Chemica Sinica Journal (Pelagia Res. Library)*, 1, 104–109.

- Sensi, S.L., Paoletti, P., Koh, J.Y., Aizenman, E., Bush, A.L., Hershfinkel, M. 2011. The neurophysiology and pathology of brain zinc. *Journal of Neuroscience*, 31, 16076–16085.
- Shankar, A.H., Prasad, A.S. 1998. Zinc and immune function: the biological basis of altered resistance to infection. *American Journal of Clinical Nutrition*, 68, 447S–463S.
- Sikkema, J., De Bont, J.A., Poolman, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiological Reviews*, 59, 201–222.
- The Japan Times. 2016. “Police launch murder investigation after deaths in Yokohama hospital” <http://www.japantimes.co.jp/news/2016/09/24/national/crime-legal/police-probe-man-died-yokohama-hospital-may-poisoned/#.WE33JWSLR60>.
- Wu, W., Bromberg, P.A., Samet, J.M. 2013. Zinc ions as effectors of environmental oxidative lung injury. *Free Radical Biology and Medicine*, 65, 57–69.
- Xia, K., Bhandari, A., Das, K., Pillar, G. 2005. Occurrence and fate of pharmaceuticals and personal care products (PPCPs) in biosolids. *Journal of Environmental Quality*, 34, 91–104.

Figure legends

Figure 1. Cytotoxicity of BZK. (A) Cytograms of cells treated with and without BZK-C16. Cytograms were obtained 2 h after the addition of BZK-C16. Each cytogram consisted of 2000 cells. Dotted lines under the cytogram indicate the population of cells exhibiting PI fluorescence (dead cells). (B) Dose-dependent changes in the percent population of dead cells by benzalkonium (BZK-C12, BZK-C14, and BZK-C16). Column and bar indicate average and standard deviation of four samples. Asterisks (**) show a statistically significant increase ($P < 0.01$) in cell lethality between control group and group of cells treated with BZK-C12, BZK-C14, or BZK-C16. (C) Dose-dependent change in cell viability by BZK-C16. Viability was estimated by WST assay. Asterisks (**) show a statistically significant decrease ($P < 0.01$) in cell viability.

Figure 2. Effect of BZK-C16 on forward (cell size) and side scatter (cell density or granularity). Column and bar indicate average and standard deviation of four samples. Asterisks (**) show a statistically significant increase ($P < 0.01$) in the intensity between control group and group of cells incubated with sublethal levels of BZK-C16.

Figure 3. Effects of DTPA and $ZnCl_2$ on cells simultaneously treated with BZK-C16 for 4 h. Column and bar indicate average and standard deviation of four samples. Asterisks (**) show a significant increase ($P < 0.01$) in cell lethality between control group and test groups. Symbols (##) indicate a significant difference ($P < 0.01$) between an arrowed pair.

Figure 4. Change in cell population by $ZnCl_2$, BZK-C16, and both. (A) Cytogram (PI fluorescence versus FITC fluorescence) of cells treated with $ZnCl_2$, BZK-C16, and both. Each cytogram was constructed with 2500 cells. N: intact living cells, A: annexin V-positive living

cells, P: annexin V-negative dead cells, AP: annexin V-positive dead cells. Effects were examined 3 h after the start of application. (B) Changes in percent population, described in the legend of Fig. 4, by treatment with ZnCl₂, BZK-C16, and both for 3 h. Column and bar indicate average and standard deviation of four samples. Asterisks (*, **) show a significant increase ($P < 0.05$, $P < 0.01$) in cell lethality between control and test groups.

Figure 5. Augmentation of FluoZin-3 fluorescence by BZK-C16. (A) Augmentation of FluoZin-3 fluorescence by BZK-C16. Effects were examined 1 h after the start of application. Column and bar indicate average and standard deviation of four samples. Asterisks (**) show a significant difference ($P < 0.01$) between control and test groups. (B) Augmentations of FluoZin-3 fluorescence by BZK-C16 in the presence of DTPA or ZnCl₂. Effects were examined 1 h after the start of application. Column and bar indicate average and standard deviation of four samples. Asterisks (**) show a significant difference ($P < 0.01$) between control and test groups.

Figure 6. Augmentation of 5-CMF fluorescence by ZnCl₂, BZK-C16, and both. Effects were examined 3 h after the start of application. Column and bar indicate average and standard deviation of four samples. Asterisks (**) show a significant difference ($P < 0.01$) between control and test groups.

Figure 7. Effects of BZK-C16 on cells simultaneously treated with H₂O₂ for 4 h. Column and bar indicate average and standard deviation of four samples. Asterisks (**) show a significant increase ($P < 0.01$) in cell lethality between control and test groups. Symbols (#, ##) indicate a significant difference ($P < 0.05$, $P < 0.01$) between an arrowed pair.

Figure 1

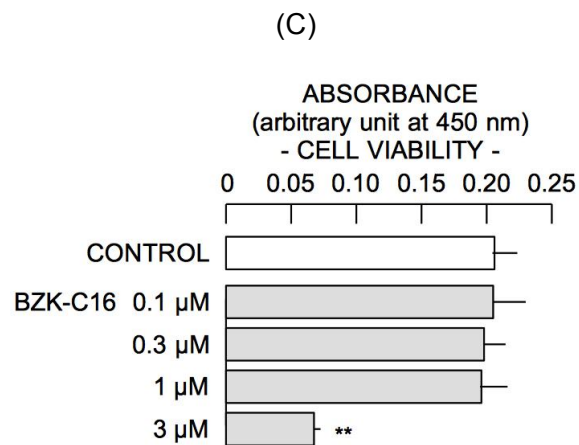
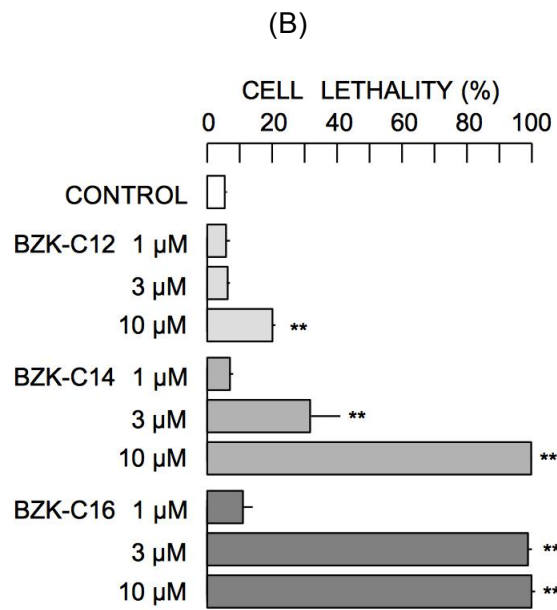
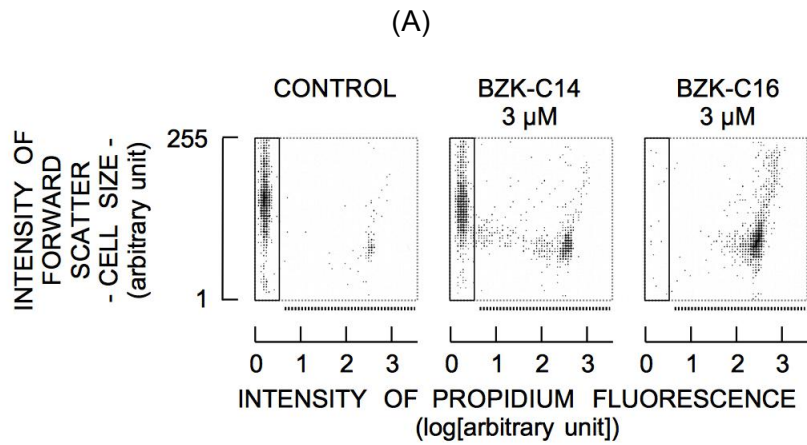


Figure 2

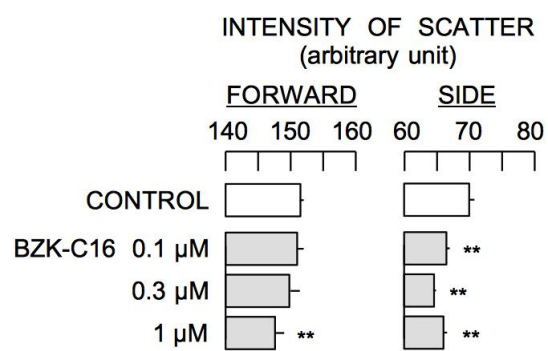


Figure 3

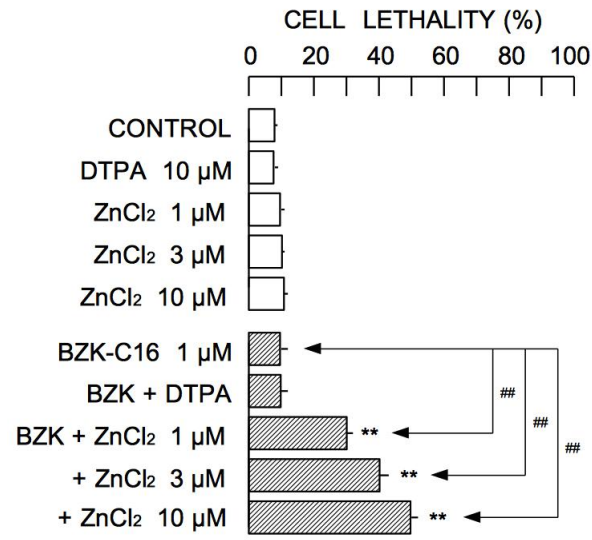


Figure 4

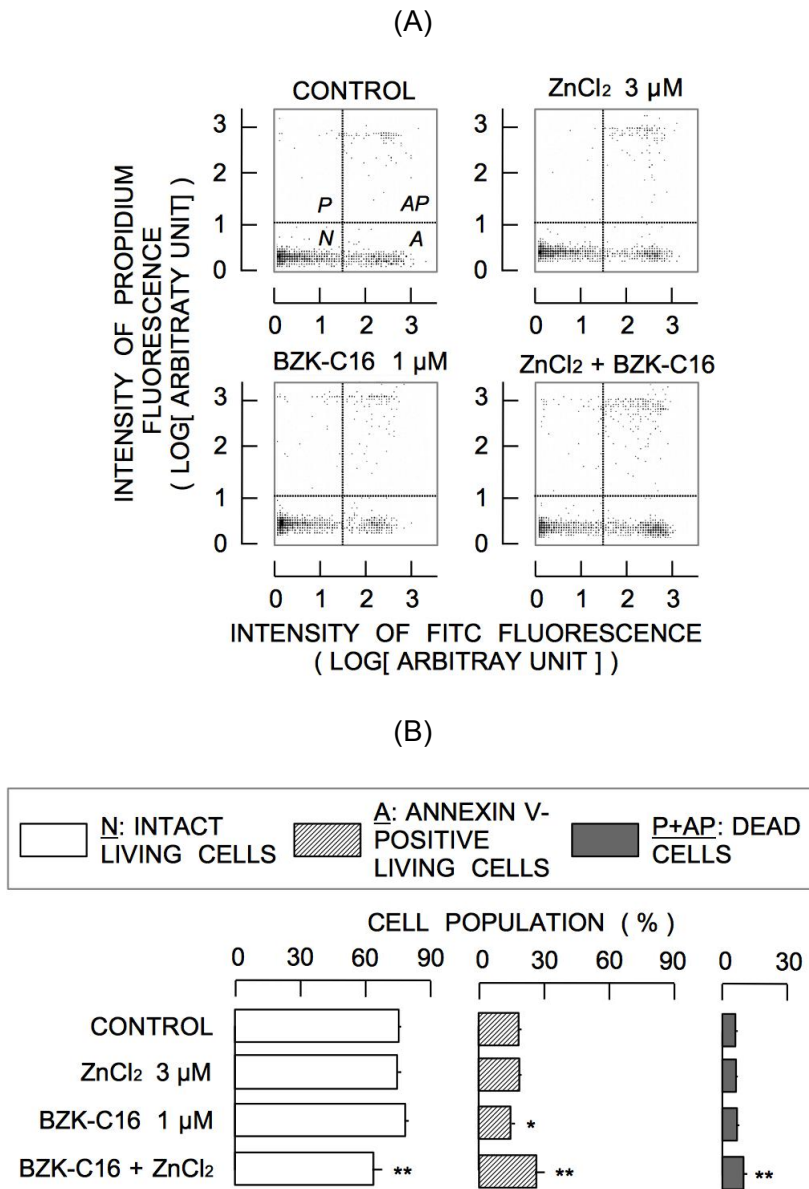


Figure 5

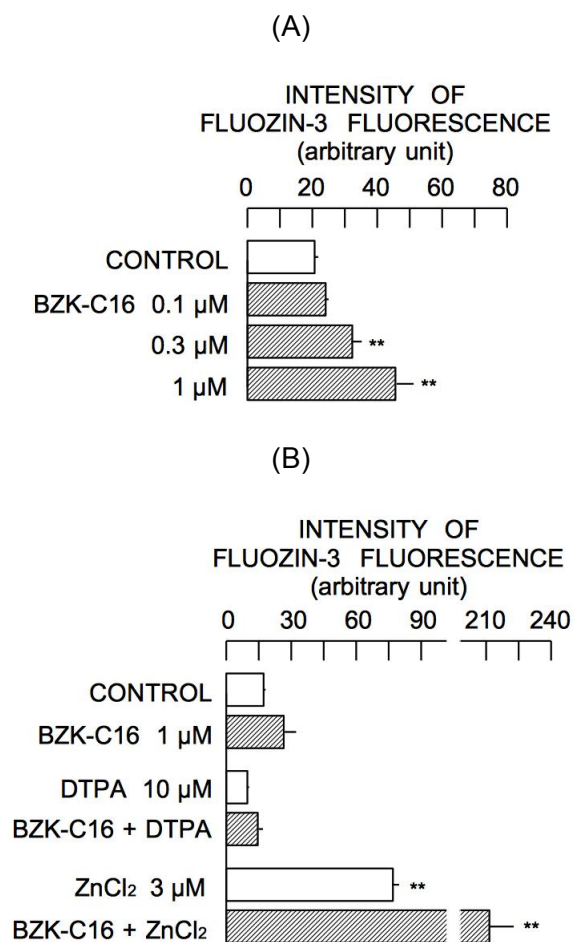


Figure 6

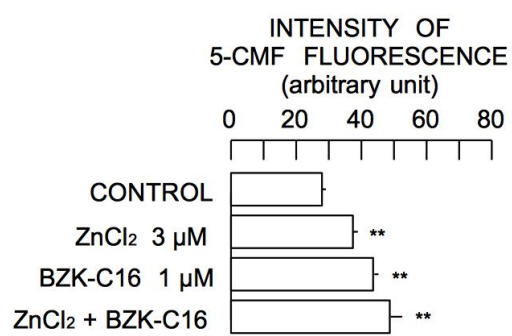


Figure 7

